Timp-1 gene regulation in response to retinoic acid and polypeptide growth factors.

Thesis

How to cite:

© 1996 Heather Frances Bigg

Version: Version of Record

Link(s) to article on publisher’s website:
http://dx.doi.org/doi:10.21954/ou.ro.0000f79e

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online’s data policy on reuse of materials please consult the policies page.
TIMP-1 GENE REGULATION IN RESPONSE TO RETINOIC ACID AND POLYPEPTIDE GROWTH FACTORS.

HEATHER FRANCES BIGG

A thesis submitted in fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy.

APRIL 1996

Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge, U.K.

Author's number : P92714760
Date of submission : April 1996
Date of award : 27th June 1996
CONTENTS

Contents
Acknowledgements
Declaration
Summary
Abbreviations and Definitions Used
Relevant Publications
Preface

CHAPTER ONE
INTRODUCTION
1.1. Retinoids
  1.1.1. Definition, structure and classification
  1.1.2. Dietary sources
  1.1.3. Absorption by the alimentary canal, storage, transport and
         uptake by cells
  1.1.4. Cellular retinoid-binding proteins
  1.1.5. Nuclear retinoic acid receptors
  1.1.6. Role in vivo and effects in vitro
         1.1.6.1. Cartilage resorption
         1.1.6.2. Differentiation of mesenchymal cells
         1.1.6.3. Synthesis of extracellular matrix components
         1.1.6.4. Cell proliferation
  1.1.7. Therapeutic uses
         1.1.7.1. Rheumatoid arthritis
         1.1.7.2. Epidermolysis bullosa
  1.1.8. Summary
1.2. Growth factors
  1.2.1. Basic fibroblast growth factor
         1.2.1.1. Definition and structure
         1.2.1.2. Synthesis and secretion
         1.2.1.3. Receptors and signal transduction
         1.2.1.4. Effects in vitro and role in vivo
  1.2.2. Epidermal growth factor
         1.2.2.1. Definition and structure
         1.2.2.2. Synthesis and secretion

page

i
vii
viii
ix
xii
xvi
xvii
1
1
2
2
4
6
10
10
14
15
15
16
16
18
18
20
20
21
21
26
27
27
28
1.2.2.3. Receptors and signal transduction 28
1.2.2.4. Effects in vitro and role in vivo 30

1.2.3. Platelet-derived growth factor 30
1.2.3.1. Definition and structure 30
1.2.3.2. Synthesis and secretion 31
1.2.3.3. Receptors and signal transduction 32
1.2.3.4. Effects in vitro and role in vivo 35

1.2.4. Transforming growth factor-β 36
1.2.4.1. Definition and structure 36
1.2.4.2. Synthesis and secretion 37
1.2.4.3. TGF-β receptors 37
1.2.4.4. Effects in vitro and role in vivo 40

1.2.5. Summary 42

1.3. Connective tissue turnover and matrix metalloproteinases 45
1.3.1. Connective tissue structure and function 45
1.3.2. Matrix metalloproteinases 46
1.3.2.1. Substrate specificity 47
1.3.2.2. Domain structure 51
1.3.2.3. Latency and activation 53
1.3.2.4. Regulation of MMP production 54
1.3.2.5. Collagenase gene expression and collagenase mRNA stability 59
1.3.3. Inhibitors of metalloproteinases 64
1.3.3.1. α2-macroglobulin 64
1.3.3.2. Tissue inhibitors of metalloproteinases 65
1.3.3.3. Regulation of TIMP-1 and TIMP-2 production 70
1.3.3.4. TIMP gene regulation 73
1.3.4. Matrix metalloproteinases and pathological connective tissue breakdown 75

CHAPTER TWO
GENERAL MATERIALS AND METHODS
2.1. Preparation of fibroblast cultures 81
2.1.1. Skin fibroblasts 81
2.1.2. Synovial fibroblasts 81
2.1.3. Tendon fibroblasts 82
2.2. General maintenance and passaging of fibroblast cultures 82
2.3. Cryopreservation of fibroblasts 83
2.4. Preparation of acid-treated foetal calf serum 83
2.5. Preparation of retinoic acid and growth factors 83
2.6. Cell assay system 85
2.7. Measurement of interstitial collagenase and TIMP-1 by ELISAs 87
   2.7.1. Free TIMP-1 ELISA 87
   2.7.2. Total TIMP-1 ELISA 88
   2.7.3. Collagenase ELISA 89
2.8. Lowry assay for protein 90
2.9. DNA assay using Hoechst 33258 90
2.10. Statistical analysis 94
2.11. Materials 94

CHAPTER THREE
THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH bFGF OR EGF ON TIMP-1 AND COLLAGENASE PROTEIN SECRETION FROM HUMAN SKIN, SYNOVIAL AND TENDON FIBROBLASTS
3.1. Introduction 96
3.2. Methods and results 98
   3.2.1. Cytotoxicity of all-trans-retinoic acid 98
   3.2.2. The effect of all-trans-retinoic acid alone on TIMP-1 protein production in human skin fibroblasts 100
   3.2.3. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human skin fibroblasts 101
   3.2.4. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human synovial fibroblasts 102
   3.2.5. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human tendon fibroblasts 109
   3.2.6. The effect of specific neutralising antibodies to bFGF or EGF on the synergistic induction of TIMP-1 in response to retinoic acid and bFGF or EGF 109
   3.2.7. The effect of all-trans-retinoic acid in combination with bFGF or EGF on collagenase production in human skin, synovial and tendon fibroblasts 114
3.3. Discussion 120

CHAPTER FOUR
THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH PDGF-BB OR TGF-β ON TIMP-1 AND COLLAGENASE PROTEIN SECRETION FROM HUMAN SKIN, SYNOVIAL AND TENDON FIBROBLASTS

4.1. Introduction 125

4.2. Methods and results 127

4.2.1. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on TIMP-1 protein production in human skin fibroblasts 127

4.2.2. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on TIMP-1 protein production in human synovial fibroblasts 128

4.2.3. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on TIMP-1 protein production in human tendon fibroblasts 128

4.2.4. The effect of specific neutralising antibodies to PDGF-BB or TGF-β on the synergistic induction of TIMP-1 in response to retinoic acid and PDGF-BB or TGF-β 135

4.2.5. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on collagenase production in human skin, synovial and tendon fibroblasts 140

4.3. Discussion 146

CHAPTER FIVE
THE EFFECT OF INTERLEUKIN-1β ON THE SYNERGISTIC INDUCTION OF TIMP-1 BY ALL-TRANS-RETINOIC ACID AND POLYPEPTIDE GROWTH FACTORS

5.1. Introduction 152

5.2. Methods and results 154

5.2.1. The effect of all-trans-retinoic acid in combination with polypeptide growth factors on TIMP-1 protein production in human skin fibroblasts in the additional presence of IL-1β 154

5.2.2. The effect of bFGF and EGF in combination with IL-1β on collagenase production from human skin and synovial fibroblasts 160

5.3. Discussion 166

CHAPTER SIX
TIME COURSE OF THE SYNERGISTIC INDUCTION OF TIMP-1 BY ALL-
TRANS-RETINOIC ACID AND GROWTH FACTORS AND THE EFFECT OF
SEQUENTIAL TREATMENT WITH THE AGENTS

6.1. Introduction 169
6.2. Methods and results 172
   6.2.1. The effect of all-trans-retinoic acid in combination with
          bFGF, EGF, PDGF-BB or TGF-β on TIMP-1 protein production
          in human skin fibroblasts at various time points after stimulation 172
   6.2.2. The effect of sequential treatment of human skin fibroblasts
          with all-trans-retinoic acid and bFGF, EGF, PDGF-BB or TGF-β 173
6.3. Discussion 194

CHAPTER SEVEN
THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH
bFGF, EGF, PDGF-BB OR TGF-β ON TIMP-1, TIMP-2 AND COLLAGENASE
STEADY-STATE mRNA LEVELS IN HUMAN SKIN FIBROBLASTS
7.1. Introduction 204
7.2. Materials and methods 207
   7.2.1. General precautions to avoid contamination of RNA
          preparations with RNA-degrading enzymes 207
   7.2.2. Cell assay system 207
   7.2.3. Preparation of total cellular RNA 208
   7.2.4. RNA gel electrophoresis 209
   7.2.5. Blotting of RNA onto nylon membrane 210
   7.2.6. Preparation of competent DH5α E. coli bacteria 211
   7.2.7. Preparation of TIMP-1, TIMP-2, interstitial collagenase and
          28S rRNA cDNA probes for Northern blot hybridization 211
   7.2.8. Preparation of labelled cDNA probes and Northern blot
          hybridization 215
   7.2.9. Measurement of total cellular protein synthesis in
          response to cycloheximide by 3H-leucine incorporation 217
   7.2.10. Materials 218
7.3. Results 220
   7.3.1. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and
          TGF-β alone and in combination on steady-state levels of TIMP-1
          mRNA 220
   7.3.2. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and
TGF-β alone and in combination on steady-state levels of TIMP-2 mRNA

7.3.3. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and TGF-β alone and in combination on steady-state levels of collagenase mRNA

7.3.4. Time course of the synergistic induction of TIMP-1 mRNA by all-trans-retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β

7.3.5. The effect of the protein synthesis inhibitor cycloheximide on the synergistic induction of TIMP-1 mRNA by all-trans-retinoic acid and bFGF

7.4. Discussion

CHAPTER EIGHT
THE EFFECT OF PROTEIN KINASE INHIBITORS ON THE SYNERGISTIC INDUCTION OF TIMP-1 PROTEIN BY ALL-TRANS-RETINOIC ACID AND bFGF

8.1. Introduction

8.2. Methods and results

8.2.1. The effect of the tyrosine kinase inhibitor HERB A on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

8.2.2. The effect of the tyrosine kinase inhibitor genistein on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

8.2.3. The effect of the protein kinase C inhibitor BIS on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

8.2.4. The effect of BIS on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and PDGF-BB

8.2.5. The effect of the p38 MAP kinase inhibitor 203580 on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

8.3. Discussion

CHAPTER NINE
FINAL DISCUSSION

References
Acknowledgements

Firstly, I would like to thank my supervisors Dr. Ian Clark and Dr. Tim Cawston for their support and enthusiasm throughout this project; particularly for critically reviewing the thesis; also for many helpful discussions and not least for their unique sense of humour. Special thanks also to Dr. Ian Clark for teaching me the cell culture and molecular biology techniques upon which the project relied. I would also like to thank all the staff at the Rheumatology Research Unit for making my time here so rewarding and enjoyable. Finally, I am grateful to the Arthritis and Rheumatism Council of Research for their funding of the work.
Declaration

This thesis is based on research performed in the Rheumatology Research Unit, Addenbrooke's Hospital, Cambridge, U.K. Except for commonly held concepts, and where specific reference is made to other work, the content of this thesis is original. No part of this thesis has been submitted for the award of any other degree.
Student: HEATHER BILLS
Pt: P9 27 4760
Sponsoring Establishment: RHEUMATOLOGY RESEARCH UNIT, ANDERSON’S HOSPITAL
Degree for which the thesis is submitted: DOCTOR OF PHILOSOPHY
Thesis title: TIMP-1 GENE REGULATION IN RESPONSE TO RETINOIC ACID AND POLYPEPTIDE GROWTH FACTORS

Part 1 Open University Library Authorisation (to be completed by all students)

I confirm that I am willing for my thesis to be made available to readers by the Open University Library and for it to be photocopied, subject to the discretion of the Librarian.

Signed: [Signature] Date: 7.6.96

Part 2 British Library Authorisation (to be completed by PhD students only)

If you want a copy of your thesis to be available on loan to the British Library Thesis Service as and when it is requested, you must sign a British Library Doctoral Thesis Agreement Form and return it to the Research Degrees Office of the University together with this form. The British Library will publicize the details of your thesis and may request a copy on loan from the University Library. Information on the presentation of the thesis is given in the Agreement form.

The University has decided that your participation in the British Library Thesis Service should be voluntary. Please tick one of the boxes below to indicate your intentions.

[ ] I am willing for the Open University to loan the British Library a copy of my thesis; a signed British Library Doctoral Thesis Agreement Form is attached.

or

[ ] I do not wish the Open University to loan a copy of my thesis to the British Library.

Signed: [Signature] Date: 7.6.96
Summary

This project has investigated tissue inhibitor of metalloproteinases-1 (TIMP-1) gene regulation in human fibroblasts by all-trans-retinoic acid in combination with each of 4 different polypeptide growth factors - basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β (TGF-β).

Initially, TIMP-1 protein secretion in response to these agents was measured using a specific enzyme-linked immunosorbent assay (ELISA) for TIMP-1. The effect of 10⁻⁸M retinoic acid in combination with 1, 10 and 100ng/ml of bFGF, EGF, PDGF-BB or TGF-β on TIMP-1 protein secretion was examined in human skin, synovial and tendon fibroblasts. Retinoic acid in combination with each of the 4 growth factors caused a potent induction of TIMP-1 protein which was greater than the additive effect of the agents by up to 4 fold. These responses occurred in all 3 types of fibroblast examined. The synergistic induction of TIMP-1 protein was dose-dependent and was not due to a general increase in cell protein synthesis or cell proliferation. The responses were also abolished by the additional presence of specific neutralizing antibodies to bFGF, EGF, PDGF-BB or TGF-β.

The effect of the same agents on collagenase (matrix metalloproteinase-1) production from the cells was also examined using a specific ELISA for this enzyme. It was found that retinoic acid potently blocked bFGF, EGF and PDGF-BB-stimulated collagenase but failed to interact in an additive manner with TGF-β to depress collagenase production.

The ability of the proinflammatory cytokine interleukin-1β (IL-1β) to modulate further the response of fibroblasts to retinoic acid in combination with growth factors was investigated. IL-1β caused a reduction in the level of synergistic interaction between retinoic acid and bFGF, EGF and PDGF-BB. However, no consistent effect was seen with IL-1β, retinoic acid and TGF-β. IL-1β in combination with bFGF or EGF also caused a synergistic induction of collagenase protein from the cells.
The mechanism(s) of the synergistic induction of TIMP-1 protein by retinoic acid and growth factors were then addressed using skin fibroblasts. The stimulation of TIMP-1 protein production continued to increase across a 72 hour time period suggesting that these effects were secondary responses to the agents. A short incubation (up to 12 hours) with bFGF alone followed by retinoic acid treatment gave a synergistic induction of TIMP-1 protein similar to that seen with both agents together. Increasingly longer incubations with bFGF were not as effective and eventually ineffective. The transient induction of an intracellular event by bFGF is therefore involved in the mechanism. Prolonged treatment with TGF-β (72 hours) alone followed by retinoic acid treatment gave an induction of TIMP-1 similar to that seen with both agents together but induction by retinoic acid and EGF required the simultaneous presence of both agents.

The synergistic induction of TIMP-1 protein by the agents was paralleled by similar effects on steady-state levels of TIMP-1 mRNA as shown by Northern blotting. The maximum levels of TIMP-1 mRNA either preceded or concurred with the peak induction of TIMP-1 protein. TIMP-1 gene regulation in response to retinoic acid and bFGF, EGF, PDGF-BB or TGF-β therefore occurs at a pretranslational level. The induction of TIMP-1 mRNA by retinoic acid and bFGF was sensitive to cycloheximide and therefore required new protein synthesis. The changes in collagenase protein in response to the agents were also paralleled by similar changes in collagenase mRNA levels. In contrast to TIMP-1, TIMP-2 mRNA was not induced by any of the factors alone or in combination except in the case of retinoic acid and TGF-β applied together in which a modest stimulation occurred.

The tyrosine kinase inhibitor genistein but not herbimycin A abolished the induction of TIMP-1 protein by retinoic acid and bFGF. This occurred in a dose-dependent manner without causing cytotoxicity. No inhibition was seen in response to the protein kinase C inhibitor bisindolylmaleimide (BIS) although BIS blocked known protein kinase C-dependent effects in the cells. BIS also partially blocked both the induction of TIMP-1 protein in response to retinoic acid and PDGF-BB and the
induction of collagenase protein in response to PDGF-BB alone. Finally, a highly specific p38 mitogen-activated protein (MAP) kinase inhibitor was found to enhance further the synergistic stimulation of TIMP-1 protein by retinoic acid and bFGF. This inhibitor also caused a dose-dependent inhibition of IL-1β-stimulated collagenase from the cells.
## Abbreviations and Definitions Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>AF-1</td>
<td>activation function 1</td>
</tr>
<tr>
<td>AF-2</td>
<td>activation function 2</td>
</tr>
<tr>
<td>ATFCS</td>
<td>acid-treated FCS</td>
</tr>
<tr>
<td>bek</td>
<td>bacterial expressed kinase</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BIS</td>
<td>bisindolylmaleimide</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CL</td>
<td>collagenase</td>
</tr>
<tr>
<td>CRABP</td>
<td>cellular retinoic-acid binding protein</td>
</tr>
<tr>
<td>CRABP[II]</td>
<td>cellular retinoic acid-binding protein type 2</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol-binding protein</td>
</tr>
<tr>
<td>CRBP[II]</td>
<td>cellular retinol-binding protein type 2</td>
</tr>
<tr>
<td>C-terminal</td>
<td>COOH-terminal</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modification of Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Erk-1</td>
<td>extracellular signal-regulated kinase-1</td>
</tr>
<tr>
<td>FCS</td>
<td>heat inactivated foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
</tbody>
</table>
FGFR-3 - FGF receptor 3
FGFR-4 - FGF receptor 4
flg - fms-like gene
flg-2 - fms-like gene-2
g - relative centrifugal force
G - guanine
GAP - GTPase activating protein
GDP - guanosine diphosphate
G-protein - GTP-binding regulatory protein
grb 2 - growth factor receptor bound 2
GTP - guanosine triphosphate
HBSS - Hanks' balanced salt solution
HEPES - N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HERB A - Herbimycin A
HRP - horse-radish peroxidase
HSPG - heparan sulphate proteoglycan
IL-1 - interleukin-1
IL-6 - interleukin-6
kb - kilobase
LB - Luria-Bertuni
LDH - lactate dehydrogenase
loading buffer - 0.25% bromophenol blue, 1mM EDTA, pH 8.0, 50% glycerol
LTBP - latent TGF-β-binding protein
LTBP-2 - latent TGF-β-binding protein-2
MAP kinase - mitogen-activated protein kinase
MEK - MAP kinase/extracellular signal-regulated kinase kinase
MMP - matrix metalloproteinase
MOPS - 3-(N-morpholino) propanesulphonic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N-terminal</td>
<td>NH₂-terminal</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PEA3</td>
<td>polyoma enhancer A3</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>Pu</td>
<td>purine</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>retinoic acid response element</td>
</tr>
<tr>
<td>RBP</td>
<td>retinol-binding protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>room temperature</td>
<td>21°C</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>running buffer</td>
<td>0.02M MOPS, 0.005M sodium acetate, 0.001M EDTA</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>RXRE</td>
<td>RXR response element</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHC</td>
<td>SH2 domain and collagen-like</td>
</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SSC</td>
<td>0.15M sodium chloride, 0.015M sodium citrate</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TBE</td>
<td>45mM Tris, 45mM boric acid, 1mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10mM Tris/hydrochloride, 10mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TIE</td>
<td>TGF-β inhibitory element</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>tissue inhibitor of metalloproteinases-1</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>tissue inhibitor of metalloproteinases-2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>tissue inhibitor of metalloproteinases-3</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>TRE</td>
<td>TPA-responsive element</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl] aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>uracil</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D₃ receptor</td>
</tr>
</tbody>
</table>
Relevant Publications


Abstracts:


Preface

Connective tissue cells in culture can be stimulated by cytokines or growth factors to secrete a family of matrix metalloproteinases which between them, are capable of degrading all components of the extracellular matrix. These same cells can also secrete specific tissue inhibitors of the metalloproteinases (TIMPs), and if these are present in excess, matrix degradation can be prevented. Collagenase (matrix metalloproteinase-1) is an enzyme capable of specifically cleaving interstitial collagens and is therefore likely to play a key role in the breakdown of matrix. It is secreted as a latent proenzyme which may be activated in vivo by plasmin and stromelysin (matrix metalloproteinase-3). The active enzyme can be inhibited by the TIMPs (TIMP-1, -2 and -3) which bind to it tightly, forming inactive enzyme-inhibitor complexes.

One possible strategy for treating disorders involving pathological connective tissue breakdown such as arthritis is to induce connective tissue cells to decrease collagenase production and/or to increase TIMP production by the use of appropriate growth factors, cytokines or other agents. Retinoic acid, a member of the vitamin A family of compounds represses collagenase and stimulates TIMP-1 production from fibroblasts. These findings suggest that retinoids may be useful in preventing pathological connective tissue breakdown. Experiments using animal models of rheumatoid arthritis showed that retinoid treatment suppressed disease activity in some cases. In apparent conflict with these findings is the observation that retinoids are also potent agents for inducing proteoglycan loss from cartilage which would be expected to exacerbate arthritic disease.

The retinoids are good candidates for drug development due to their simple chemical structure and a large number of natural and synthetic retinoids already exists. Some of these compounds are currently used in other clinical situations which means that information on side-effects and toxicity is already available. The structure-activity...
relationship of many retinoids has also been studied. This has shown that it is possible to modify the structure of these compounds so that beneficial effects are enhanced and undesirable side-effects are diminished. Thus it may be possible to design a retinoid which selectively suppresses collagenase and stimulates TIMP-1 production from cells.

The aim of this project is to increase our understanding of the effect of retinoids on collagenase and TIMP-1 secretion from connective tissue cells. Whilst there are many reports of single growth factors or cytokines modulating the expression of the MMPs or their inhibitors, considerably less is known about interactions between 2 or more such factors and the mechanisms of any such interactions are not understood. Cytokines, growth factors and other agents do not necessarily act independently of one another when both are present simultaneously and phenomena such as synergism and antagonism have been reported. Indeed, it has been shown that basic fibroblast growth factor and epidermal growth factor synergize with transforming growth factor-\(\beta\) to superinduce TIMP-1 production from human fibroblasts. Since cells in vivo are exposed to many different modulatory factors at once, it is important to understand these interactions. The use of TIMP-1 stimulation in cells as a therapeutic goal in the treatment of connective tissue breakdown requires a detailed understanding of TIMP-1 gene expression and protein production in cells. Preliminary investigations in the Rheumatology Research Unit have indicated that retinoic acid and mononuclear cell factor synergistically stimulate the production of TIMP-1 from fibroblasts. Mononuclear cell factor is a mixture of various cytokines and growth factors and it would be useful to pinpoint which factor or factors are causing the effect in order to further our understanding of TIMP-1 gene regulation. This project has therefore focused on the effect of retinoic acid in combination with several different growth factors on TIMP-1 production from fibroblasts.
1.1. RETINOIDS

1.1.1. Definition, structure and classification

Vitamin-A-active substances or retinoids are compounds other than carotenoids, that exhibit some or all of the biological activities of trans-retinol which is the parent vitamin A compound. Its naturally occurring oxidation products are trans-retinal and trans-retinoic acid. The common structure of these 3 compounds includes a cyclic end group (the trimethylcyclohexenyl ring); a dimethyl-substituted, all-trans, tetraene chain; and a polar hydroxyl, aldehyde or carboxyl end group (see figure 1.1. below).

Figure 1.1

Structural formulas of some naturally occurring retinoids (from Blomhoff et al., 1990)
IUPAC-IUB therefore defined retinoids as 'diterpenoids derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion'. Each region of the retinoid molecule can be chemically modified in many ways, and the resulting almost unlimited number of natural and synthetic compounds are collectively called retinoids. Because retinoids possess four carbon-carbon double bonds in the side-chain each of which can potentially exist in the \textit{cis} or the \textit{trans} configuration this gives rise to various optical isomers of retinol, retinal, retinoic acid and other retinoids. There are also a large number of compounds whose structure does not fit the classical IUPAC-IUB definition but which are retinoids because they display vitamin A activity. Good examples are the tricyclic or tetracyclic retinoidal benzoic acid derivatives such as TTNPB which have been shown to be more than a thousand times more potent than retinol and its closely related derivatives in biological assays (Loeliger et al., 1980, Newton et al., 1980, Strickland et al., 1983, Dawson et al., 1983).

1.1.2. Dietary sources

Vitamin A cannot be synthesized endogenously by animals and must be supplied in the diet. The ultimate sources of vitamin A are the provitamin carotenoids which are biosynthesized exclusively by photosynthetic microorganisms and plants. Humans obtain their vitamin A by metabolism from ingested plant carotenoids and from ingestion of animal products that contain the vitamin in the form of retinyl esters.

1.1.3. Absorption by the alimentary canal, storage, transport and uptake by cells

Dietary retinyl esters and carotenoids are dispersed and emulsified in the stomach during the gastric phase of lipid digestion. Retinyl esters are then enzymatically hydrolysed to retinol in the intestinal lumen before absorption by the intestinal cells (enterocytes) (Ganguly, 1969). In contrast \(\beta\)-carotene is first absorbed by the cells
and then enzymatically cleaved to give two retinal molecules which are then reduced to retinol (Goodman & Huang, 1965, Glover et al., 1948, Wolf, 1984).

In the enterocytes, retinol reacts with long chain fatty acids to form retinyl esters before incorporation into chylomicrons (Huang & Goodman, 1965, Goodman et al., 1965a). Chylomicrons reach the general circulation by way of the intestinal lymph and chylomicron remnants are formed in the blood capillaries (Green & Glickman, 1981). The chylomicron remnants, which contain most of the absorbed retinol are cleared from the circulation primarily by the liver (Goodman et al., 1965a, Blomhoff et al., 1982, Goodman et al., 1965b) although some uptake also takes place in the bone marrow and spleen.

In the liver, parenchymal cells (hepatocytes) are responsible for the uptake of chylomicron remnant retinyl esters (Blomhoff et al., 1982). The retinyl esters are hydrolysed at the plasma membrane or in early endosomes by a retinyl ester hydrolase (Harrison and Gad, 1989). Retinol is then transferred to the endoplasmic reticulum where it binds to retinol-binding protein (RBP) and is subsequently exported from the cell (Blomhoff et al., 1985a, Hendriks et al., 1988, Rask et al., 1983, Ronne et al., 1983).

Retinol is subsequently taken up by the hepatic stellate cells (Blomhoff et al., 1984) which store retinol as retinyl esters. In mammals, about 70 to 95% of the total body vitamin A is normally stored in this manner in large cytoplasmic lipid droplets (Blomhoff et al., 1985b, Blaner et al., 1985, Wake, 1980).

Retinol can be mobilized from the stellate cells to the general circulation as retinol-RBP. It is the task of the stellate cells to control this mobilization such that the blood plasma retinol remains close to 2μM in spite of normal fluctuations in daily vitamin A intake (Wolf, 1984, Goodman & Blaner, 1984). RBP solubilizes the water-insoluble retinol molecule and protects it from oxidative damage while it is transported in the circulation. The majority of retinol-RBP in plasma is reversibly complexed with transthyretin also known as plasma prealbumin which renders it less susceptible to filtration by kidney glomeruli (Wolf, 1984). Due to its amphipathic nature, free
retinol may interact with membrane lipids and cause membrane labilization. Binding of retinol to transport proteins lessens its toxic surface-active effects on cell membranes (Dingle et al., 1972b).

The mechanism for cellular uptake of retinol from plasma is not yet fully elucidated. Since a small amount of free retinol may be in equilibrium with retinol-RBP in plasma, retinol may partition into the plasma membrane of cells without the use of a cell surface receptor (Fex & Johannesson, 1988) or may enter cells as a result of fluid-phase endocytosis. Various studies (Bok & Heller, 1976, McGuire et al., 1981, Gjøen et al., 1987, Senoo et al., 1990, Heller, 1975, Rask & Peterson, 1976, Eriksson et al., 1986) have indicated that there is a cell surface receptor or transporter for RBP-bound retinol in vivo. It is not yet clear whether RBP delivers retinol to tissues via receptor-mediated endocytosis or whether the RBP receptor acts as a transporter for retinol.

In addition to 2μM retinol-RBP, there is a 5 to 10nM plasma concentration of retinoic acid bound to albumin (Goodman, 1984, Wolf, 1984, Goodman & Blaner, 1984) which can move into cells by diffusion. There is also some uptake of retinoids by cells from chylomicron remnant retinyl esters.

Retinol serves as the metabolic precursor of biologically active retinoic acids which include but are not limited to all-trans-retinoic acid, dihydroretinoic acid and the 9-cis-retinoic acid isomer (Napoli et al., 1993). After cellular uptake retinol can undergo reversible oxidation to retinal by alcohol dehydrogenase and retinal can be further irreversibly oxidised to retinoic acid (Olson, 1972). More recent evidence has suggested that retinol can also be metabolised to retinoic acid independently of alcohol dehydrogenase and without the appearance of retinal as an intermediate (Posch et al., 1989, Siegenthaler et al., 1990) Retinoic acid may also be synthesised from β-carotene in several tissues since in some species, including man, some β-carotene enters the circulation from the intestine and is stored in the tissues rather than being metabolized to retinol in the intestinal enterocytes (Napoli & Race, 1988).

1.1.4. Cellular retinoid-binding proteins
The uptake, storage, remobilization and metabolism of retinoids requires the presence of cellular retinoid-binding proteins. Four well-characterized cellular retinoid-binding proteins have been found to be members of a larger protein family of small, intracellular carriers of hydrophobic ligands. These are the cellular retinol-binding protein (CRBP), cellular retinol-binding protein type 2 (CRBP[II]), cellular retinoic acid-binding protein (CRABP) and cellular retinoic acid-binding protein type 2 (CRABP[II]). As the names suggest, the CRBPs bind retinol and retinal but reject retinoic acid (Saari et al., 1982, Levin et al., 1988, Li et al., 1991) although another report showed that CRBP did not bind retinal (MacDonald and Ong, 1987). The main ligand for the CRABPs is retinoic acid (Saari et al., 1982, Siegenthaler et al., 1990, Fiorella and Napoli, 1991, Bailey and Siu, 1988).

The organ and cellular distribution of the members of each pair differs suggesting that there are functional differences between them. CRBP is found in almost all organs, and is particularly abundant in cells known to process considerable amounts of vitamin A such as the stellate cells of the liver (Ong et al., 1994). In contrast, CRBP[II] is found only in the villous-associated enterocytes of the small intestine. This suggests that CRBP[II] is particularly concerned with the trafficking and metabolism of vitamin A which occurs during absorption from the intestinal lumen, but that CRBP is better suited for all other cells of the body that handle vitamin A (Ong, 1994). CRABP and CRABP[II] are also distributed differently; CRABP is widespread while CRABP[II] is found mainly in the skin (Ong et al., 1994).

One of the functions of all the binding proteins is to serve as a cellular reservoir of retinoids, protecting the cell from the detergent-like properties of the compounds and simultaneously protecting the labile retinoids from degradation (Ong., 1994). It has also been suggested that the retinoid-binding proteins are involved in the transfer of retinoids in and out of cells (such as the small intestine enterocyte and liver hepatocytes) and cellular compartments. In addition, they have also been shown to interact with the enzymes involved in vitamin A metabolism. CRBP and CRBP[II] direct the metabolism of their ligands so that they are unavailable for reaction with
some enzymes but still accessible to others (see for example Ong, 1993, Napoli et al., 1991, Napoli, 1993, Ong, 1994).

CRABP is thought to sequester retinoic acid in the cytoplasm and to be involved in its metabolism to more polar compounds (Fiorella & Napoli 1991) which reduces the amount of retinoic acid available to regulate gene expression in the nucleus (Boylan and Gudas, 1991). However, others have suggested that the CRABPs have a role in delivering retinoic acid to the nuclear retinoic acid receptors (Ong, 1994). Neither CRABP nor CRABP-II bind 9-cis-retinoic acid which suggests the existence of a distinct metabolic pathway for the 9-cis-retinoids (Allenby et al., 1993, Fogh et al., 1993). However, another report has indicated that 9-cis-retinoic acid does bind both proteins although with a lower affinity than all-trans-retinoic acid. (Fiorella et al., 1993).

1.1.5. Nuclear retinoic acid receptors

Although the cellular retinoid-binding proteins are important in the intracellular transport and metabolism of retinoids, they do not have any direct role in eliciting the response of target cells to the hormone-like effects of retinoids. This is carried out by a completely separate set of proteins found in the nucleus of the cell which function as retinoid receptors in a similar fashion to steroid/thyroid hormone receptors for steroid/thyroid hormones.

These proteins actually belong to the steroid/thyroid hormone nuclear receptor superfamily and share with them a modular structure of 6 domains designated A to F (see figure 1.2., page 11). Binding of retinoic acid to a dimerized receptor converts it into an active transcription factor that is able to regulate transcription from gene promoters containing specific nucleotide sequences.

The first retinoic acid receptor (RARα) was cloned in 1987 (Petkovich et al., 1987, Giguère et al., 1987) and in 1988 a hepatitis B virus integration site in a human hepatocellular carcinoma was shown to be a different retinoic acid receptor (RARβ, Brand et al., 1988). Finally, a third human retinoic acid receptor (RARγ) and the
mouse counterparts of all 3 were cloned (Krust et al., 1989, Zelent et al., 1989). Comparison of the amino acid sequences of the 3 human receptors with the mouse receptors showed that the interspecies conservation of a member of the RAR subfamily is much higher than the conservation of all 3 receptors within a given species, suggesting that the 3 receptors have their own specific function (Krust et al., 1989, Zelent et al., 1989). In addition, the expression of the receptors has been shown to be tissue specific.

Another set of nuclear retinoic acid receptors have also been described which are substantially different in primary sequence from the RARs. These receptors are termed RXRα, RXRβ and RXRγ and represent a separate family of retinoid receptors (Mangelsdorf et al., 1990, Yu et al., 1991, Rowe et al., 1991, Leid et al., 1992a) which are also expressed in a tissue-specific manner. The RXRs have only 5 structural domains (A-E), the F domain being absent (reviewed by Pemrick et al., 1994).

All 3 of the RAR genes have been shown to encode more than 1 protein (Leroy et al., 1992). These different forms arise from differential usage of the gene promoters and alternative splicing of the primary mRNA transcript (reviewed by Leid et al., 1992b). Although most of the investigation into receptor isoforms has been done in the mouse, it is likely that the human RAR genes encode a similar range of proteins (Krust et al., 1989). Detailed information of this sort is not yet available for the RXR receptors although already, 2 different species of human RXR-β mRNA have been described (Fleishhauer et al., 1992).

The DNA-binding area of the RARs and RXRs is the C domain (see figure 1.2., page 11) which consists of 66 amino acids (Yang et al., 1991). These form 2 cysteine-type zinc fingers in which 4 cysteine residues tetrahedrally coordinate a zinc ion. The sequences of nucleotides to which retinoid receptors specifically bind are called retinoic acid response elements (RAREs or RXREs) and these are characterised by the presence of a core sequence PuG(G/T)TCA. This sequence is believed to represent a minimal site for the binding of 1 receptor molecule, so that the binding of receptor dimers requires the presence of 2 such sequences. The core sequences may be present
in the same or opposite orientation with respect to each other. The orientation of the core sequences, the exact sequence composition, and the spacing between them affects the affinity of receptor binding (Näää et al., 1991, Umesono et al., 1991, Leid et al., 1992a) which allows differential activation of target genes by different receptor types. It has been suggested that each receptor type has its own unique high affinity binding sequence. RARs bind more strongly to directly repeated core sequences separated by 5 base pairs (Umesono et al., 1991) although direct repeats of the core motif separated by 2 base pairs can also operate efficiently as RAREs (Williams et al., 1992). Most natural RAREs characterized to date consist of repeated motifs spaced by either 2 or 5 base-pairs. In contrast, RXREs consist of directly repeated core sequences separated by 1 base pair (e.g. Mangelsdorf et al., 1991). It appears that retinoid receptors bind as dimers to DNA both in the absence and presence of retinoic acid but activate transcription only in the presence of ligand (Hoffmann et al., 1990, Yang et al., 1991, Perlmann and Vennström, 1995). The binding of ligand to RARs appears to release the receptors from interactions with co-repressor molecules which silence transcription (reviewed by Perlmann and Vennström, 1995).

Binding of ligand to RARs and RXRs is mediated by the E domain of the receptor which contains large numbers of hydrophobic residues forming a hydrophobic pocket in which the ligand sits (Green & Chambon, 1988). The RAR and RXR classes of receptor share no significant amino acid identity in their ligand binding domains and consequently have different retinoid-binding specificities. All-trans-retinoic acid and 9-cis-retinoic acid bind to RARs with high affinity while the RXRs bind tightly to 9-cis-retinoic acid but not to all-trans-retinoic acid (Levin et al., 1992, Heyman et al., 1992, Allenby et al., 1993). The amino acid sequence identity of the E domains of the RAR-α, RAR-β and RAR-γ proteins is high (85-90%) but the differences suggest that these proteins have different affinities for retinoid ligands and this has indeed been shown to be the case (Crettaz et al., 1990).

Members of the retinoic acid receptor family contain two additional regions that can influence the transcription of target genes. These are the activation function-1 (AF-1)
region, which is located within the N-terminal A and B domains and the AF-2 region which is located in the E domain (Green & Chambon, 1988, Nagpal et al., 1992) (see figure 1.2). These regions can enhance transcriptional transactivation in certain cell types and from certain promoters presumably by interacting with other proteins in the cell. Differences in the amino acid sequences of these regions in the various receptor types and protein isoforms are thought to lead to differential transactivation characteristics for each receptor leading to differential regulation of retinoid responsive genes (Nagpal et al., 1992).

The retinoid receptors bind to DNA as dimers and dimerization interfaces have been characterized in both the DNA and the ligand binding domains (Forman & Samuels, 1990, Perlmann et al., 1993). However, homodimers of RAR proteins bind very poorly to RAREs and in order to bind with high affinity, RARs must form heterodimers with RXRs (Yu et al., 1991, Kliewer et al., 1992, Leid et al., 1992a). It has been shown that RXR occupies the 5'-upstream half site and RAR the 3'-downstream half-site of RAREs (Predki et al., 1994, Perlmann et al., 1993, Kurokawa et al., 1993). However, a report has suggested that homodimers of RAR can also mediate gene expression (Schrader et al., 1993).

In addition, the RXRs appear to be able to function independently, such that in the presence of 9-cis-retinoic acid RXRs form homodimers that bind strongly to RXREs (Zhang et al., 1992). In some instances the activation of genes containing RXREs can be inhibited by the presence of RARs (Mangelsdorf et al., 1991). Changes in the levels of RARs and RXRs within a cell can therefore shift the equilibrium between RAR-RXR heterodimers and RXR-RXR homodimers and thus alter the pattern of target gene activation that results.

Thyroid hormone nuclear receptors (TRs) and vitamin D3 receptors (VDRs) are structurally more similar to the RARs and RXRs than to other members of the steroid/thyroid hormone receptor superfamily. It has been shown that RXRs are able to form heterodimers with TRs resulting in an increase in thyroid hormone-dependent transcriptional transactivation from promoters containing thyroid response elements.
(Yu et al., 1991, Kliewer et al., 1992, Leid et al., 1992a). RXRs can also form heterodimers with VDRs, increasing the efficiency of transcriptional transactivation in the presence of 9-cis-retinoic acid and vitamin D3 from promoters containing vitamin D3 response elements (Yu et al., 1991, Kliewer et al., 1992). Similar effects have also been seen with the peroxisome proliferator receptor (Kliewer et al., 1992). There is also evidence to suggest that heterodimer formation can occur between RARs and TRs (Glass et al., 1989). The interaction of RARs and RXRs with TRs and VDRs indicates that retinoic acid, thyroid hormone and vitamin D3 control the expression of overlapping networks of genes.

Clearly, the response of cells to retinoids is highly complex and is controlled by the presence of cell-specific combinations of RXR and RAR types and isoforms which vary in their ability to activate different subsets of retinoid-responsive promoters. The activity of receptor heterodimers can also be regulated by cell-specific levels of all-trans- and 9-cis retinoic acid which are in turn regulated by cellular levels of CRABPs and enzymes which metabolize retinoids. Further diversity in the cellular response is generated by the overlap of the retinoid signalling system with those of thyroid hormone and vitamin D3. Such complexity provides a basis for the highly pleiotropic effect of the retinoids.

1.1.6. Role in vivo and effects in vitro

The retinoids have many diverse effects in vitro and in vivo (e.g reproduction, growth, differentiation, vision) for which there is insufficient space for discussion in this introduction. Of particular relevance to this thesis is the marked effect of retinoids on cartilage which is discussed below. Other effects of retinoids which are of direct relevance to connective tissue turnover are also discussed.

1.1.6.1. Cartilage Resorption

Fell and Mellanby showed in 1952 that in organ cultures of cartilagenous bone rudiments, vitamin A caused a marked loss of cartilage proteoglycan. Similar effects
Figure 1.2. The intracellular metabolism and signal transduction mechanism of retinoids.
have also been demonstrated in the cartilage of young rabbits dosed with excess vitamin A (Thomas et al., 1960) and in adult bovine articular cartilage treated with retinoic acid (Campbell and Handley, 1987). The early investigations suggested that hydrolytic enzyme activity was involved in these changes, due to the similarity of these effects on cartilage to that of papain (Fell & Thomas, 1960, Thomas et al., 1960). In addition, the work of Dingle et al., (1961a) demonstrated that cartilagenous limb-bone rudiments grown in the presence of excess vitamin A produced more proteolytic activity than control explants.

Since then, an enormous number of different reports investigating the mechanism and the enzymes involved in retinoid-induced cartilage breakdown have appeared. These reports have included investigating the roles of cathepsins, metalloproteinases and serine proteinases in this process. The resorption of cartilage by retinoids is clearly a complex phenomenon, since as yet, no satisfactory explanation has been put forward which is consistent with all the available evidence.

In the 1960s, experiments were carried out to find out whether normal cartilage contained an enzyme capable of producing an effect on the matrix like that of vitamin A (Lucy et al., 1961). It was found that an enzyme or group of enzymes degraded cartilage in bone rudiments from chick embryos after hypo-osmotic treatment of the tissue and that the optimum pH of this (these) enzyme(s) was in the acid region. Vitamin A was found to release an enzyme from rat liver lysosomes with an acid pH optimum similar to that of the bone rudiment enzyme (Dingle, 1961b). Another investigation showed that rabbit ear cartilage contained a proteolytic enzyme which could degrade cartilage with an optimum activity at pH 5.0 (Ali, 1964). Further experiments demonstrated that vitamin A could release an acid protease from cartilage rudiments, that a protease preparation from lysosomes could cause cartilage breakdown and that vitamin A could release a protease from a cartilage particulate preparation (Fell & Dingle, 1963). These various reports suggested that the lysosomal system played a key role in the catabolism of cartilage proteoglycan in response to
vitamin A by releasing enzymes which were active at low pH. It was postulated that 
these enzymes were cathepsins D and B (Woessner, 1959, Ali, 1964).
Subsequent work showed that cathepsin D was capable of degrading cartilage and that 
a specific antibody to cathepsin D produced an almost complete inhibition of the 
atolysis of chick and rabbit cartilage at pH 5.0 (Dingle et al., 1971). It was also 
shown that human adult articular cartilage and rabbit ear cartilage degradation at pH 
5.0 could be potently inhibited by pepstatin (Dingle et al., 1972a). Furthermore, the 
release of 35S-labelled material from limb-bone rudiments treated with vitamin A in 
organ culture was decreased by 35-50% in the presence of anti-(chicken cathepsin D) 
sera (Dingle et al., 1971).
More recent work, however has indicated that neither antibodies to nor inhibitors of 
cathepsin D activity could prevent the breakdown of cartilage caused by vitamin A in 
chick limb bone organ cultures (Hembry et al., 1982). Furthermore, the pH of the 
extracellular fluid of cartilage is normally about neutral (Sapolsky et al., 1973) and 
cathepsin D has no detectable hydrolytic effect on cartilage proteoglycans above pH 6 
(Woessner, 1973a, Woessner, 1973b). Thus the action of this enzyme must be limited 
to proteoglycans taken into digestive vacuoles or immediately at the cell surface 
where an acid pH might be produced at the expense of metabolic energy. This 
prompted investigations into the role of matrix metalloproteinases in retinoid-
stimulated cartilage breakdown since these act at neutral pH and there is good 
evidence for their presence in cartilage (Sapolsky et al., 1976, Ehrlich et al., 1977). It 
was found that retinoid-stimulated rabbit articular cartilage breakdown could be 
inhibited with metalloproteinase inhibitors (Caputo et al., 1987). A metalloproteinase 
inhibitor has also been shown to inhibit retinoid-stimulated proteoglycan release from 
bovine nasal and bovine articular cartilage (Buttle et al., 1993) while a cathepsin B 
inhibitor failed to inhibit this breakdown.
The role of serine proteinases such as the plasmin/plasminogen activator system in 
cartilage resorption induced by retinoids has also been investigated. It has been 
shown that retinoic acid and retinol can stimulate the production of plasminogen
activator by cultured human chondrocytes (Meats et al., 1985). This effect resulted from a stimulation of enzyme production and activity which remained bound to the cell membrane. These observations correlate with work which suggested that cartilage breakdown is limited to the pericellular environment of the chondrocytes (Dingle and Dingle, 1980). They also showed that retinoic acid stimulated the breakdown of bovine nasal cartilage proteoglycan but that when plasminogen was not present then this resorption was partially abrogated. This suggested that the effect of retinoic acid on cartilage is caused partly by the activation of plasminogen. The authors also suggested that the residual breakdown observed when plasminogen is not present is due to a direct effect of plasminogen activator on cartilage which bypasses the effect of plasmin.

It is not clear whether retinoids also cause collagen breakdown as well as proteoglycan degradation in cartilage. In one report, treatment of chick limb-bone rudiments with excess retinol resulted in increased release of both hexosamine and hydroxyproline into the culture medium, suggesting that this is the case (Dingle et al., 1966). Treatment of porcine articular cartilage with retinol also resulted in the breakdown of both proteoglycan and collagen and was accompanied by the presence of collagenolytic activity produced by the cartilage (Jubb and Fell, 1980). Dingle et al., (1975), however, found an almost complete disappearance of proteoglycan from porcine articular cartilage in the presence of retinol but only a slight loss of collagen in some experiments.

1.1.6.2. Differentiation of mesenchymal cells

Retinoic acid inhibits chondrogenesis in mesenchymal cells derived from prechondrogenic limb buds of mouse embryos and differentiation of rat chondrogenic cell lines (Hassell et al., 1978, Lewis et al., 1978, Pennypacker et al., 1978, Lau et al., 1993). These cells fail to form cartilagenous nodules and do not synthesize the sulfated glycosaminoglycans characteristic of cartilage. The administration of vitamin
A to pregnant mice also interferes with chondrogenesis in the developing embryo (Kochhar, 1973).

Retinoids also affect the mature chondrocyte, inducing transformation into dedifferentiated fibroblastic cells which no longer synthesize type II collagen (Shapiro and Poon, 1976, Benya and Padilla, 1986). A recent report has also demonstrated that inhibition of the cartilage phenotype in rat epiphyseal chondrocytes is associated with positive regulation of metalloproteinase genes (Ballock et al., 1994).

1.1.6.3. Synthesis of extracellular matrix components
Retinoids can also influence glycosaminoglycan and proteoglycan production of mesenchymal cells although different cell types respond in different ways. Undifferentiated mesenchymal cells and mature chondrocytes display a decreased glycosaminoglycan synthesis (Vasan and Lash, 1975, Shapiro and Poon, 1976, Pennypacker et al., 1978) whereas fibroblasts, dermal and epidermal cells exhibit enhanced glycosaminoglycan synthesis when treated with retinoids (Jetten et al., 1979, Shapiro and Poon, 1978). However, a recent report has shown that retinoic acid reduces decorin gene expression in human skin fibroblasts (Kähäri et al., 1995). Retinoic acid has also been shown to decrease the production of type I collagen in human fibroblasts (Oikarinen et al., 1985, Krupsky et al., 1994).

1.1.6.4. Cell Proliferation
Many contradictory reports have appeared on the effect of retinoids on fibroblast proliferation. Murine and human fibroblasts respond to retinoid treatment with a decrease in exponential growth rate and a decrease in saturation density (Lacroix et al., 1981, Jetten et al., 1979, Schroder et al., 1982) while other reports using hamster, chick, human and murine fibroblasts have shown a stimulation of cell division in response to retinoic acid (Harper and Burgoon, 1982, Lasnitzki, 1955, Dicker and Rozengurt, 1979).
1.1.7. Therapeutic uses

The relatively simple chemical structure of retinoids has made them good candidates for drug development. However, their clinical use has been limited by the teratogenic action of retinoids and other side effects associated with retinoid toxicity including hair loss, mucocutaneous effects, bone lesions, elevated serum levels of cholesterol and triglycerides, hepatotoxicity and musculoskeletal effects (Kaplan and Haettich, 1991, Klinkhoff et al., 1989). Another problem is that continuous treatment of patients with retinoids such as all-trans-retinoic acid leads to the development of retinoid resistance due to a gradual reduction in plasma levels of retinoic acid (Muindi et al., 1992). This is thought to be caused by the induction of catabolic enzymes and increased expression of CRABPs leading to increased drug clearance (reviewed by Pemrick et al., 1994). A major challenge for drug discovery is to design a drug which circumvents these problems, either by designing novel drug delivery systems (e.g. liposomal encapsulation) (Drach et al., 1993) or by employing drugs which do not bind to CRABP such as 9-cis-retinoic acid.

Retinoids have been used in a number of diverse clinical situations which will not be discussed here; especially in oncology (e.g. acute promyelocytic leukaemia, skin cancer) and dermatology (e.g. psoriasis, acne); of particular interest to this thesis is the possible use of retinoids in the treatment of rheumatoid arthritis.

1.1.7.1. Rheumatoid arthritis

Rheumatoid arthritis is a proliferative and invasive disease in which synovial tissue consisting of immune lymphocytes, monocytes/macrophages, polymorphonuclear leucocytes and fibroblast-like cells organizes into a mass which invades and destroys articular cartilage, bone and tendons. The aetiology of rheumatoid arthritis is not understood, but a favoured hypothesis is that an unknown antigen elicits an immune response within the joints of genetically susceptible individuals. This immune response leads to a chronic inflammation and proliferation of the synovial lining tissue which then invades and destroys the joint cartilage. Interleukin-1 (IL-1) is well-
characterized for its ability to stimulate both synovial cell proliferation and connective tissue breakdown, the latter occurring because IL-1 induces the synthesis and secretion of large quantities of collagenase by the synovial cells (Mizel et al., 1981, Dayer et al., 1986). The collagenases are the only enzymes able to initiate breakdown of the interstitial collagens at neutral pH, and the role of these enzymes in the joint destruction accompanying rheumatoid arthritis is well recognized (Harris, 1985). IL-1 can also stimulate the production of prostaglandin E\(_2\) from rheumatoid synovial cells (Mizel et al., 1981, Dayer et al., 1986) and this contributes to disease progress by enhancing oedema and pain and modulating inflammation (Lewis, 1986).

Retinoids inhibit collagenase production by synovial cells (Brinckerhoff et al., 1980, Brinckerhoff & Harris, 1981) and antagonize the proliferative action of growth factors in cell culture (Brinckerhoff et al., 1985). This information suggests that retinoids may be of therapeutic use in the treatment of rheumatoid arthritis. However, as discussed above, retinoids have been shown to induce the loss of proteoglycan and (in some experiments) collagen from cartilage suggesting that they would probably exacerbate the disease.

Rheumatoid arthritis is an illness peculiar to humans, however, several animal models have been developed to help to understand the pathophysiology of the disease. Collagen-induced arthritis, streptococcal cell wall-induced arthritis and adjuvant arthritis are 3 commonly used rat models and the effect of retinoids (13-cis-retinoic acid or N-(4-hydroxyphenyl)-retinamide) on these diseases has been tested. Collagen-induced arthritis, essentially an autoimmune disease was exacerbated (Trentham & Brinckerhoff, 1982) while adjuvant arthritis, an inflammatory disorder with immunological aspects was suppressed (Brinckerhoff et al., 1983). Streptococcal cell wall-induced arthritis was also suppressed by retinoid treatment (Brinckerhoff et al., 1985). Collagenase production by adherent cells dissociated from the synovium of retinoid-treated animals was decreased compared to that in untreated rats in all disease states. Prostaglandin E\(_2\) production was suppressed in collagen-induced arthritis and in streptococcal cell wall-induced arthritis. The reason for the disparate effects of
retinoid treatment in the different models are unclear but could result from differences in the type and dosage of retinoid used or differences in the mediators of the disease in the various types of arthritis. The results encourage more research into this potential therapeutic use of retinoids but the potentially destructive effect of retinoids on cartilage must be taken into account in the design of retinoid-based drugs to treat this disease.

Retinoids, particularly etretinate have been shown to be effective in the treatment of psoriatic arthritis (Klinkhoff et al., 1989) although a major drawback was the high incidence of side effects.

1.1.7.2. Epidermolysis Bullosa

Both all-trans-retinoic acid and 13-cis-retinoic acid have been shown to inhibit the production of collagenase and gelatinase in skin fibroblast cultures derived from normal skin and from patients with recessive dystrophic epidermolysis bullosa (Bauer et al., 1982, 1983). These data suggest that retinoids may be useful therapeutic agents in recessive dystrophic epidermolysis bullosa, a disease in which pathological blistering is partly related to collagen destruction caused by excessive synthesis of collagenase.

1.1.8. Summary

Vitamin A-active substances or retinoids are compounds other than carotenoids that exhibit some or all of the biological activities of trans-retinol. Vitamin A has been implicated in a wide range of biological functions including reproduction, growth, differentiation and vision.

Vitamin A derived from dietary retinyl esters and carotenoids is present in the general circulation mainly as retinol bound to retinol-binding protein at a concentration of approximately 2μM. Excess vitamin A is stored in the liver. Following uptake by cells, retinol serves as the metabolic precursor of biologically active retinoids including all-trans- and 9-cis-retinoic acid. The uptake, storage, remobilization and
metabolism of retinoids is facilitated by the cellular retinoid-binding proteins. These also serve as a cellular reservoir of retinoids and regulate the availability of biologically active retinoids to the nuclear receptors.

The nuclear retinoid receptors are responsible for eliciting the response of target cells to the hormone-like effects of retinoids. These proteins belong to the steroid/thyroid hormone nuclear receptor superfamily. Binding of retinoic acid to a dimerized receptor converts it into an active transcription factor that is able to regulate transcription from gene promoters containing retinoic acid response elements (RAREs and RXREs). Two classes of retinoid receptors have been characterized; the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). All-trans-retinoic acid and 9-cis-retinoic acid bind to and activate RARs while the RXRs are only bound and activated by 9-cis-retinoic acid. The RARs bind to RAREs as heterodimers with members of the RXR family whereas the RXRs can function independently and bind as homodimers to RXREs.

Retinoids have been shown to have some efficacy in suppressing experimentally-induced arthritis in animals; however in apparent contradiction to this, retinoids can also dramatically resorb cartilage in vitro. However, it may be possible to design a synthetic retinoid in which the beneficial effects are enhanced and the undesirable effects on cartilage resorption are diminished.
1.2. GROWTH FACTORS

1.2.1. Basic fibroblast growth factor

1.2.1.1. Definition and structure

Basic fibroblast growth factor (bFGF) is a member of a family of fibroblast growth factors (FGFs) which are involved in soft tissue growth and regeneration. The family of FGFs presently includes 9 members (FGFs-1 to -9) that share a varying degree of homology at the protein level and with one exception have a similar broad mitogenic spectrum including a variety of mesodermal and neuroectodermal cells. Because they all bind to heparin, heparan sulphate proteoglycans and glycosaminoglycans, they have also been called the heparin-binding growth factors.

Human bFGF (FGF-2) is expressed in 4 forms (Mr 18 000, 22 000, 22 500 and 24 000) arising from initiation of translation at alternative codons. The high Mr forms of bFGF contain the same amino acid sequence as the 18 000-Mr form (which has 155 amino acids) but have additional N-terminal extensions of varying lengths (Florkiewicz and Sommer, 1989, Prats et al., 1989). The different forms of bFGF seem to be correlated with differences in subcellular distribution. The 18 000-Mr form lacks a typical signal sequence for secretion and is retained in the cell, primarily in the cytosol, or is exported onto the cell surface, but is not released into the surrounding culture medium. The higher Mr forms are found in the nuclear and ribosomal fractions (Renko et al., 1990, Florkiewicz et al., 1991). The higher Mr forms appear to contain a nuclear translocation sequence in the N-terminal extensions (Bugler et al., 1991, Quarto et al., 1991). In addition to endogenous nuclear forms of bFGF, translocation of exogenous 18 000-Mr bFGF to the nucleus has also been described (Bouche et al., 1987, Baldin et al., 1990). The significance of nuclear forms of bFGF is not yet clearly understood but may be involved in cell cycle progression and growth regulation.
1.2.1.2. Synthesis and secretion

The cellular source of bFGF is uncertain. It has been identified in many tissues and organs and also in serum (Baird et al., 1986). Expression of mRNA (messenger ribonucleic acid) for bFGF is particularly high in the brain. It is synthesized by cultured fibroblasts, endothelial cells, glial cells and smooth muscle cells (Connolly et al., 1987, Gospodarowicz et al., 1988, Hatten et al., 1988, Moscatelli et al., 1986) and since these cell types are ubiquitous, they may be the source of bFGF in organs. It is presumed to be produced at low levels by the cells and to subsequently accumulate in the extracellular matrix. However, the lack of a signal sequence on the bFGF molecule makes its secretion from cells extremely inefficient. It has therefore been proposed that bFGF is released only from dead or dying cells as a response to tissue destruction or from small nonlethal disruptions of the plasma membrane (McNeil et al., 1989). However, there is increasing evidence that healthy cells do release small quantities of bFGF despite the lack of signal sequence (Mignatti et al., 1991) although the mechanism of this release is not understood.

1.2.1.3. Receptors and signal transduction

bFGF binds to 2 types of cell surface receptors. One of these is a low affinity receptor, which is widely distributed with a large number of sites per cell and with a binding affinity of 2 to 10nM. These receptors are heparan sulphate proteoglycans (HSPGs). The other type of receptor are the high affinity receptors (Kd ~ 10-100pM) with a lower number of sites per cell and a Mr of 110 000-160 000. These receptors have intrinsic tyrosine kinase activity and form a family of receptors known as receptor tyrosine kinase subclass IV. Both high and low affinity binding sites for bFGF occur on fibroblasts, endothelial cells, myoblasts and tumour cells (reviewed by Burgess and Maciag, 1989).

There are at least 5 different high affinity FGF receptors called flg (fms-like gene), bek (bacterial expressed kinase), FGFR-3, (FGF receptor 3) FGFR-4 (FGF receptor 4) and flg-2 (fms-like gene-2) (reviewed by Jaye et al., 1992). Flg and bek are widely
expressed while the expression of the other 3 is much more limited. They share a
common structure of a signal peptide, 3 immunoglobulin-like loops flanked by
cysteines and a hydrophobic transmembrane region. The extracellular domain is
glycosylated and the sugar residues are important for ligand binding (Feige and Baird,
1988). The intracellular domain includes the catalytic tyrosine kinase domain and a
long C-terminal tail. cDNAs (complementary deoxyribonucleic acid) encoding
soluble receptors, truncated receptors, intracellular receptors and other variants have
also been described (Jaye et al., 1992). Receptor isoforms generated by alternative
splicing result in receptor variants with different ligand-binding specificities and
affinities (Friesel and Maciag, 1995). It is possible that the secreted receptors play a
significant role in secretion, binding or storage of FGFs extracellularly and that the
cytoplasmic receptors may be activated upon binding of FGFs intracellularly.

The various high affinity receptors appear to have overlapping specificity for ligand
binding but with preferential affinity for 1 ligand or another. flg appears to bind
acidic FGF (FGF-1) and bFGF with similar high affinity and FGF-4 with about 15-
fold lower affinity while bek binds all 3 with similar high affinity (Dionne et al.,
1990, Mansukhani et al., 1990). A detailed analysis of binding specificities of FGFR-
3, -4 and flg-2 is not yet available but it has been shown that FGFR-3 is activated by
both acidic and bFGF while FGFR-4 binds to acidic FGF but not to basic FGF.

The interaction of bFGF with the heparan sulphate moieties of the low affinity HSPG
receptors or with soluble heparin or extracellular matrix heparan sulphate increases
the affinity of the growth factor for the high affinity receptors (reviewed by Basilico
and Moscatelli, 1992). Some studies have shown that the HSPGs are essential for the
binding of bFGF to the high affinity receptors and the generation of a biological
response. It is currently debatable whether FGFs can activate FGFRs in the absence
of any proteoglycan. Cells also use the extracellular matrix as a temporary storage
site for bFGF and can draw on this reserve of growth factor to greatly increase their
response to a brief exposure to bFGF (Flaumenhaft et al., 1989). In addition, the
HSPGs may present multiple FGF molecules to receptors and thus facilitate receptor
dimerization. Binding of bFGF to heparin, heparan sulphate and to the cell membrane HSPGs protects the growth factor from thermal or acid denaturation and proteolytic degradation (Gospodarowicz and Cheng, 1986, Saksela et al., 1988, Sommer and Rifkin, 1989). However, in some cases, heparan sulphate has an inhibitory effect on the biological activity of bFGF possibly by sequestering it in an inactive or unavailable form (Damon et al., 1988). This may prevent extracellular matrix-bound bFGF from interacting with cell membranes except in special circumstances such as extracellular matrix breakdown. Chondroitin sulphate can also interact with and modify the activity of bFGF (Damon et al., 1988).

The high affinity FGF receptors are believed to dimerize following ligand binding (Ullrich and Schlessinger, 1990) and different FGF receptors can create heterodimers (Jaye et al., 1992). Binding of ligand results in activation of receptor tyrosine kinase activity and autophosphorylation of the cytoplasmic domain by an intermolecular mechanism occurring between dimerized receptors (Bellot et al., 1991).

Receptor autophosphorylation is followed by the phosphorylation and resultant activation of various intracellular substrates. The intracellular substrates interact with specific phosphorylated tyrosine residues in the cytoplasmic domain of the receptor (Jaye et al., 1992). The best characterised of these is phospholipase C\(\gamma\) (Rhee et al., 1989, Burgess et al., 1990, Wahl and Carpenter, 1991) which hydrolyses phosphatidylinositol 4,5-bisphosphate to produce inositol trisphosphate and diacylglycerol. Inositol trisphosphate acts as a second messenger molecule to liberate stored calcium from the endoplasmic reticulum and thereby activates calcium-requiring enzymes or processes. Diacylglycerol is an activator of protein kinase C. Protein kinase C activation is believed to lead to the activation of c-jun (reviewed by Angel and Karin, 1991).

Activated FGF receptors also interact with the regulatory subunit of phosphatidylinositol 3'-kinase (Mason, 1994). This enzyme catalyses the addition of a phosphate group to position 3 of inositol in phosphatidylinositol forming a unique phosphoinositide which cannot be hydrolysed by any known phospholipase. In vivo,
the preferred substrate appears to be phosphatidylinositol 4,5-bisphosphate which is converted to phosphatidylinositol 3,4,5-trisphosphate (Claesson-Welsh, 1994). It is thought that these phospholipids may therefore serve an alternate function such as cofactors for membrane-bound enzymes or that they may have a role in protein sorting.

Activated FGF receptors also interact with Src which is thought to lead to the eventual activation of mitogen-activated protein kinase (MAP kinase) a serine/threonine kinase via several intermediary proteins in the following signal transduction cascade (reviewed by Friesel and Maciag, 1995). Activated Src results in the tyrosine phosphorylation of the adaptor protein SHC (SH2 domain and collagen-like). Further propagation of this signalling cascade results from the binding of SHC to grb 2 (growth factor receptor bound 2) which appears in a stable complex with son of sevenless (SOS) a guanine nucleotide exchange factor. This results in the conversion of the membrane-bound protein Ras from an inactive GDP-bound state to an active GTP-bound state. Ras activation culminates in the activation of MAP kinase via Raf and MEK (MAP kinase/extracellular signal-regulated kinase kinase). MEK phosphorylates MAP kinase on threonine and tyrosine residues (Crews and Erikson, 1992). MAP kinase then phosphorylates specific transcription factors including c-jun and c-myc and results in the expression of Ras-inducible genes including c-fos. This scheme of events is illustrated in figure 1.3 overleaf. The Ras pathway is one of the most broadly implicated avenues of nuclear signalling from growth factor receptors. MAP kinase is also believed to phosphorylate S6 kinase which in turn phosphorylates ribosomal protein S6 (Pelech et al., 1986, Sturgill et al., 1988). This sequence of events may alter ribosomal function and the translation of proteins. Differences occur in the degree to which the various FGF receptors are able to activate these various cellular substrates (Mason, 1994).

Binding of bFGF to the high affinity receptors also causes rapid downregulation of the receptor with subsequent internalization of the ligand (reviewed by Burgess and Maciag, 1989). Internalized ligand is eventually degraded in the lysosomes but is
Figure 1.3. Intracellular growth factor signalling pathways

DAG - diacylglycerol  
grb 2 - growth factor receptor bound 2  
I-1,4,5PPP - inositol 1,4,5-trisphosphate  
MAP-K - mitogen activated protein kinase  
MEK-1 - MAP kinase/extracellular signal-regulated kinase kinase-1  
P - inorganic phosphate  
PI-3' kinase - phosphatidylinositol 3'-kinase  
PI-4,5PP - phosphatidylinositol 4,5-bisphosphate  
PI-3,4,5PPP - phosphatidylinositol 3,4,5-trisphosphate  
PKC - protein kinase C  
PLC - phospholipase Cγ  
SOS - son of sevenless  
shc - SH2 domain and collagen-like  
TI - topoisomerase  
TF - transcription factor  
TK - tyrosine kinase  

25
remarkably resistant to degradation. bFGF is also translocated to the nucleus in a receptor-dependent manner as discussed above, where it has been shown to bind to casein kinase II (Mason, 1994, Friesel and Maciag, 1995). Casein kinase II is found in both the nucleus and the cytoplasm and is capable of phosphorylating a number of nuclear proteins including enzymes that modify DNA topology and several transcription factors such as c-myc (reviewed by Carpenter and Cohen, 1990).

Later effects of stimulation of cells with bFGF include the transient expression of c-fos mRNA and c-myc mRNA (Müller et al., 1984, Kruijer et al., 1984) and induction of mRNA for actin (Rybak et al., 1988).

1.2.1.4. Effects *in vitro* and role *in vivo*

Many of the effects of bFGF are relevant to connective tissue turnover. bFGF is a potent mitogen for a variety of cells including fibroblasts, chondrocytes, osteoblasts and endothelial cells (Gospodarowicz et al., 1987). Upon the re-initiation of growth in quiescent cells, there is a prereplicative lag phase exceeding the length of the G phase of dividing cells before the commencement of the S phase in which DNA replication occurs. This lag phase is known as the G phase (Baserga et al., 1973) and growth factors are required both for the transition from G to G and for progression through G until the last hours before the onset of the S phase. These 2 separate cell cycle events are termed competence and progression respectively (Pledger et al., 1977). Competence is induced by factors such as platelet-derived growth factor in responsive cells while progression requires the presence of further factors present in plasma such as epidermal growth factor and somatomedins (Pledger et al., 1977, Stiles et al., 1979). bFGF has been shown to act as an inducer of cellular competence (Stiles et al, 1979).

bFGF has a major role in angiogenesis. It induces an invasive phenotype in cultured endothelial cells enabling them to penetrate basement membranes via the stimulation of plasminogen activator and collagenase (Mignatti et al., 1989, Presta et al., 1986). bFGF also stimulates the proliferation of endothelial cells (Presta et al., 1986) and has
been shown to stimulate angiogenesis in vivo (e.g. Hayek et al., 1987). bFGF has also been shown to accelerate wound healing (e.g. McGee et al., 1988). bFGF is known to modulate proteoglycan turnover in cartilage although opposite effects are seen depending upon the concentration of bFGF used and the age of the cartilage (Osborn et al., 1989, Sah et al., 1994).

bFGF has also been implicated in the pathology of rheumatoid arthritis by virtue of its ability to stimulate the proliferation of synovial fibroblasts and to act as an angiogenic factor (new blood vessel formation and hyperplasia of the synovial connective tissues are prominent features of rheumatoid synovium). Synovial fibroblasts have been shown to synthesize, release, bind and respond to bFGF in vitro (Melvynk et al., 1990). bFGF has been detected by immunohistochemistry in rheumatoid arthritis synovial tissue associated with monocytes and macrophages and with areas of new blood vessel formation. mRNA for bFGF is also present in synovial tissue suggesting that bFGF is synthesized in situ (Qu et al., 1990).

bFGF has also been shown to regulate the production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in connective tissue cells. Discussion of these effects is deferred to later in this chapter.

1.2.2. Epidermal growth factor

1.2.2.1. Definition and structure

Epidermal growth factor (EGF) is a member of a family of EGF-like molecules, all of which are encoded by distinct genes. It consists of a single polypeptide chain with a Mr of 6 000. The other members of this family are transforming growth factor-α, the pox virus growth factors and amphiregulin (Carpenter and Wahl, 1990). These EGF-like molecules are characterised by high affinity binding to the EGF receptor, production of mitogenic responses in EGF sensitive cells and the presence in the primary structure of 6 cysteines which form 3 disulphide bonds (Carpenter and Wahl, 1990).
Studies investigating the 3-dimensional structure of EGF have shown that the polypeptide has 1 major and 1 minor anti-parallel β-sheet structures, but little or no α-helical conformation therefore giving it a very compact structure (Cooke et al., 1987, Mayo et al., 1989). Site-directed mutagenesis has demonstrated that the disulphide bonds are essential for biological activity (Carpenter and Wahl, 1990).

1.2.2.2. Synthesis and secretion

EGF is found in almost all body fluids under normal physiological conditions (Carpenter and Zendegui, 1986) and is produced by multiple cell types. It is synthesized as a large precursor molecule (Gray et al., 1983, Scott et al., 1983, Carpenter and Zendegui, 1986) with a Mr of approximately 130 000 which in addition to the sequence of EGF also contains a signal peptide, 7 EGF-like units and a hydrophobic sequence near the C-terminus characteristic of an integral membrane protein. Studies have demonstrated that prepro-EGF can exist as a glycosylated biologically active membrane protein or as a soluble protein (Mroczkowski et al., 1989). It has been suggested that proteolytic activity at the cell surface may give rise to free growth factor (Carpenter and Zendegui, 1986). EGF is capable of binding to and activating membrane receptors and inducing biological responses while in the precursor form (Mroczkowski et al., 1989). It is also possible that the precursor may function as a receptor for an unidentified ligand (Carpenter and Zendegui, 1986). The means by which EGF is processed from the precursor molecule is not known.

1.2.2.3. Receptors and signal transduction

Epidermal growth factor receptors are present on all cell membranes except for cells of the haematopoietic system. The EGF receptor has a molecular weight of 170 000 and is glycosylated (Cummings et al., 1985). It is a member of the receptor tyrosine kinase subclass I family of receptors. A single hydrophobic membrane anchor sequence separates an extracellular ligand-binding domain with 2 cysteine-rich regions from a cytoplasmic domain that encodes an EGF-regulated tyrosine kinase
The extracellular domain of the receptor binds EGF and EGF-like ligands with high affinity. The region between the 2 cysteine-rich clusters in the extracellular portion of the receptor is thought to be involved in ligand binding (Lax et al., 1988).

Stimulation with EGF results in the autophosphorylation of 4 tyrosine residues located in the cytoplasmic portion of the receptor close to the C-terminus (Downward et al., 1984, Margolis et al., 1989) although it is likely that other tyrosines are phosphorylated as well. EGF is thought to induce oligomerization of the receptors which is coupled to intermolecular phosphorylation (Schlessinger, 1988) although it is possible that the mechanism is intramolecular.

Several intracellular signalling molecules which are thought to be involved in EGF signal transduction have been identified. These include phospholipase Cγ (Meisenhelder et al., 1989), phosphatidylinositol 3'-kinase, GAP (an activating protein for the GTPase activity of Ras leading to the inactivation of Ras) Raf-1 (Morrison et al., 1988) and MAP kinase (Rossomando et al., 1989) (see figure 1.3.) (reviewed by Carpenter and Cohen, 1990). Casein kinase II is also phosphorylated and activated by EGF by an unknown mechanism (Ackerman and Osheroff, 1989, Ackerman et al., 1990). Localization studies show that casein kinase II is found in both the nucleus and the cytoplasm and phosphorylation studies indicate that casein kinase II phosphorylates and activates a range of nuclear proteins including topoisomerases and transcription factors (Carpenter and Cohen, 1990).

Other effects of EGF stimulation on cells include increased alkalinization of the cytoplasm due to Na⁺/H⁺ exchange, increased K⁺ transport, stimulation of glucose and
amino acid transport, increased glycolytic activity, increased protein and RNA synthesis, expression of c-fos c-jun and c-myc and ultimately DNA synthesis and mitosis (reviewed by Fox et al., 1982).

The formation of EGF-receptor complexes on the cell surface is followed by clustering of the receptors, rapid internalization and degradation of the ligand and receptor in the lysosomes. No recycling of receptors back to the cell surface appears to occur following internalization. The role of this is uncertain but it may be to limit the response of the cells to EGF. Internalization and degradation of EGF receptors also occurs in the absence of EGF followed by new receptor synthesis but at a reduced rate compared to when ligand is present (Carpenter and Cohen, 1976, Stoscheck and Carpenter, 1984, reviewed by Fox et al., 1982).

1.2.2.4. Effects in vitro and role in vivo

EGF stimulates the growth of epithelial, epidermal and mesenchymal cells and stimulates the differentiation and regeneration of epithelial tissues (reviewed by Carpenter and Cohen, 1979, Horn and Maisel, 1992). EGF stimulates wound healing by accelerating epithelialization, neovascularization and collagen accumulation (reviewed by Horn and Maisel, 1992). It is chemotactic for fibroblasts, endothelial and epithelial cells and also stimulates the proliferation of all these cell types (reviewed by Horn and Maisel, 1992). It acts as a 'progression factor' (see above) in regulation of the cell cycle to stimulate mitosis (Stiles et al., 1979).

1.2.3. Platelet-derived growth factor

1.2.3.1. Definition and structure

Platelet-derived growth factor (PDGF) was discovered in 1974 when it was observed that material released from platelets could stimulate the growth of cells in culture and was the principal source of mitogens present in whole blood serum (Ross et al., 1974, Kohler and Lipton, 1974). PDGF is a 30 000-Mr polypeptide connective tissue
growth factor composed of 2 related chains, A and B of about equal size which are disulphide-linked (Heldin et al., 1985, Ross et al., 1986). It is a glycosylated basic protein which is extremely stable to heat, pH and denaturing agents due to the presence of a large number of disulphide bonds (Stiles, 1983, Raines and Ross, 1985). The dimer structure is important for biological activity since reduction irreversibly inactivates PDGF. All 3 dimeric isoforms of the 2 chains (AA, AB and BB) have been isolated from natural sources (Bowen-Pope et al., 1989). Other PDGF-like polypeptides have also been detected (e.g. Matsuoka and Grotendorst, 1989).

Both the A and the B chains are synthesized as precursor molecules that undergo proteolytic processing after synthesis and dimerization (Betsholtz et al., 1986, Josephs et al., 1984, Östman et al., 1988). There are 2 forms of the A chain precursor due to differential splicing of the mRNA transcript (Bonthron et al., 1988). Both the A chain and the B chain precursors contain signal sequences and PDGF-AA, PDGF-AB and PDGF-BB accumulate in the extracellular space as 30 000-Mr dimers. However, relatively small amounts of the BB dimer are secreted and a 24 000-Mr form of PDGF-BB remains associated with the cell (Östman et al., 1988).

The mature parts of the 2 chains show about 60% identity between the A and B chain with perfect conservation of the 8 cysteine residues. The 2 subunits of the dimer are joined by 2 disulphide bonds encompassing the second and fourth cysteine residues such that the second residue bridges with the fourth in the other chain. The remaining cysteine residues form intrachain disulphide bonds. Each subunit is folded into 2 sets of twisted anti-parallel pairs of β-strands, thus forming 3 loops at each end of the molecule (Oefner et al., 1992). The receptor binding epitopes are thought to be located in these loop regions.

1.2.3.2. Synthesis and secretion

Although PDGF was first isolated from human platelets, it has since been shown to be produced by a number of cell types including monocytes, macrophages, endothelial
cells, smooth muscle cells, mitogen-stimulated fibroblasts and many neoplastically transformed or tumour cells (reviewed by Heldin et al., 1993).

Normal human plasma contains undetectable levels of PDGF and the response of connective tissue cells in the body is likely to depend upon local synthesis and secretion rather than on circulating levels. Expression of the PDGF genes may be regulated by specific factors released locally by activated cells; for example it has been shown that PDGF A chain gene expression is stimulated by transforming growth factor-β and by PDGF itself in human fibroblasts (Paulsson et al., 1988). The supply of active PDGF is also regulated by the presence of intracellular PDGF which is released in response to stimulation such as in the case of platelets. These cells store PDGF in their α-granules and release it in response to stimuli such as thrombin (Witte et al., 1978).

PDGF has an extremely short half-life in the circulation (Bowen-Pope et al., 1984) and this may be due to its highly cationic and hydrophobic nature (Raines and Ross, 1985) which causes nonspecific binding to matrix proteins such as acidic glycosaminoglycans. However, in vitro studies show that PDGF continues to be available as a mitogen when bound to extracellular matrix components (Smith et al., 1982) and this effect in vivo could cause a local increase in its concentration and/or prolong its activity. PDGF can also bind specifically to plasma proteins (Raines and Ross, 1985) which may prevent it from binding to its receptors thus limiting the activity of the growth factor.

1.2.3.3. Receptors and signal transduction

High affinity receptors of Mr 164 000-185 000 for PDGF have been found on fibroblasts, smooth muscle cells and glial cells but not on epithelial or blood cells (Heldin et al., 1985). Two receptors for PDGF have been identified: α, which binds all 3 isoforms of PDGF with high affinity, and β, which binds only PDGF-BB with high affinity although PDGF-AB is bound with low affinity (Hart and Bowen-Pope, 1990). Fibroblasts express both receptor types. Receptor dimerization appears to be
necessary for receptor activation and signal transduction in response to PDGF binding (Williams, 1989a, Hammacher et al., 1989, Hart and Bowen-Pope, 1990) The dimeric isoform of the PDGF ligand determines the α/β-receptor subunit combination of the dimer suggesting that each subunit in the dimeric ligand binds 1 receptor molecule. PDGF-AA will bind only to α-α receptor dimers, whereas PDGF-AB will bind to either α-α or α-β dimers and PDGF-BB will bind to any α- and/or β-receptor dimer combination (Hart and Bowen-Pope, 1990). This means that the level and ratio of subunit expression by a cell will in turn regulate the responsiveness of the cell to the 3 forms of PDGF. Human fibroblasts bind PDGF-BB and -AB well but PDGF-AA poorly.

PDGF receptors are members of the receptor tyrosine kinase subclass III family of growth factor receptors (Ullrich and Schlessinger, 1990) which have a glycosylated ligand-binding domain on the outside of the cell membrane with 5 immunoglobulin-like domains and an effector domain on the inside with a tyrosine kinase sequence interrupted by a kinase insert domain (Williams, 1989a, Williams, 1989b). The binding of PDGF to its receptor stimulates a series of intracellular changes and ultimately leads to DNA synthesis and cell division (reviewed by Remmers et al., 1991). PDGF binding is rapidly followed by receptor autophosphorylation or transphosphorylation between dimers. Ligand binding, receptor dimerization and accompanying conformational changes allow a basal kinase activity to phosphorylate a critical tyrosine residue, thereby 'unlocking' the kinase, leading to full enzymatic activity directed toward other tyrosine residues in the receptor molecules and towards other substrates for the kinase (Claesson-Welsh, 1994). The tyrosine phosphorylation sites in the receptor create binding sites for downstream signal transduction molecules, which in many cases are also substrates for the kinase. This leads to a physical association of the receptor with, and phosphorylation of signalling molecules including the tyrosine kinase Src and its close relatives Fyn and Yes (Eisenman and Cooper, 1995) phospholipase Cγ (Meisenhelder et al., 1989, Williams, 1989a), phosphatidylinositol 3'-kinase, Raf, and GAP (Heldin and Westermark, 1990,
Williams, 1989a). Different signalling molecules are believed to interact with specific phosphotyrosine residues in the cytoplasmic part of the receptor (Peters et al., 1992). The Src family kinases have recently been implicated in the induction of myc gene expression and in PDGF-induced mitogenic signaling (Claesson-Welsh, 1994, Eisenman and Cooper, 1995). The Ras pathway is activated by PDGF receptors but in a slightly different way to the pathway shown for bFGF (figure 1.3) (Claesson-Welsh, 1994). PDGF receptors interact directly with grb 2 which is found in a stable complex with SOS. Complex formation with the receptor serves to bring grb 2-SOS to the membrane in close contact with Ras resulting in its activation. In addition PDGF receptors also interact with SHC and with protein tyrosine phosphatase 1D; both of these in turn bind to Grb 2-SOS leading to activation of Ras. Activation of Ras is likely to represent a major pathway for PDGF-induced mitogenesis.

Early responses to high affinity PDGF binding also include reorganization of actin filaments causing morphological changes (Hammacher et al., 1989), intracellular alkalization (Cassel et al., 1983), fluxes in intracellular calcium ion levels, migration of cells towards the growth factor and receptor downregulation by internalization and degradation which allows the cells to regulate their responsiveness to further stimulation (Williams, 1989b). Receptor internalization and degradation also occurs in the absence of ligand but at a slower rate. Protein kinase C activation and calcium release which occur as a result of activation of phospholipase C (see above) are thought to be important in transduction of the mitogenic signal in cells following PDGF stimulation.

Early effects of PDGF binding include the induction of mRNA from c-fos and c-myc genes (Kruijer et al., 1984, Müller et al., 1984), chemotaxis, stimulation of cholesterol, lipid, and glycosaminoglycan synthesis, an increase in protein synthesis and appearance of new proteins. Most of these effects are related to, or required for increased mitosis. Later effects include increased collagen and collagenase synthesis, DNA synthesis, and cell division. PDGF is also known to lower the binding of EGF.
to cells; this is mediated via binding of PDGF to its own receptor and is due to a decreased affinity of the EGF receptor for its ligand (Bowen-Pope et al., 1983).

1.2.3.4. Effects in vitro and role in vivo

PDGF is a key mitogen for mesenchymal cells such as fibroblasts, smooth muscle cells and glial cells and is a potent 'competence factor' (see above) for these cells. It has no direct effect on the growth of cells of epithelial or endothelial origin (Heldin et al., 1985).

PDGF plays a key role in wound healing. Its release from the platelets in response to wounding and from injured endothelium causes the migration of monocytes and neutrophils and later fibroblasts to the site of injury. It subsequently stimulates fibroblast proliferation and the production of collagen and collagenase by the fibroblasts. After the initial release of PDGF by the platelets, PDGF continues to be released in the wound area by activated monocytes and macrophages so that the process of repair continues. Clinical trials have shown that PDGF can promote wound healing in patients with chronic pressure ulcers (Robson et al., 1992).

Increasing evidence indicates that PDGF is involved in the pathogenesis of rheumatoid arthritis (reviewed recently by Remmers et al., 1991). mRNA transcripts for PDGF-A and PDGF-B, immunoreactive polypeptides and PDGF receptors are present in rheumatoid arthritis synovia. Levels of expression are significantly higher in rheumatoid arthritis synovia than in osteoarthritis synovia and correlate with the extent and intensity of mononuclear cell infiltration. These and other observations strongly support the view that PDGF is involved in stimulating the proliferative and invasive phenotype of rheumatoid arthritis synovial fibroblasts. PDGF produced primarily by platelets and infiltrating activated monocytes and macrophages could drive fibroblast proliferation and collagenase secretion and in conjunction with other growth factors stimulate these cells to display an invasive phenotype resembling transformed cells. PDGF may also contribute to the recruitment and activation of monocytes at the site of inflammation.
1.2.4. Transforming growth factor-β

1.2.4.1. Definition and structure
Transforming growth factor-β (TGF-β) is the prototypic member of the TGF-β superfamily of growth and differentiation factors (Massagué, 1990). Based on their structural and biological similarities, the members of this family can be subdivided into 3 groups which are the TGF-βs, the activins and inhibins and the bone morphogenetic proteins.

Several distinct TGF-βs have been identified (Massagué, 1990) including 3 closely related mammalian forms designated TGF-β1, 2 and 3. TGF-β4 and TGF-β5 have been identified in chicken and *Xenopus laevis* respectively. Mammalian forms of TGF-β4 and TGF-β5 have not yet been described. It has been suggested that the different mammalian isoforms of TGF-β have distinct activities *in vivo* although, with a few exceptions, cells respond almost identically to all forms of TGF-β *in vitro* (Roberts and Sporn, 1993). However, it has been shown that the different forms vary in their affinity for TGF-β signalling receptors with the TGF-β1 and TGF-β3 forms having a higher affinity than the TGF-β2 form (Cheifetz et al., 1987, Franzén et al., 1993).

The mature biologically active forms of TGF-β are composed of homodimers of 2 12-15 000-M_r subunits linked by a single disulphide bond. Only the nonreduced dimeric form of the peptide is biologically active. Although TGF-βs exist primarily as homodimers, heterodimers such as TGF-β1.2 have also been identified (Cheifetz et al., 1987). The polypeptide originally described as TGF-β is TGF-β1 (Cheifetz et al., 1987) and consists of 2 identical β1 subunits of 112 amino acids (Sporn et al., 1986) and this is the most abundant form in mammals (Roberts and Sporn, 1993). The X-ray crystallographic structure of TGF-β2 (Daopin et al., 1992) and the NMR structure of TGF-β1 (Archer et al., 1993) show that TGF-β monomers have an elongated...
structure, with 1 cysteine participating in an interchain disulphide bond, while the other 8 are involved in intrachain disulphide bonds.

1.2.4.2. Synthesis and secretion
Essentially, all cells synthesize one or another isoforms of TGF-β (Sporn et al., 1987). The most abundant sources are the platelets and bone (Assoian et al., 1983, Seyedin et al., 1985).

Following cleavage of the signal peptide, TGF-β is released as a biologically inactive precursor consisting of a dimer of the N-terminal pro-region, or latency-associated peptide, associated with the dimeric C-terminal mature protein (Gentry et al., 1987, Gentry et al., 1988). In some cases, this complex also contains a latent TGF-β-binding protein (LTBP) of 125-160 000-Mr (Miyazono et al., 1993). LTBP is not essential for maintaining TGF-β latency and its function remains unclear. A second LTBP called LTBP-2 has recently been characterized (Moren et al., 1994). Release of the biologically active TGF-β dimer can be achieved in vitro by a variety of means including the enzymatic activity of plasmin, cathepsin D and glycosidases or alternatively by chemical means such as heating or extreme pH (Miyazono et al., 1993). The in vivo relevance of these various means of activating TGF-β is not yet clear but local areas of low pH or increased proteolytic activity associated with different physiological and pathological processes may be important. The activation of TGF-β is important in the control of TGF-β activity in vivo due to the widespread occurrence of TGF-β and TGF-β receptors.

1.2.4.3. TGF-β receptors
TGF-β interacts with high affinity (in the subnanomolar range) with several distinct membrane proteins. The receptors which are most widely expressed are the type I, type II and type III (betaglycan) receptors (Massagué, 1990).

TGF-β receptors type I and type II are glycoproteins of approximately 53 and 70 000-100 000-Mr respectively (Franzén et al., 1993) and are present at low levels in most
mammalian cells. So far, only 1 TGF-β type II receptor has been identified while 2 TGF-β type I receptors have been characterised. In contrast to receptors for other growth factors, TGF-β receptors show either none or limited downregulation in response to high concentrations of ligand (Massagué and Like, 1985, Frolik et al., 1984).

The type III (betaglycan) receptor is a 280 000-Mr proteoglycan and is widely although not ubiquitously expressed. Other TGF-β-binding membrane-associated proteins such as the type IV and type V receptor display a much more restricted expression pattern suggesting a cell-specific function. (Cheifetz et al., 1988a, O'Grady et al., 1992).

The TGF-β type I and type II receptors are both transmembrane proteins composed of a short cysteine-rich extracellular domain, a single membrane-spanning region and an intracellular kinase domain with predicted specificity towards serine/threonine residues. The type II receptor has a longer C-terminal tail than the type I receptor (Lin et al., 1992, Franzén et al., 1993). The type I receptor is a member of a subfamily of closely related serine/threonine kinase receptors that display 60-90% identity among their kinase domains (ten Dijke et al., 1993).

The betaglycan (type III) receptor is a membrane-anchored proteoglycan with a large extracellular domain containing heparan sulphate and chondroitin sulphate glycosaminoglycan chains (Cheifetz et al., 1988b, López-Casillas et al., 1991, Wang et al., 1991, Andres et al., 1991). Betaglycan is able to bind to all 3 of the mammalian TGF-β isoforms with high affinity (Andres et al., 1991); the glycosaminoglycan chains are not required for this interaction (Cheifetz et al., 1988b, López-Casillas et al., 1991). The extracellular domain is followed by a single transmembrane segment and a short cytoplasmic region rich in serines and threonines but with no obvious signalling structure.

It appears that the role of membrane-bound betaglycan is to bind the TGF-β and to present it to the type II signalling receptor. (Moustakas et al., 1993, López-Casillas et al., 1993). Betaglycan increases the affinity of TGF-β for the type II receptor and this
is particularly important in the case of TGF-β2 which has a lower affinity than TGF-β1 and TGF-β3 for the type II receptor (Lin et al., 1992). The type II receptor forms a stable ternary complex with TGF-β and betaglycan (López-Casillas et al., 1993, Moustakas et al., 1993).

TGF-β signalling takes place via the formation of a heteromeric receptor complex composed of both a type I and type II receptor (Wranâ et al., 1992, Franzén et al. 1993, Inagaki et al., 1993). The type II receptor is required for ligand binding which is mediated by betaglycan as discussed above while the type I receptor generates the signal (Wranâ et al., 1992). The type II receptor is a constitutively active kinase and is able to phosphorylate the type I receptor on serine and threonine residues following ligand binding and the formation of the heteromeric complex. This then results in the generation of an intracellular signal by the type I receptor (reviewed by Wranâ et al., 1994). The type II receptor is able to interact with different type I receptor isoforms and the specificity of the biological response to TGF-β in a given cell type appears to be defined by the particular type I receptor engaged in the complex, thus providing a basis for the multifunctional nature of TGF-β (Wranâ et al., 1994). It has also been suggested that the heteromeric complex is in fact a tetramer consisting of a type II homodimer and two type I receptors (Yamashita et al., 1994).

At present, very little is known about the TGF-β signalling pathway beyond the formation of the heteromeric receptor complex. Although evidence suggests that the intracellular domain of the receptor complex functions as a kinase (Ohtsuki and Massagué, 1992) the substrates of this kinase activity remain obscure. However, it has been shown that TGF-β effects on DNA synthesis are pertussis-toxin sensitive, implicating G-proteins (GTP-binding regulatory proteins) as signal transducers (Murthy et al., 1988). There is also some evidence to suggest that TGF-β acts by modulating the activity of Ras in cells (Howe et al., 1993, Mulder et al., 1992). TGF-β also induces protein phosphatase 1 and tyrosine phosphatase activity and phosphorylation of the transcription factor cAMP response element binding protein (Gruppuso et al., 1991, Kramer et al., 1991).
In addition to interacting with cell surface receptors, TGF-β also binds to a number of soluble proteins. These include α₂-macroglobulin, soluble betaglycan, decorin and biglycan, thrombospondin, β-amyloid precursor protein and α-fetoprotein (Massagué, 1990, Miyazono et al., 1993). The extracellular domain of betaglycan is shed by cells as a soluble form presumably by the action of proteolytic enzymes on 1 of 2 potential cleavage sites close to the transmembrane domain (López-Casillas et al., 1991). Soluble betaglycan acts as an antagonist of TGF-β binding and actions (López-Casillas et al., 1994) in contrast to the membrane-bound form which enhances cellular responsiveness to the ligand as discussed above. This suggests that the ratio of soluble versus membrane-bound forms of betaglycan may be an important determinant of TGF-β potency. Binding of TGF-β to α₂-macroglobulin and decorin also results in inactivation of TGF-β and α₂-macroglobulin may act as a scavenger to remove excess TGF-β from the circulation (Miyazono et al., 1993). The biological relevance of TGF-β binding to the other proteins is not certain, however these proteins could alter the availability of ligand for the signalling receptors.

1.2.4.4. Effects in vitro and role in vivo.

TGF-β is a multifunctional growth factor with wide ranging and often opposite effects on many cellular processes. In many types of cells including fibroblasts it has an inhibitory effect on growth. TGF-β appears to act negatively on the cell cycle by interfering with progression through the late portion of the G₁ phase (Laiho et al., 1990). However, it is also known to have a stimulatory effect on proliferation in some cell types including human embryo fibroblasts and rat osteoblasts (Massagué, 1990). It can also stimulate the proliferation of rabbit synovial fibroblasts in the additional presence of EGF (Brinckerhoff et al., 1985). When TGF-β does stimulate proliferation in cells, this is believed to be accomplished by production of a secondary growth factor such as PDGF (Leof et al., 1986).

TGF-β is also known to regulate extracellular matrix composition and cellular adhesiveness. TGF-β increases the synthesis and deposition of extracellular matrix
components such as fibronectin, proteoglycan and collagen (Ignotz and Massagué, 1986, Bassols and Massagué, 1988). It also increases the expression of cell adhesion protein receptors and facilitates adhesion of cells to the extracellular matrix (Ignotz and Massagué, 1987). TGF-β increases proteoglycan synthesis in cartilage explant cultures (Morales and Roberts, 1988), but both inhibitory and stimulatory effects on glycosaminoglycan and proteoglycan synthesis have been observed in chondrocyte cultures (Skantze et al., 1985, Redini et al., 1988a, Rosen et al., 1988, O'Keefe et al., 1988). Synthesis of cartilage specific type II collagen is decreased by TGF-β in chondrocytes (Rosen et al., 1988).

In addition, the synthesis of extracellular matrix degrading enzymes such as collagenase, stromelysin, elastase, plasminogen activator and a thiol protease are decreased by TGF-β (Laiho et al., 1986, Edwards et al., 1987, Overall et al., 1989, Matrisian et al., 1986, Chiang and Nilsen-Hamilton, 1986, Redini et al., 1988b) while the synthesis of inhibitors of these enzymes such as plasminogen activator inhibitor-1 and TIMP-1 are increased (Laiho et al., 1986, Overall et al., 1989). TGF-β thus strengthens the stability of extracellular matrix by several different means including the enhanced synthesis, deposition and attachment of the matrix components and decreased degradation due to the limitation of proteolytic enzyme activity.

TGF-β may also have both an endogenous role and possible therapeutic applications in the pathophysiology of arthritis (reviewed by Roberts and Sporn, 1993). TGF-β is found in synovial fluid from patients with rheumatoid arthritis and osteoarthritis, it is produced by rheumatoid synovial tissue in vitro and TGF-β mRNA is expressed by synovial fibroblasts and macrophages in vivo (Wahl et al., 1990, Fava et al., 1989, Brennan et al., 1990, Lafyatis et al., 1989). There is evidence to suggest that TGF-β in combination with EGF contributes to synovial fibroblast proliferation causing these cells to display a phenotype resembling transformed cells (Brinckerhoff et al., 1985). Locally administered TGF-β exacerbates an ongoing inflammatory lesion as demonstrated in an experimental model of synovial inflammation and tissue destruction (Wahl et al., 1991). However, systemic administration of TGF-β is
beneficial in prevention of disease in animal models of rheumatoid arthritis (Kuruvilla et al., 1991). In addition, TGF-β is known to inhibit cartilage proteoglycan degradation (Chandrasekhar and Harvey, 1988, Morales and Roberts, 1988, Andrews et al., 1989). Patients with rheumatoid arthritis may therefore benefit from systemically applied TGF-β.

1.2.5. Summary

bFGF, EGF and PDGF are 3 polypeptide growth factors which have many similarities in their actions on mesenchymal cells and in their intracellular mechanism of action although each is a member of a different growth factor family.

bFGF, EGF and PDGF are all potent mitogens for cells of mesenchymal origin; PDGF being exclusively a mesenchymal cell mitogen. bFGF and PDGF are competence factors whereas EGF is a progression factor. bFGF, EGF and PDGF have all been implicated in wound healing by virtue of their ability to act as mitogenic and chemotactic agents for cells such as fibroblasts, monocytes, neutrophils and endothelial cells and to initiate extracellular matrix remodelling. bFGF and PDGF have also both been implicated in rheumatoid arthritis for the same reasons.

bFGF, EGF and PDGF are all believed to elicit their effects on cells via high affinity receptors with intrinsic tyrosine kinase activity. These receptors oligomerize in response to binding of growth factor and the tyrosine kinase is activated. This results in autophosphorylation of the cytoplasmic domain followed by phosphorylation and activation of various intracellular substrates and pathways including phospholipase Cγ, phosphatidylinositol 3′-kinase, the Ras pathway, casein kinase II and GAP. Differences occur between the different growth factors in their ability to activate these various substrates and in the pathways used for activation. Each of the 3 growth factors also causes the the induction of early genes such as c-fos and c-myc. It should be stressed that the scheme shown in figure 1.3. is only a working model many aspects of which are still poorly understood.
In addition to these similarities between bFGF, EGF and PDGF, these growth factors also show some important differences in their characteristics. bFGF is unique in its ability to bind to heparin, HSPGs and glycosaminoglycans and is believed to interact with low affinity HSPG receptors in order to activate the high affinity tyrosine kinase receptors. Its mechanism of release from cells is not clearly understood since in contrast to EGF and PDGF, it has no signal sequence. Exogenous bFGF can be translocated to the nucleus where it may trigger a nuclear signalling pathway. This has not been demonstrated for EGF or PDGF.

EGF is unique in its capacity to be secreted as a large precursor molecule which can exist as a glycosylated membrane protein. PDGF is characterized by the fact that it exists as 3 distinct dimeric isoforms which vary in the types of receptor dimers to which they will bind and activate.

TGF-β is another polypeptide growth factor from a fourth family of growth factors which is distinct from the other 3 in its effects on connective tissue cells and in its mechanism of action. Several forms of TGF-β exist and the most abundant form is TGF-β1 which is found ubiquitously. It is secreted as an inactive precursor and the extracellular activation of TGF-β is important in controlling the activity of the growth factor due to the widespread occurrence of TGF-β and TGF-β receptors. There are 3 main types of TGF-β receptor; and the type III betaglycan receptor which has no signalling structure is believed to bind TGF-β and present it to the type II receptor. Signalling takes place via the formation of a heteromeric complex between a type I and a type II receptor. In contrast to bFGF, EGF and PDGF, the signalling type I and type II receptors are not tyrosine kinases but are believed to phosphorylate serine and threonine residues. In addition, TGF-β receptors are not acutely downregulated in response to growth factor binding in contrast to the receptors for bFGF, EGF and PDGF which do exhibit such downregulation. While bFGF, EGF and PDGF are all mitogenic growth factors, TGF-β has an inhibitory effect on growth in most cell types. TGF-β also has a unique role in strengthening the extracellular matrix by
enhancing the synthesis, deposition and attachment of the matrix components and decreasing degradation by proteolytic enzymes.
1.3. CONNECTIVE TISSUE TURNOVER AND MATRIX METALLOPROTEINASES

1.3.1. Connective tissue structure and function

Connective tissue provides a support system to the many structures and functions of the body including the skin, bone, cartilage and tendon (Buckley et al., 1988, Kornblit and Gutman, 1988, Matrisian, 1990) and is continually remodelled in normal tissue turnover. The resorption of connective tissue is associated with normal processes such as cell migration, angiogenesis, tissue morphogenesis and growth, uterine involution and cervical softening (Murphy et al., 1991a, Sodek and Overall, 1988). In certain pathological conditions however, such as the arthritides, periodontal diseases, tumour invasion and metastasis, excessive degradation occurs, leading to connective tissue destruction (Sodek and Overall, 1988, Buckley et al., 1988).

Collagen is one of the major components of connective tissue and its role is to provide the tensile strength required for tissue support. At least 17 different types of collagen have been identified to date, designated types I to XVII (Van der Rest and Garrone, 1991). Type I collagen is seen in virtually all the major connective tissues including skin, bone, tendon and cornea while the distribution of type II collagen is more restricted being confined mainly to articular cartilage (Murphy and Reynolds 1985, Heinegård and Oldberg, 1989).

Collagen molecules are made up of 3 polypeptide chains (α-chains) which can be identical or nonidentical. Each individual chain is coiled in a left-handed helix that contains a repeating sequence glycine-X-Y and is rich in proline and hydroxyproline. In type I collagen, 2 of the peptide chains are identical (called α-I (I) chains) and the third chain has a different grouping of peptides (α-II (I) chain). These 3 α-chains are then combined together in a right-handed triple helix to form a rod-shaped molecule. Type II collagen is made up of 3 identical polypeptide chains called α-I (II) chains. The coiling of the α-chains to form a triple helix occurs within the cell and this collagen precursor is called pro-collagen. The pro-collagen is then exported from the
cell and cleavage of the terminal peptides occurs with the formation of a rod-like tropocollagen molecule. The tropocollagen molecules then aggregate to form the larger collagen fibrils. Within the collagen fibril, the tropocollagen molecules are arranged axially, staggered by about 1/4 of their length resulting in a repeating banded pattern. Cross-linking of amino acids within the molecules also occurs increasing the tensile strength of the collagen.

Other components of connective tissue include elastin, proteoglycans, laminin and fibronectin (Kornblith and Gutman, 1988). The proteoglycans in articular cartilage are large protein-polysaccharide molecules. The proteoglycan monomer is composed of keratan sulphate and chondroitin sulphate glycosaminoglycan chains bound covalently to a protein core molecule. The monomers have a 'bottle-brush'-like structural arrangement with the glycosaminoglycans radiating from the protein core. In articular cartilage most of the proteoglycan monomers are associated with hyaluronate which is a derivative of hyaluronic acid, a large polymer of repeating identical disaccharide units. The proteoglycan and hyaluronate combine to form large proteoglycan-hyaluronate aggregates. A link protein binds simultaneously to both the hyaluronic acid and the proteoglycan monomer to stabilize the aggregate. Smaller non-aggregating proteoglycan molecules containing dermatan sulphate are also present in cartilage.

Proteoglycan enables the cartilage to function as a 'cushion' or 'shock absorber' due to its ability to retain water (Heinegård and Oldberg, 1989). The retention of water causes resting cartilage to swell thus allowing it to be compressed when a mechanical load is applied. The proteoglycan aggregates are surrounded by a dense network of collagen fibres which provides tensile strength and acts to resist the swelling pressure caused by the proteoglycans. Proteoglycan also provides a low-friction surface in the joints (Heinegård and Oldberg, 1989).

1.3.2. Matrix metalloproteinases
Specific extracellular proteases have been characterized which are capable of degrading the extracellular matrix. These enzymes play a crucial role in normal and pathological connective tissue breakdown. It is currently thought that the initial step in the degradation of connective tissues is an extracellular process involving the matrix metalloproteinase (MMP) family of enzymes (Murphy and Reynolds, 1985). In certain special environments, acid pH cysteine proteinases are also active extracellularly, and in inflammatory or rapidly resorbing systems, serine proteinases are released by invading cells. Mechanical disruption and the presence of free radicals also augment the enzymatic processes. The MMPs however, appear to have the most ubiquitous role in matrix turnover and are believed to be the normal, physiologically relevant mediators of matrix degradation. This is because they are secreted proteins, placing them in the proper location for ECM degradation and their enzymatic activities are most potent at pH values close to neutrality.

Most connective tissue cells, at least in vitro including fibroblasts, chondrocytes, osteoblasts and endothelial cells can be stimulated to synthesize and secrete a family of MMPs (see table 1.1., pages 49-50) which between them have the ability to degrade all the components of the extracellular matrix (Murphy and Docherty, 1988). They are all (with the exception of the neutrophil enzymes) secreted rapidly from the cell rather than being stored intracellularly. They are all Zn$^{+2}$- and Ca$^{+2}$-requiring enzymes and are secreted in a latent pro-form, requiring activation. The active enzymes are specifically inhibited by members of the family of tissue inhibitors of MMPs (TIMPs) as discussed below and also by chelating agents but not by inhibitors for serine, cysteine and aspartic proteinases.

1.3.2.1. Substrate specificity (see table 1.1.)

The collagenases are the most specific of the MMPs cleaving the helix of the native fibrillar collagens (collagens I, II and III) at a single locus to yield fragments of approximately 3/4 and 1/4 the size of the original molecule. Collagenase action on the type I collagen molecule results in cleavage of a glycine-isoleucine peptide bond
in the \( \alpha-1 \) (I) chain and a glycine-leucine bond in the \( \alpha-2 \) (I) chain which are regions of low helix stability in relation to the rest of the molecule (reviewed by Woolley, 1984). At temperatures greater than 33°C the cleaved triple helical collagen fragments then spontaneously denature to random coil fragments and these can then be further degraded either by the collagenases or by other proteases. Although the collagenases hydrolyse other substrates in addition to the fibrillar collagens, the cleavage of intact fibrillar collagens at neutral pH is specifically limited to these enzymes. This means that these enzymes are likely to have a unique rate-limiting role in collagen degradation in vivo. However, a recent report has shown that 72 000-M\(_r\) gelatinase (MMP-2) can also cleave triple helical collagen (Aimes and Quigley, 1995). The cathepsins B and N have also been shown to have collagenolytic properties but only at low pH. Since fibrillar collagens form the basic architecture of connective tissue, the dismantling of collagen by collagenases is believed to be the rate-limiting step in irreversible connective tissue degradation. In cases in which the collagen is cross-linked, the action of stromelysins is also required to cleave the cross-links which then allows the subsequent action of the collagenases (Wu et al., 1991). Interstitial collagenase (MMP-1) is produced particularly by fibroblasts and macrophages while the expression of neutrophil collagenase (MMP-8) is restricted to cells of the neutrophil lineage. Recently, a third collagenase from tumour cells has been characterized (collagenase-3, MMP-13) (Freije et al., 1994). References to collagenase in this thesis always refer to interstitial collagenase (MMP-1) unless otherwise stated.

The gelatinases degrade type IV, V, and VII collagens (although some reports suggest that native type IV collagen is a poor substrate for these enzymes), fibronectin and elastin (Mackay et al., 1990, Nagase et al., 1991, Collier et al., 1988, Senior et al., 1991) and may act synergistically with collagenases in the degradation of fibrillar collagens since they efficiently degrade gelatins (denatured collagens) (Collier et al., 1988, Nagase et al., 1991). The stromelysins have a broad pH optimum and substrate specificity and are able to degrade many extracellular matrix proteins including
THE MATRIX METALLOPROTEINASE (MMP) FAMILY AND THEIR SUBSTRATES
(continued overleaf)

The collagenases
Interstitial collagenase (MMP-1)  Collagen types I, II, III, VII, X
Vertebrate/fibroblast collagenase  gelatins

Neutrophil collagenase (MMP-8)  Collagen types I, II, III

Collagenase-3 (MMP-13)  Collagen type I, II, III, gelatin

The gelatinases
Gelatinase-A (MMP-2)  Gelatin types I, II, III
72 000-M₉ gelatinase  collagen types I, IV, V, VII, X
Type IV collagenase  fibronectin, elastin

Gelatinase-B (MMP-9)  Gelatins type I and V
d (MMP-9)  collagen IV and V
92 000-M₉ gelatinase  Type V collagenase

The stromelysins
Stromelysin-1 (MMP-3)  Proteoglycan, link protein, fibronectin, laminin, collagen types III, IV, V, IX
Transin  gelatins types I, III, IV, V, activates
Proteoglycanase  pro-collagenase, pro-collagen peptides
Pro-collagenase activator  elastin?

Stromelysin-2 (MMP-10)  Gelatin types I, III, IV, V, fibronectin, collagen types III, IV, V, activates
Transin-2  pro-collagenase

Stromelysin-3 (MMP-11)  N-terminal domain cleaves casein

Matrilysin (MMP-7)  Gelatin types I, III, IV, V, proteoglycan, fibronectin, activates pro-collagenase
PUMP-1
Uterine metalloproteinase

Table 1.1.
### The membrane-bound metalloproteinases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-1 MMP (MMP-14)</td>
<td>Activates pro-gelatinase-A and possibly other MMPs</td>
</tr>
<tr>
<td>MT-2 MMP (MMP-15)</td>
<td>not known</td>
</tr>
<tr>
<td>MT-3 MMP (MMP-16)</td>
<td>activates pro-gelatinase-A and possibly other MMPs</td>
</tr>
<tr>
<td>MT-4 MMP (MMP-17)</td>
<td>not known</td>
</tr>
</tbody>
</table>

### Other enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metalloelastase (MMP-12)</td>
<td>Elastin</td>
</tr>
</tbody>
</table>

Table 1.1, continued
proteoglycans, gelatins, fibronectin, laminin, elastin, type IV collagen and type IX collagen (reviewed by Emonard and Grimaud, 1990). These enzymes can also activate pro-collagenase. In addition, MMP-3 can act as a pro-collagen peptidase for types I, II and III pro-collagens (Galloway et al., 1983; Okada et al., 1986). A metalloelastase has also been recently identified (Shapiro et al., 1992) and this enzyme may represent a fourth elastin-degrading subclass of MMPs. In addition, a family of membrane-bound metalloproteinases, 2 of which are known to be involved in the activation of 72 000-Mr progelatinase have been described.

1.3.2.2. Domain structure

A comparison of the primary amino acid sequences of various members of the MMP family demonstrates that these proteins are divided into several distinct domains which are conserved among family members (Matrisian, 1990; Woessner, 1991). This is illustrated in figure 1.4. (page 52). All metalloproteinases are produced as pre-pro-peptides the pre-domain being a leader sequence which targets the protein for secretion. Human interstitial collagenase for example is synthesized as a pre-pro-enzyme of Mr 54 092 (Goldberg et al., 1986) with a signal peptide of 19 amino acids. The primary secretion products of interstitial collagenase consist of a minor glycosylated form of Mr 57 000 and a major unglycosylated polypeptide of Mr 52 000 (Goldberg et al., 1986). The pro-domain of metalloproteinases is removed when the enzymes are activated (see below). MMP catalytic domains are essentially spherical with a deep substrate cleft that contains the active site zinc atom. The catalytic domain contains the conserved sequence His-Glu-X-X-His-X-X-Gly-X-X-His (where X is any amino acid) which is the catalytic zinc binding site. In interstitial collagenase, the catalytic zinc cation is bound to the enzyme via the 3 histidine residues in the conserved motif (Borkakoti et al., 1994) and to a cysteine residue in the pro domain. There is also a second structural zinc cation which is ligated by 3 additional histidine residues and an aspartic acid residue. The glutamic acid residue in the conserved motif is believed to be part of the catalytic site. The catalytic domain
### Stromelysins

<table>
<thead>
<tr>
<th>MMP</th>
<th>Name</th>
<th>Domain Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-3</td>
<td>Stromelysin 1</td>
<td>Propeptide</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin 2</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin 3</td>
<td>Hinge</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin</td>
<td>C-terminal domain</td>
</tr>
</tbody>
</table>

### Collagenase

<table>
<thead>
<tr>
<th>MMP</th>
<th>Name</th>
<th>Domain Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase</td>
<td>Propeptide</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Neutrophil collagenase</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase 3</td>
<td>Hinge</td>
</tr>
</tbody>
</table>

### Gelatinase

<table>
<thead>
<tr>
<th>MMP</th>
<th>Name</th>
<th>Domain Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>Propeptide</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>N-terminal domain</td>
</tr>
</tbody>
</table>

### Other Enzymes

<table>
<thead>
<tr>
<th>MMP</th>
<th>Name</th>
<th>Domain Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-12</td>
<td>Metalloelastase</td>
<td>Propeptide</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Membrane metalloproteinase</td>
<td>N-terminal domain</td>
</tr>
</tbody>
</table>

---

**THE DOMAIN STRUCTURE OF THE MMPS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>■</td>
<td>Zn binding domain</td>
</tr>
<tr>
<td>□</td>
<td>Gelatin binding domain</td>
</tr>
<tr>
<td>□</td>
<td>α2V collagen domain</td>
</tr>
<tr>
<td>▯</td>
<td>Trans-membrane domain</td>
</tr>
<tr>
<td>■</td>
<td>Furin insert</td>
</tr>
</tbody>
</table>

---

**Figure 1.4.**
also contains 3 calcium-binding sites (Li et al., 1995); calcium is required to stabilize the enzyme.

With the exception of matrilysin, the enzymes also contain a C-terminal domain with sequence similarity to haemopexin, a haem-binding protein and to the extracellular matrix component vitronectin. The C-terminal domain is separated from the catalytic domain in most of the enzymes by a proline-rich hinge sequence. The C-terminal domain is required in interstitial collagenase for the specific binding and cleavage of fibrillar collagen (Clark and Cawston, 1989, Nagase et al., 1991, Murphy et al., 1992a) and constitutes a 4-bladed β-propeller structure (Li et al., 1995). The gelatinases contain additional domains with sequence similarity to matrix proteins. In both the gelatinases, there are 3 repeats of a sequence homologous to the gelatin-binding region of fibronectin located before the zinc binding site in the catalytic domain. A domain with similarity to type V collagen is found only in the 92 000-Mr gelatinase attached at the C-terminal side of the catalytic domain.

1.3.2.3. Latency and activation

Since all MMPs are secreted as inactive zymogens, the activation of these enzymes is one level at which control over their activity can be exerted. All MMPs are activated by organic mercurial compounds (Cawston et al., 1981a), chaotrophic agents (Birkedal-Hansen et al., 1975, Shinkai et al., 1977) and with the exception of MMP-2 by protease treatment (Cawston et al., 1981a, Nagase et al., 1991). MMP-2 can be activated by the presence of intact cells or cell membranes (Ward et al., 1991a, Brown et al., 1990)

Proteases such as trypsin, tryptase, chymotrypsin, cathepsin G, plasmin, plasma kallikrein, neutrophil elastase and thermolysin (Nagase et al., 1991) perform an initial cleavage producing a short-lived intermediate with a molecular weight between that of the latent and fully active enzyme. The exact cleavage site at which this takes place depends upon the activating enzyme but is located within the sequence Glu^{35}-Lys-Arg-Arg-Asn^{37} in interstitial collagenase (Nagase et al., 1991). In vivo, this cleavage is
probably carried out by plasma kallikrein or plasmin (from the activation of plasminogen with plasminogen activator) depending upon the physiological situation (Werb et al., 1977, Nagase et al., 1981). When interstitial pro-collagenase is treated with trypsin, plasmin or plasma kallikrein in vitro, it is initially cleaved to a 46 000-Mr intermediate which is then converted to a second intermediate of 43 000-Mr by autolytic cleavage of the Thr\(^{64}\)-Leu\(^{65}\) bond. Treatment with the mercurial 4-aminophenylmercuric acetate results in the generation of a 43 000-Mr intermediate by intramolecular cleavage of the Val\(^{67}\)-Met\(^{68}\) bond. The 43 000-Mr intermediates possess only about 15% of full collagenolytic activity. Full expression of collagenolytic activity can be achieved with stromelysin which cleaves the Gln\(^{80}\)-Phe\(^{81}\) and His\(^{82}\)-Phe\(^{83}\) bonds in protease-cleaved collagenase. A recent report has shown that MMP-2 can also cleave the Gln\(^{80}\)-Phe\(^{81}\) bond in collagenase leading to a substantial increase in its collagenolytic activity (Crabbe et al., 1994).

It is thought that a highly conserved region of the pro-domain (Pro-Arg-Cys-[Val/Asn]-Pro-Asp-[Val/Leu]-[Ala/Gly]) is responsible for the maintenance of enzyme latency due to the interaction of the cysteine residue of this sequence (cysteine\(^{73}\) in interstitial collagenase) with the active site zinc cation (Springman et al., 1990). Treatment of the zymogen with proteases or chaotrophic agents alters the conformation of the enzyme resulting in the dissociation of the cysteine from the zinc and the substitution of a water molecule in its place. Mercurials are thought to act by complexing with the cysteine residue and therefore dissociating it from the zinc cation. All of these processes result in the exposure of the active site and the enzyme then autoactivates by the intramolecular cleavages detailed above which renders it irreversibly active. This activation mechanism has been referred to as the 'cysteine switch'. Further 'superactivation' can then take place by stromelysin.

The autocatalytic intramolecular cleavage but not the initial cleavage by proteases can be blocked by TIMP-2 in interstitial collagenase (DeClerck et al., 1991).

1.3.2.4. Regulation of MMP production

54
In addition to regulation at the level of enzyme activation, MMP activity can also be regulated by control over enzyme production within the cell. Quiescent connective tissue cells produce low levels of MMPs and this production can be markedly stimulated by agents such as cytokines, growth factors and hormones. Several of these effector molecules are synthesized by connective tissue cells suggesting that balanced autocrine regulatory mechanisms may be operating in healthy tissues in vivo. Others are products of monocytes and macrophages and are likely to be of importance in inflammatory conditions in which connective tissue is infiltrated by these cells. Regulation of production of the collagenases is of particular importance given the pivotal role of these enzymes in controlling connective tissue breakdown as discussed above. Several different factors have been shown to stimulate the production of interstitial collagenase (MMP-1) from connective tissue cells and this induced collagenase is predominantly proenzyme which must therefore be subsequently activated before it can elicit its effects.

The proinflammatory cytokine interleukin-1 (IL-1) has been shown to stimulate the production of interstitial collagenase from human synovial and skin fibroblasts (Dayer et al., 1986, Postlethwaite et al., 1983). IL-1 also increases collagenase production and levels of collagenase mRNA in human articular chondrocytes in monolayer culture (Stephenson et al., 1987).

PDGF stimulates the production of collagenase protein in human skin fibroblasts (Bauer et al., 1985). Furthermore, IL-1 or tumour necrosis factor-α (TNF-α) and PDGF in combination act to synergistically induce the production of collagenase protein and collagenase mRNA in these cells (Circolo et al., 1991). In rabbit articular chondrocytes in monolayer culture, it has been shown that PDGF-BB alone has no effect on the production of collagenase from these cells but that it potentiates the effect of IL-1α and IL-1β on collagenase production (Smith et al., 1991, Harvey et al., 1993). However another report using bovine nasal chondrocytes in monolayer culture has shown that PDGF-BB inhibits the stimulatory effect of IL-1α and TNF-α on collagenase production (Smith et al., 1992).
TNF-α has been shown to stimulate the production of collagenase from human fibroblasts (Dayer et al., 1985) by increasing collagenase gene transcription (Brenner et al., 1989). Collagenase mRNA levels and collagenase production are also increased in porcine articular chondrocytes in response to TNF-α and this effect is at least partially due to increased collagenase gene transcription (Mitchell and Cheung, 1991). EGF induces the production of collagenase from human foreskin and foetal lung fibroblasts (Chua et al., 1985, Edwards et al., 1987) and increases the steady-state level of collagenase mRNA in human foetal lung fibroblasts (MRC-5 cells) (Edwards et al., 1987). In porcine articular chondrocytes, however mRNA for collagenase is increased only minimally by EGF and there is no increase in collagenase protein production (Mitchell and Cheung, 1991). In periosteal explants from rabbit calvariae, it has been shown that EGF alone has no effect on collagenase production but that it synergizes with IL-1α to stimulate collagenase production (Van der Zee et al., 1993). bFGF also increases the steady-state level of collagenase mRNA and collagenase production in human foetal lung fibroblasts (Edwards et al., 1987). It has also been shown that IL-1 and bFGF can synergistically increase the production of collagenase from chondrocytes (Phadke, 1987). There is also evidence to indicate that interleukin-6 (IL-6) further augments the stimulatory effect of IL-1 on collagenase protein production from connective tissue cells (Ito et al., 1992, Sato et al., 1990, Smith et al., 1992) although it has no effect on its own. A contradictory report, however showed no effect in the presence of IL-1 (Lotz and Guerne, 1991).

In addition to stimulation by cytokines and growth factors, collagenase production can also be induced by a variety of chemical agents. The phorbol ester tumour promoter phorbol myristate acetate (PMA) is the best studied of these chemical inducers and has been shown to increase collagenase production in rabbit synovial fibroblasts, human skin fibroblasts and human articular chondrocytes (Brinckerhoff and Harris, 1981, Clark et al., 1985, Stephenson et al., 1987). Collagenase production in connective tissue cells can also be stimulated by cytochalasin B, colchicine, lipopolysaccharide, calcium ionophore A23187, concanavalin A, and crystals such as
urate, hydroxyapatite and calcium pyrophosphate (reviewed by Emonard and Grimaud, 1990).

In addition to stimulatory factors, a smaller number of agents have been shown to repress collagenase production from connective tissue cells. TGF-β inhibits the production of collagenase from human fibroblasts. A report by Edwards et al., (1987) indicated that TGF-β alone did not modulate collagenase produced by human foetal lung fibroblasts. However, TGF-β could inhibit bFGF and EGF-stimulated collagenase from these cells both at the mRNA level and protein level primarily by a decrease in transcription from the collagenase gene. Overall et al. (1989, 1991) subsequently showed that TGF-β alone could inhibit the production of collagenase protein and mRNA from human gingival fibroblasts again by a transcriptional effect. Another report by Wright et al., (1991a) showed the same effect on collagenase protein in human synovial and skin fibroblasts and that TGF-β could also inhibit IL-β-stimulated collagenase in these cells. The effect of TGF-β on collagenase gene expression in chondrocytes appears to depend upon the culture conditions used. In rat epiphyseal chondrocytes in 3-dimensional pellet culture TGF-β treatment results in a decrease in steady-state levels of collagenase mRNA but in low density monolayer culture, collagenase mRNA levels are increased by TGF-β (Ballock et al., 1993).

Retinoids can also suppress collagenase protein production from fibroblasts. This has been shown by several different workers using human or rabbit synovial, skin, gingival and lung fibroblasts (Brinckerhoff et al., 1980, Brinckerhoff et al., 1982, Bauer et al., 1982, Bauer et al., 1983, Clark et al., 1987). Clark et al. (1987) additionally showed that levels of mRNA for collagenase were reduced in retinoic acid-treated human skin fibroblasts. This report also found that low concentrations caused a mild stimulation of collagenase production while higher concentrations caused a potent inhibition. It has been shown that retinoic acid can also inhibit PMA-stimulated or mononuclear cell factor-stimulated collagenase production in human or rabbit synovial cells (Brinckerhoff and Harris, 1981, Brinckerhoff et al., 1982, Wright et al., 1991b). However in 1 human synovial cell line, a low concentration of retinoic
acid potentiated the effect of mononuclear cell factor on collagenase production while higher concentrations of retinoid were inhibitory. This report and the report of Clark et al (1987) mentioned above indicate that retinoic acid may have a biphasic action on collagenase production from fibroblasts.

The inhibitory effect of retinoids on collagenase production from fibroblasts is difficult to reconcile with the potent effect of retinoids on cartilage resorption discussed earlier. This apparent contradiction may be due the different cell types involved. The effect of retinoids on collagenase from cultured chondrocytes has therefore been investigated. A study using human articular chondrocytes has shown that retinol inhibits collagenase production from cells stimulated with mononuclear cell factor (McGuire-Goldring et al., 1983). In monolayer culture, chondrocytes quickly lose their cartilage-specific phenotype and dedifferentiate into a fibroblast-like morphology in which type II collagen synthesis is replaced by type I collagen production (von der Mark et al., 1977). It is therefore possible that these experiments do not reliably reflect the effect of retinoids on collagenase production from chondrocytes in intact cartilage.

In a study carried out on rat epiphyseal chondrocytes in monolayer culture, it was shown that retinoic acid caused an increase in mRNA for collagenase in these cells (Ballock et al., 1994). However, it should be noted that rodent collagenase is not analogous to the human enzyme since the rodent enzyme has recently been identified as MMP-13 (collagenase-3) in contrast to the human enzyme which is MMP-1 (Quinn et al., 1990, Freije et al., 1994). Retinoic acid also stimulates rather than represses the production of collagenase (MMP-13) in rodent bone cells (Heath et al., 1990, Connolly et al., 1994). It is therefore possible that the effect of retinoic acid on cartilage can be explained by the induction of MMP-13 synthesis in chondrocytes.

Interferon-γ has an inhibitory effect on basal and IL-1β-stimulated collagenase production from human articular chondrocytes in monolayer culture (Andrews et al., 1990) but in human dermal fibroblasts, collagenase production is stimulated by interferon-α, -β and -γ (Duncan and Berman, 1989).
Progesterone inhibits collagenase production from uterine explants (Jeffrey, 1981) although another report suggested that only the activation of collagenase is altered rather than the production of collagenase protein (Tyree et al., 1980). Finally, the glucocorticoid dexamethasone reduces the production of collagenase from unstimulated human and PMA-stimulated rabbit synovial fibroblasts (Werb et al., 1977, Brinckerhoff and Harris, 1981, Brinckerhoff et al., 1982). Retinoic acid and the glucocorticoid prednisolone were also shown to inhibit PMA-stimulated collagenase production from rabbit synovial fibroblasts in an additive manner (Brinckerhoff et al., 1982).

Most of these various effector agents are thought to regulate collagenase production at the level of transcription from the collagenase gene or stability of the collagenase mRNA and the identification and characterisation of the regulatory components of the collagenase gene is therefore an area of great interest.

1.3.2.5. Collagenase gene expression and collagenase mRNA stability

It is thought that the collagenase gene has 2 start sites of transcription located between -24 and -31; a major and a minor one separated by 5 nucleotides (Angel et al., 1987a). The regulation of collagenase gene expression by cytokines and other factors is complex and at present only partially understood. However progress has been made in elucidating the mechanism by which the phorbol ester tumour promoters 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and PMA induce collagenase gene expression. Angel et al. (1987a) have demonstrated that a 32 base-pair enhancer sequence at positions -73 to -42 in the human gene directs induction of transcription by TPA in transformed or tumour cell lines. However, deletion of sequences upstream of -73 resulted in a substantial loss in TPA responsiveness suggesting that other TPA-responsive elements are present upstream of -73. Further work demonstrated that the -73 to -42 TPA-responsive element (TRE) is the binding site for the transcription factor AP-1 (Angel et al., 1987b). AP-1 was originally defined as a DNA-binding activity recognizing the TRE and responsible for transcriptional
induction of a number of genes in response to activation of protein kinase C (Angel and Karin, 1991). It is a complex of several polypeptides and contains the proto-oncogene proteins $c$-$jun$ and $c$-$fos$ (e.g. Halazonetis et al., 1988, reviewed by Curran and Franzka, 1988) which can heterodimerize and bind to the AP-1 site. $c$-$jun$ but not $c$-$fos$ can also form homodimers in the absence of $c$-$fos$ but these homodimers bind the AP-1 site much less efficiently than the $c$-$fos$/$c$-$jun$ heterodimer (Halazonetis et al., 1988). There are several other proteins closely related to either $fos$ (FRA-1, FRA-2 and $fosB$) or $jun$ (junB and junD) (Angel and Karin, 1991). However, these other proteins may have opposite effects on transcription to that of $fos$ and $jun$; for example, junB may downregulate transcription from AP-1 sites (Chiu et al., 1989, Angel and Karin, 1991).

The exact manner in which the AP-1 complex is activated in response to phorbol ester stimulation is not yet understood but the $fos$ and $jun$ proteins appear and are turned over with different kinetics. There is evidence to suggest that $fos$ activity is regulated primarily at the level of transcription (Schöenthal et al., 1988) and that AP-1 activity is also regulated by a posttranslational modification resulting in increased binding to the TRE (Angel et al., 1987b).

Other studies using the rabbit fibroblast collagenase gene in rabbit synovial fibroblasts indicate that in this system, the TRE sequence is necessary for phorbol inducibility by PMA but is insufficient on its own (Auble and Brinckerhoff, 1991). At least 2 additional sequences appear to be required; a polyoma enhancer A3 (PEA3)-like element (-94 to -87) and a sequence that includes 5'-TTCA-3' (-109 to -106). PEA3 is a transcription factor whose activity is regulated by the expression of a number of oncogenes. Gutman and Wasylyk (1990) have shown that it binds specifically to the human collagenase promoter and that PEA3 and AP-1 interact synergistically to achieve maximum levels of transcriptional activation by TPA.

In the rabbit collagenase gene, Auble and Brinckerhoff (1991) have demonstrated increased responsiveness to phorbol esters when an additional 36 base-pair region located between -182 and -149 is included. This region contains an additional AP-1
site which binds the c-fos and c-jun proteins in the area -182 to -161 (Chamberlain et al., 1993). Hence it appears that a series of sequences are responsible for the phorbol ester inducibility of the collagenase gene. In addition to increased gene transcription, phorbol esters also act by increasing the stability of collagenase mRNA. It appears that the half-life of the mRNA is directly proportional to the level of collagenase mRNA induction by PMA such that higher levels of induction are associated with a longer half-life (Brinckerhoff et al., 1986).

Some work has also been done on the mechanism by which IL-1 induces collagenase production. The work of Lafyatis et al (1990) suggested that the TRE is important in IL-1 induction of the human collagenase gene. Furthermore, IL-1 increased mRNA for c-fos and c-jun and the induction of c-fos was obligatory for induction by IL-1 (Lafyatis et al., 1990). Another report indicated that in human synovial fibroblasts, the induction of collagenase mRNA by IL-1 is caused by a transient increase in transcription with no significant effect on message stability (McCachren et al., 1989). Further work using rabbit synovial fibroblasts suggested that in this case, transcriptional activation of the rabbit collagenase gene made only a modest contribution to the induction of collagenase mRNA by IL-1 and that increased mRNA stability played a more important role (Vincenti and Brinckerhoff, 1993, Vincenti et al., 1994). These reports also showed surprisingly, that a construct including the TRE gave a very poor response to IL-1 although a larger construct did respond to IL-1 suggesting the presence of as yet unidentified IL-1-responsive upstream elements (Vincenti and Brinckerhoff, 1993, Vincenti et al., 1994). IL-1 was also shown to cause only a minimal induction of AP-1-binding activity and this may be caused at least in part by poor activation of the c-jun gene (Vincenti et al., 1994).

The TRE is also important in mediating the effect of TNF-α on human collagenase gene transcription in human tumour cells and human fibroblasts and TNF-α has been shown to cause prolonged activation of jun gene expression and transient induction of fos gene expression (Brenner et al., 1989).
In the case of EGF, collagenase mRNA levels appear to be regulated primarily by increased collagenase mRNA stability (Delany and Brinckerhoff, 1992). It is thought that the degradation of collagenase and other metalloproteinase mRNAs may involve specific conformational features and/or the primary sequence of the RNAs. An AU-rich destabilizing sequence (AUUUA) has been characterized (Shaw and Kamen, 1986) which is repeated 3 times in the 3' untranslated region of human collagenase mRNA (Vincenti et al., 1994). This element is also present in the untranslated regions of many other mRNAs such as that for GM-CSF (Shaw and Kamen, 1986). Proteins which bind to the 3' untranslated region of unstable mRNAs have been identified and are thought to be involved in mRNA degradation (Huang et al., 1993, Zhang et al., 1993). It has been shown that the AUUUA elements have a role in destabilizing human collagenase mRNA (Vincenti et al., 1994). Agents which modulate collagenase mRNA stability may do so partly via a specific mRNA degradation pathway mediated by the AU-rich sequences.

The mechanism by which retinoic acid represses collagenase production in fibroblasts has also been studied. Retinoic acid has no effect on collagenase mRNA stability and appears to act entirely at the level of transcription to decrease basal and PMA-induced collagenase in rabbit synovial fibroblasts (Brinckerhoff et al., 1986). It has been shown in human synovial fibroblasts that the TRE is important in mediating the inhibitory effect of retinoic acid on TPA or IL-1-stimulated collagenase (Lafyatis et al., 1990). Retinoic acid was also found to repress IL-1 and TPA-stimulated c-fos mRNA levels and basal c-fos levels although another report failed to show this (Yang-Yen et al., 1991) but to have no effect on c-jun mRNA levels (Lafyatis et al., 1990). In another report, retinoic acid decreased the levels of phorbol-induced mRNA for fos and jun and decreased the binding of nuclear proteins to an AP-1 oligonucleotide (Pan et al., 1992). It appears, therefore that the mechanism of action of retinoic acid is via the inhibition of c-fos and c-jun gene expression rather than via a direct effect on the collagenase gene. This is consistent with the lack of steroid hormone receptor
response elements in the 527 base-pairs 5' to the start site of the human gene (Lafyatis et al., 1990).

An alternative mechanism has also been suggested. Schüle et al., (1991) found that RAR-α specifically inhibited binding of c-jun to target DNA. RAR-α did not bind directly to the TRE suggesting that the mechanism may be the formation of a non-productive complex with c-jun. More recent evidence shows that nuclear extracts from fibroblasts treated with retinoic acid contain proteins which bind to the collagenase TRE and that these proteins include RARs and RXRs (Pan and Brinckerhoff, 1994, Pan et al. 1995). Since there appears to be no direct binding of RARs to the TRE (Schüle et al., 1991, Yang-Yen et al., 1991) this binding may be mediated by RXRs or via other proteins. Finally, retinoic acid-induced nuclear proteins also specifically bind to the sequence -182 to -161 of the collagenase promoter (Pan et al., 1992) showing that sequences in addition to the TRE play a role in mediating retinoic acid repression. The proteins which bind to this region appear to include RARs and RXRs (Pan et al., 1995). Clearly, retinoic acid-mediated suppression of the collagenase gene is complex and involves more than 1 mechanism.

In summary, retinoids may act by suppressing mRNA levels for fos and jun and retinoid receptors may also bind one or both of these proteins therefore preventing them from interacting with gene regulatory sequences. A direct interaction between RARs and RXRs and these DNA sequences is also possible. The TRE is clearly important in mediating the inhibitory effect of retinoids but the evidence indicates the importance of additional sites.

The role of the different retinoic acid receptors in mediating collagenase gene repression have been investigated. Only RAR-γ1 represses basal collagenase gene expression while RAR-α1, RAR-β2 and -γ1 all suppress phorbol-induced collagenase expression (Pan et al., 1992). The mechanisms suppressing basal versus phorbol-induced transcription are therefore different and indicate distinct roles for each RAR. However, another report has shown that all 3 RARs repressed basal collagenase gene expression although this result was from HeLa cells instead of fibroblasts (Schüle et
al., 1991). In addition, retinoic acid treatment of cells increases levels of RAR mRNAs which are constitutively low but has no such effect on RXR mRNA which is constitutively higher (Pan et al., 1995).

The effect of TGF-β on collagenase gene expression has been localized to a DNA sequence in the promoter region of the gene called the TGF-β inhibitory element (TIE) which has the sequence GAATTGGAGA and is present at position -246 in the human collagenase promoter (Kerr et al., 1990). A TGF-β-inducible protein complex containing c-fos has been shown to bind to this sequence (Kerr et al., 1990) suggesting that the induction of c-fos is involved in the inhibitory effect of TGF-β on collagenase. TGF-β has been shown to stimulate c-fos expression in cells (e.g. Liboi et al., 1988). In addition to the TIE at -246, the human collagenase gene promoter also contains other TIE-like elements further upstream (Kerr et al., 1990) but the exact role of these in mediating TGF-β inhibition of the gene has not yet been established.

1.3.3. Inhibitors of metalloproteinases

1.3.3.1. α₂-macroglobulin

The final level at which metalloproteinase activity can be regulated is by inhibition of the active enzymes by metalloproteinase inhibitors. α₂-macroglobulin which inhibits most endopeptidases accounts for most of the anti-metalloproteinase activity of human plasma (Woolley et al., 1976, Werb et al., 1974). α₂-macroglobulin is a large glycoprotein of Mr 725 000 consisting of 4 identical polypeptide chains which form a tetramer (Barrett, 1981). The conformation of the tetramer changes upon interaction with proteinases, which then become trapped within the molecule (Barrett and Starkey, 1973). With a few exceptions, the ability of the entrapped enzyme to digest high molecular weight substrates is abolished or greatly reduced although the breakdown of low molecular weight substrates is unaffected or only weakly inhibited. Latent enzyme is not trapped since the formation of the complex is initiated by proteolysis in a specific region of each of the 4 subunits called the 'bait' region.
Collagenase, despite its narrow substrate specificity can cleave the bait region of \( \alpha_2 \)-macroglobulin and is believed to cleave in a region with sequence similarity to the collagen cleavage site which is adjacent to the sequence normally cleaved by other proteinases (Werb et al., 1974, Mortensen et al., 1981). The role of \( \alpha_2 \)-macroglobulin as a metalloproteinase inhibitor 
\textit{in vivo} is not clear. In inflammation where significant amounts of \( \alpha_2 \)-macroglobulin are found within the tissues, it may have a role to play in limiting tissue breakdown. However, because of its size it is thought normally to be restricted to the vasculature and therefore plays little part in physiological processes within the tissues (Woolley et al., 1976). In these situations, it is thought that control over metalloproteinase activity is carried out by the smaller tissue inhibitors of metalloproteinases (TIMPs).

\textbf{1.3.3.2. Tissue inhibitors of metalloproteinases}

All active matrix metalloproteinases can be inhibited by the TIMPs but these inhibitors do not inhibit serine, cysteine or aspartic proteinases; neither do they inhibit the bacterial metalloproteinases. The first TIMP to be characterised (TIMP-1) was purified from human tendon and rabbit bone (Vater et al., 1979, Cawston et al., 1981b) but is now known to be widely distributed. It is produced by human fibroblasts of diverse tissue origin, smooth muscle cells and osteoblasts (Welgus and Stricklin, 1983) and human macrophages (Welgus et al., 1985) and has additionally been shown to be present in several body fluids including plasma, serum, synovial fluid and amniotic fluid (Welgus and Stricklin, 1983, Stricklin and Welgus, 1983, Clark et al., 1993, Kodama et al., 1989). TIMP-1 is produced by the same cell types as collagenase (Welgus et al., 1985, Murphy and Docherty, 1988, Welgus and Stricklin, 1983) suggesting an important role in controlling its activity and immunofluorescent studies have shown that an individual cell can secrete both collagenase and TIMP-1 simultaneously (Murphy et al., 1985a).

The amino acid sequence of TIMP-1 has been determined from the nucleic acid sequence (Docherty et al., 1985). It has 184 residues and is thought to be glycosylated.
by N-linked glycosylation at Asn^{30} and Asn^{78}. Removal of the carbohydrate portion does not have any known effect on the inhibitory activity of TIMP-1 (Kishi and Hayakawa, 1984). The molecular weight of the glycosylated protein is approximately 28 500 (Cawston et al., 1981b, Stricklin and Welgus, 1983). It has 12 cysteines all of which are involved in disulphide bonds (Kishi and Hayakawa, 1984) which is consistent with its loss of activity on reductive alkylation (Cawston et al., 1981b, Kishi and Hayakawa, 1984). This arrangement gives a 2 domain structure with a peptide linker between Cys^{124} and Cys^{127}, each domain being formed from 3 loops of varying size. Structural analysis of TIMP-1 by tryptic peptide mapping suggests that the C-terminus and the Arg^{115}-Lys^{118} region are exposed on the surface of the molecule; the loop extending from Lys^{23} to Arg^{59} also appears to be exposed to a lesser extent (Williamson et al., 1993b). Preliminary investigations into the 3-dimensional structure of TIMP-1 have shown that it is mainly in a β-sheet conformation with significant amounts of α-helix and β-turn (Hodges et al., 1994). This structure is consistent with the high thermal stability of TIMP-1 (Cawston et al., 1981b, Hodges et al., 1994). TIMP is also stable to acid pH but is destroyed by proteolytic cleavage (Stricklin and Welgus, 1983, Cawston et al., 1981b).

The exact mechanism by which TIMP-1 inhibits the MMPs is not yet understood. TIMP-1 has been shown to bind tightly but non-covalently to collagenase with a 1:1 molar stoichiometry and a K_{d} of 1.4 x 10^{-10}M (Cawston et al., 1981b, Cawston et al., 1983, Stricklin and Welgus, 1983). Enzyme activity is required for TIMP-1 to bind since it does not bind to pro-collagenase. TIMP-1 also forms stable inactive complexes with active stromelysin and gelatinase (Galloway et al., 1983, Murphy et al., 1985b). TIMP-1 is not cleaved by the MMPs and retains its activity on dissociation from the enzyme-inhibitor complex (Murphy et al., 1989). Thus, TIMP-1 is not thought to act as a substrate analogue that is cleaved, leading to irreversible complex formation (Lelièvre et al., 1990). It is also unlikely to form covalent bonds within the active site of the enzymes, preventing substrate access (Lelièvre et al., 1990). Instead, the interaction may involve non-covalent interactions between
enzyme and inhibitor and possibly a direct interaction with the active site zinc atom. The MMP binding and inhibitory activity of TIMP-1 appears to reside mainly in the N-terminal domain (residues 1-126) of the molecule (Murphy et al., 1991). Chemical modification studies have indicated that the histidine residues His⁹⁵, His⁴⁴ and His¹⁶⁴ are involved in inhibition (Williamson et al., 1993a). However, these observations have not been confirmed by mutagenesis studies in which these residues are substituted or deleted (Murphy et al., 1991b, O'Shea et al., 1992). The work of O'Shea et al. (1992) has instead indicated that the region between Cys¹ and Cys¹₃ is important for inhibitory activity, in particular His⁷ and Gln⁹ although the chemical modification studies failed to demonstrate this (O-Shea et al., 1992, Williamson et al., 1993a). In the light of these conflicting reports, it is likely that the interaction of TIMP-1 with the metalloproteinases is complex and involves multiple sites on the molecule.

There are at least 2 other members of the TIMP family. TIMP-2 was initially found in human melanoma and bovine aortic cells (Stetler-Stevenson et al., 1989, DeClerk et al., 1989). More recently, a third TIMP has been identified which is one of 5 or more metalloproteinase inhibitors found in chickens (chIMPs) (Pavloff et al., 1992) and has been named TIMP-3 (Yang and Hawkes, 1992). An analogous protein is also produced by mouse and human cells (Leco et al., 1994, Apte et al., 1994, Kishnani et al., 1994). In contrast to TIMP-1 and TIMP-2 which are easily isolated from tissue fluids, TIMP-3 is found exclusively in the extracellular matrix.

TIMP-2 is a non-glycosylated protein of 194 amino acid residues (M, 20 400-21 600) (Stetler-Stevenson et al., 1989, DeClerck et al., 1989, Boone et al., 1990) with 41% amino acid identity with TIMP-1 and a further 29% of residues which are conservative substitutions (Stetler-Stevenson et al., 1989). The conserved residues include the 12 disulphide-bonded cysteines giving TIMP-2 a 2 domain structure each consisting of 3 loops similar to that of TIMP-1. The inhibitory activity of TIMP-2 appears to reside mainly in the N-terminal domain as for TIMP-1 (DeClerck et al., 1993). The structure of this N-terminal domain has been analysed and has been
shown to contain a 5-stranded antiparallel β-sheet that is rolled over on itself to form a closed β-barrel, and 2 short helices which pack close to one another on the same barrel face (Williamson et al., 1994). TIMP-2 has essentially similar properties to TIMP-1 (trypsin sensitivity, acid and heat resistance and inactivation by reduction-alkylation) (DeClerk et al., 1989) and inhibits the activity of the MMPs by binding with similar affinities and with similar inhibitory activities (Stetler-Stevenson et al., 1989, Goldberg et al., 1989, Ward et al., 1991b). The inhibition of interstitial collagenase by TIMP-2 is stoichiometric and consistent with the formation of a 1:1 molar ratio complex as for TIMP-1 (DeClerck et al., 1991). However, another report has shown that TIMP-2 inhibits the gelatinases more effectively than TIMP-1 while TIMP-1 is a more effective inhibitor of interstitial collagenase (Howard et al., 1991a). Contradictory reports in the literature concerning the efficiency with which the different TIMPs inhibit specific metalloproteinases may possibly be explained by differences in the substrate assayed and the sources of the proteins.

In addition to binding to and inhibiting active metalloproteinases TIMP-2 also forms a tightly bound complex specifically with the pro-form of MMP-2 (Stetler-Stevenson et al., 1989, Goldberg et al., 1989, Ward et al., 1991b, Howard and Banda, 1991, Curry et al., 1992) while TIMP-1 forms a complex with the proform of MMP-9 (Wilhelm et al., 1989, Goldberg et al., 1992). TIMP-2 is frequently present in cell culture medium as a complex with MMP-2 (e.g. Stetler-Stevenson et al., 1989, Goldberg et al., 1989). However, TIMP-1 does not bind to pro-MMP-2 and TIMP-2 does not bind to pro-MMP-9 (Howard and Banda, 1991). This suggests that there is some specificity in TIMP-MMP interactions and that certain members of the TIMP family may show selective affinities for specific members of the MMP family. It has been speculated that the presence of both TIMP-1 and TIMP-2 is necessary to achieve complete inhibition of connective tissue breakdown (DeClerck et al., 1989) which may be due to the selective effects of the 2 inhibitors on different MMPs. Such a theory, however requires further substantiation. In addition, the high degree of sequence conservation between TIMP-2s from different species suggests that TIMP-2 performs an essential
biological function and does not merely mimic the effects of TIMP-1 (Leco et al., 1992, Santoro et al., 1994).

The C-terminal domain of TIMP-1 is required for binding to pro-MMP-9 (Murphy et al., 1991b). TIMP-2 interacts with the C-terminal domain of pro-MMP-2 (Murphy et al., 1992b) and the addition of TIMP-2 to pro-MMP-2 prevents its membrane activation (Ward et al., 1991a). The binding of TIMP-1 to pro-MMP-9 has been shown to prevent activation of this enzyme by stromelysin (Goldberg et al., 1992). It would therefore appear that one purpose of the TIMPs binding to the pro-gelatinases is to regulate the activation of these enzymes. Activation of pro-MMP-2 complexed to TIMP-2 by mercurials yields active MMP-2 still bound to TIMP-2 which is less active than MMP-2 which autoactivates in the absence of bound TIMP-2 (Goldberg et al., 1989, Howard et al., 1991b). The binding of TIMP-2 to MMP-2 therefore does not prevent gelatinolysis and a second molecule of TIMP-1 or TIMP-2 must bind to the N-terminal active site area to inhibit enzyme activity (Goldberg et al., 1989).

Activated MMP-9 still bound to TIMP-1 can also be inhibited by the binding of a second molecule of TIMP-1 or TIMP-2 to the active site. Apart from the specific binding of TIMP-1 and TIMP-2 to pro-MMP-9 and pro-MMP-2 respectively, the TIMPs have not been shown to exhibit any other binding to pro-forms of the MMPs. TIMP-2 can also inhibit the autoactivation of interstitial procollagenase by the formation of a complex with partially activated enzyme (DeClerck et al., 1991). In addition, both the TIMPs when present in excess of enzyme can inhibit the activation of pro-MMP-2, pro-MMP-3 and pro-MMP-9 by mercurials although TIMP-1 appears to be more effective (Ward et al., 1991b). TIMP-2 has also been shown to inhibit mercurial-induced activation of pro-MMP-2 complexed to TIMP-2 when added in a stoichiometric quantity (Goldberg et al., 1989). The TIMPs are therefore able to control the extracellular activity of MMPs at 2 levels - the activation of the pro-enzyme and the activity of the activated enzyme. TIMP-1 and TIMP-2 also have growth factor-like properties (Hayakawa, 1994, Hayakawa et al., 1994) although the physiological relevance of such properties is not yet understood.
1.3.3.3. Regulation of TIMP-1 and TIMP-2 production

TIMP-1 production from connective tissue cells can be regulated in a similar manner to MMP production and in many cases, the same factors which induce metalloproteinase secretion can also induce inhibitor secretion. The reason for this may be to allow limited extracellular matrix breakdown by the enzymes before enzyme inactivation by TIMP-1. There is evidence to suggest that there are differences in the kinetics of induction of the enzymes and TIMP-1, with inhibitor being produced more slowly, thus allowing this limited extracellular matrix degradation (Murphy et al., 1985a).

The production of TIMP-1 protein is stimulated in human foetal lung, synovial and skin fibroblasts and from rabbit periosteal explants by IL-1 (Murphy et al., 1985a, van der Zee et al., 1993, Ito et al., 1992). However, another report showed that in rabbit uterine cervical fibroblasts TIMP-1 protein production is suppressed by IL-1α (Ito et al., 1988). In addition, a study using human synovial fibroblasts showed that neither IL-1 nor TNF had any significant effect on TIMP-1 production or on TIMP-1 mRNA levels (MacNaul et al., 1990). It therefore appears that the effect of IL-1 on fibroblast TIMP-1 production is highly variable and possibly depends upon the species and tissue origin of the cells. These discrepancies may also result from differences in experimental protocol in the various studies which leads to the differential presence of other growth factors. The tumour promoters TPA and PMA also have a stimulatory effect on TIMP-1 protein production from human foetal lung fibroblasts and human skin fibroblasts (Murphy et al., 1985a, Clark et al., 1985) although another report showed that PMA had a transient effect on TIMP-1 mRNA in human synovial fibroblasts and no significant effect on TIMP-1 protein production (MacNaul et al., 1990).

The effect of growth factors on TIMP-1 production has also been investigated. bFGF and EGF both cause an increase in steady-state levels of TIMP-1 mRNA and TIMP protein production in human foetal lung fibroblasts (Edwards et al., 1987). TGF-β
had no significant effect on TIMP-1 production alone but when applied in combination with bFGF or EGF a synergistic 'superinduction' of TIMP-1 mRNA and TIMP-1 secretion protein occurred. In the case of bFGF, these effects are caused at least partially by an increase in TIMP-1 gene transcription. TIMP-1 protein production from rabbit periosteal explants is also stimulated by EGF (van der Zee et al., 1993). Although Edwards et al. (1987) showed that TGF-β alone does not stimulate TIMP-1 production from human foetal lung fibroblasts, other reports have shown that TIMP-1 is stimulated by TGF-β in other types of human fibroblast. This has been demonstrated in human skin, synovial and gingival fibroblasts although in some cases the additional presence of IL-1β was required (Overall et al., 1989, Wright et al., 1991a). In mouse fibroblasts, TGF-β alone induces TIMP-1 mRNA and a 'superinduction' of TIMP-1 mRNA is seen with TGF-β and PMA (Leco et al., 1994). A study using chondrocytes in a 3-dimensional culture system showed that TGF-β has a slight inhibitory effect on TIMP-1 expression in these cells (Ballock et al., 1993).

PDGF has also been shown to increase TIMP-1 mRNA levels and TIMP-1 protein production from human skin fibroblasts (Circolo et al., 1991). Retinoic acid also induces TIMP-1 protein production from human fibroblasts derived from various tissues (adult skin, foetal skin, adult lung, foetal lung, synovial and gingival) which is accompanied by increased levels of TIMP-1 mRNA (Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991). Using human skin fibroblasts it was shown that all-trans and 13-cis-retinoic acid gave the greatest stimulation of TIMP-1 protein production while retinal and retinol resulted in more moderate effects (Clark et al., 1987). The synthetic retinoid etretinate and its metabolite etretin failed to produce any effect on TIMP-1 production. Recent work has demonstrated that TIMP-1 mRNA is reduced by retinoic acid in rat osteoblast cultures (Overall, 1994) indicating that retinoic acid may have opposite effects on TIMP-1 production depending upon the cell type or species. This was shown to be due to a decrease in TIMP-1 gene transcription without any posttranscriptional regulation of mRNA stability. In human articular chondrocytes in monolayer, retinol stimulated the production of TIMP-1 and
restored levels of TIMP-1 to normal which were repressed by mononuclear cell factor (McGuire-Goldring et al., 1983).

IL-6 stimulates the production of TIMP-1 protein from human uterine cervical, synovial and skin fibroblasts and from human articular chondrocytes (Sato et al., 1990, Ito et al., 1992, Lotz and Guerne, 1991). In addition, IL-1α and IL-6 stimulate the production of TIMP-1 protein from human synovial fibroblasts in an additive manner (Ito et al., 1992). The lymphocyte- and monocyte-derived cytokine oncostatin M also increases steady-state levels of mRNA for TIMP-1 and TIMP-1 protein production in human lung and synovial fibroblasts (Richards et al., 1993). Interleukin-11 also increases TIMP-1 protein production from human chondrocytes and synovial fibroblasts (Maier et al., 1993).

There are very few factors which are known to suppress the production of TIMP-1 from cells. However, concanavalin A has been reported to have this effect in fibroblasts (Overall and Sodek, 1990) and rat osteoblast cultures (Overall, 1994). In addition, in mouse fibroblasts dexamethasone inhibits TIMP-1 mRNA induction by EGF and PMA (Leco et al., 1994). However, the addition of dexamethasone alone to human skin fibroblasts has no appreciable effect on either TIMP-1 protein production or TIMP-1 mRNA levels (Clark et al., 1987).

Considerably less is known about the control of TIMP-2 production from cells. Northern blot analysis of RNA from human melanoma, fibrosarcoma and foetal lung fibroblast cells have shown that there are two distinct transcripts of TIMP-2 mRNA of 1.0 and 3.5 kilobases (kb) of which the 3.5kb form predominates (Stetler-Stevenson et al., 1990). The origin and function of these 2 distinct transcripts remains to be determined although it has been suggested that alternative 5' or 3' untranslated regions may account for it. TIMP-2 mRNA levels remained unchanged after treatment of the melanoma cells with TPA and were reduced in the fibrosarcoma cells, while TIMP-1 mRNA levels were induced in both of these cell lines by TPA. In human foetal lung fibroblasts, TPA caused only a modest increase in TIMP-2 mRNA levels while a much greater induction of TIMP-1 mRNA was seen. TGF-β caused a decrease in
both 3.5 and 1.0 kb TIMP-2 mRNA levels in all cell lines except the foetal lung fibroblasts while increasing TIMP-1 mRNA levels in all the cells. In rat hepatocytes, it has been shown that dexamethasone downregulates steady-state levels of TIMP-1 mRNA but upregulates TIMP-2 mRNA (Roeb et al., 1993, Roeb et al., 1995). A synergistic upregulation of TIMP-2 was also observed in response to dexamethasone combined with various cytokines (Roeb et al., 1995). Another report has shown that TIMP-2 expression is not inducible by IL-1, PMA and TNF-α treatment (Mackay et al., 1992). Northern blot analyses of human colorectal tissue showed that TIMP-1 mRNA is elevated in tumour tissue compared to normal tissue while TIMP-2 mRNA levels remain unchanged (Stetler-Stevenson et al., 1990).

The expression of TIMP-1 and TIMP-2 mRNAs has also been compared in normal and transformed murine fibroblasts (Leco et al., 1992). TIMP-1 mRNA was highly inducible by serum while TIMP-2 mRNA was largely constitutive and TIMP-1 but not TIMP-2 mRNA expression was sensitive to transformation. The trend that has therefore emerged is that TIMP-1 expression is highly stimulus-responsive and transformation-sensitive, whereas TIMP-2 expression is largely constitutive. When TIMP-2 expression is influenced by stimuli, the response is often opposite to that of TIMP-1. However another report has shown that TIMP-1 and TIMP-2 expression are coordinately regulated in response to concanavalin A in rat osteoblasts since both are downregulated (Overall, 1994). TIMP-1 and TIMP-2 expression are also coordinately induced in response to follicle-stimulating hormone but not to TPA which stimulated TIMP-1 expression only in rat Sertoli cells (Ulisse et al., 1994).

It has been suggested that the purpose of TIMP-2 production may be to protect the extracellular matrix from chronic attack by MMPs while the expression of TIMP-1 may be an acute response to remodelling pressure (Leco et al., 1992).

1.3.3.4. TIMP gene regulation.

Early work on regulation of TIMP-1 gene expression identified a murine gene homologous to the human TIMP-1 gene (Edwards et al., 1986). The mouse gene was
deduced to have a structure consisting of 5 exons and 4 introns extending over 4.3kb of DNA although a later report indicated that there were in fact 6 exons and 5 introns (Gewert et al., 1987, Coulombe et al., 1988). The murine TIMP-1 gene appears to have at least 7 transcription start sites, one of which is the major site (Coulombe et al., 1988). Such indetermination of initiation of transcription is often observed for genes which have no TATA box or whose TATA box deviates widely from the consensus sequence. Sequences homologous to the transcription start sites are found in the human gene (Campbell et al., 1991).

Intron 1 contains several potential regulatory features including 11 repeats of an interferon-like regulatory element and a region homologous to the SV40 enhancer core. In addition, a binding site for the general transcription factor SP-1 and a degenerate Hogness box have been characterized 5' to the first exon of the gene (Coulombe et al., 1988). The murine TIMP-1 gene therefore has promoter/enhancer elements both 5' to the gene and within the first intron.

Further work has identified a promoter and an enhancer element responsive to serum, TPA and TGF-β in the 5' region of the murine TIMP-1 gene. The enhancer is a TRE-like element that is likely to bind proteins of the AP-1 family. It is also conserved within the human gene. An inverted repeat immediately downstream of the TRE and a downstream SP-1 site within exon 1 were found to further enhance transcription from a marker gene containing the TRE-like element although no protein binding to these sequences could be demonstrated. The inverted repeat and the SP-1 binding site are also conserved in the human TIMP-1 gene. The sequence upstream of the TRE may also be important as this contains the additional SP-1 site identified earlier (Coulombe et al., 1988) which is also conserved in the human gene (Campbell et al., 1991).

The work of Edwards et al. (1992) has also demonstrated the importance of this TRE-like element as an enhancer and has shown that this element binds c-fos and c-jun and additional proteins that do not bind a consensus collagenase TRE. This study also identified the inverted repeat adjacent to the TRE as a PEA3 binding site which is
involved in serum induction of murine TIMP-1 gene expression. Serum stimulation of cells was shown to enhance the binding of nuclear factors to both the AP-1 and PEA3 sites and these 2 elements appear to interact in an additive manner to induce transcription. An upstream enhancer containing a second consensus AP-1-binding site was also identified.

The mouse TIMP-1 gene therefore possesses multiple regulatory elements which act in a combinatorial fashion to effect transcriptional response of the gene to stimulatory agents. It also appears that binding sites for AP-1 and PEA3 transcription factors are involved in the regulation of both collagenase and TIMP-1 gene transcription. This suggests that the coordinated induction of TIMP-1 and collagenase may be achieved through the actions of a shared set of nuclear transcription factors. However, differences between the subsets of proteins which bind the collagenase and TIMP-1 TREs may allow for differential regulation of these genes.

A recent study has investigated the TIMP-2 promoter and has revealed several differences compared with the TIMP-1 promoter consistent with the differential regulation of TIMP-1 and TIMP-2 in cells. This report showed that the TIMP-2 gene 5' flanking region has several features of housekeeping genes including a high G-C content, inclusion in a typical CG island and a TATA-like element. It also contains regulatory sequences including several SP-1 sites, one AP-2 motif, an AP-1 consensus sequence and an unidentified negative regulatory sequence (De Clerck et al., 1994).

1.3.4. Matrix metalloproteinases and pathological connective tissue breakdown

The involvement of the matrix metalloproteinases and the TIMPs in physiological and pathological processes is indicated by their presence in tissue and body fluids (Murphy et al., 1981, Welgus and Stricklin, 1983, Cawston et al., 1984, Mercer et al., 1985, Kodama et al., 1989). However, it is pertinent to ask what other evidence is available to implicate them in pathological situations such as arthritis.

In rheumatoid arthritis, it is believed that the degradation of cartilage is caused by the invading synovium which produces collagenase. This is consistent with observations
that the most severe cartilage damage occurs at the cartilage-synovium interface although does not explain why much of the articular destruction takes place without visible synovial encroachment. Immunolocalisation studies show collagenase at the cartilage-synovium junction (Woolley et al., 1977) suggesting that this enzyme is involved in cartilage degradation. It has been shown that collagenase mRNA is expressed more abundantly in rheumatoid arthritis synovial tissue compared to osteoarthritis synovial tissue. There was no significant difference in the expression of TIMP-1 mRNA. In addition, both collagenase and TIMP-1 mRNA were shown to correlate with the degree of synovial inflammation (Firestein et al., 1991). Another study has shown that mRNA for collagenase and TIMP-1 is expressed in the synovium of patients with active rheumatoid arthritis and that in highly inflammatory rheumatoid arthritis, collagenase gene expression predominates over that of TIMP-1 (McCachren, 1991). Collagenase and TIMP-1 are also present in synovial fluid and levels of collagenase and to a lesser extent TIMP-1 are greater in rheumatoid compared to osteoarthritis synovial fluid. Collagenase-TIMP-1 complexes are present more frequently in synovial fluid from patients with rheumatoid arthritis compared to those from patients with osteoarthritis (Clark et al., 1993). In addition, collagenase mRNA levels in rheumatoid arthritis synovium are reduced by therapy with methotrexate or corticosteroids (Firestein et al., 1991, Firestein et al., 1994). TIMP mRNA levels were also reduced by corticosteroid treatment (Firestein et al., 1991). These studies therefore suggest that drugs which are commonly used in the treatment of arthritis act at least in part by decreasing the expression of synovial collagenase.

There is evidence to indicate that enzymes produced by the chondrocytes also play a role in cartilage destruction in arthritis. Collagenase is produced by cartilage in increased amounts in both rheumatoid and osteoarthritis (Martel-Pelletier et al., 1994). The same report also showed that cartilage from patients with active rheumatoid arthritis had higher levels of collagenase expression than those with inactive disease. Studies on cartilage from osteoarthritic joints show an increase in collagenase
associated with disease, particularly in the centre of erosions (Ehrlich et al., 1978, Pelletier et al., 1983, Martel-Pelletier and Pelletier 1987).

Cartilage breakdown can be stimulated by IL-1 which induces cartilage collagenase production (Martel-Pelletier et al., 1994, Gowen et al., 1984, Ellis et al., 1994) and this cytokine has been isolated from human joint effusions (Wood et al., 1983) and is produced by human synovial tissue in vitro (Wood et al., 1985).

Further evidence for the role of metalloproteinases in cartilage breakdown is provided by studies showing that natural and synthetic metalloproteinase inhibitors are effective in inhibiting cartilage breakdown in vitro (Caputo et al., 1987, Caputo et al., 1988, Seed et al., 1991, Nixon et al., 1991, Andrews et al., 1992, Ellis et al., 1994). In addition, the non-steroidal anti-inflammatory drug nimesulide has been shown to reduce the production of collagenase from human articular cartilage in vitro when used at concentrations within the therapeutic range (Pelletier and Martel-Pelletier, 1993).

Recessive dystrophic epidermolysis bullosa is a condition characterised by blister formation in response to minor trauma and it has been found that skin fibroblasts from patients with this condition secrete increased amounts of collagenase (Bauer et al., 1983). Collagenase has also been implicated in corneal disease (Gordon et al., 1980, Kao et al., 1982) and in gingival disease (Woolley and Davies, 1981, Overall et al., 1987).

Although the involvement of collagenase and TIMPs in these diseases needs to be better understood, there seems to be little doubt that they are important. It therefore may be possible to treat these conditions by controlling the activity of collagenase. One level at which this can be done is by controlling enzyme production by the use of appropriate cytokines, growth factors and other factors which are known to inhibit collagenase gene expression. Enzyme activation is another possible point of intervention since collagenase does not digest collagen provided that it is maintained in the latent form. Once the enzyme is activated, control rests with inhibition by the use of natural or synthetic metalloproteinase inhibitors. It may be difficult for protein
inhibitors such as the TIMPs to gain access to the required areas due to instability, lack of cartilage penetration and rapid metabolism *in vivo* (Andrews et al., 1992, Wearley, 1991). Although low Mr chemical inhibitors may be effective (Caputo et al., 1987, Caputo et al., 1988, Seed et al., 1991, Nixon et al., 1991, Andrews et al., 1992, Ellis et al., 1994), difficulties may be caused by the lack of biostability of these inhibitors. An alternative approach is to stimulate the production of TIMPs by the cells of the diseased tissue which would by-pass these problems.

However, the prerequisite for this therapeutic goal is a detailed understanding of the manner in which gene expression and protein production of the TIMPs are regulated in connective tissue cells. Whilst there are many reports of single growth factors or cytokines modulating the expression of the MMPs or their inhibitors, considerably less is known about interactions between 2 or more such factors and the mechanisms of any such interactions are not understood. Cytokines, growth factors and other agents do not necessarily act independently of one another when both are present simultaneously and phenomena such as synergism and antagonism have been reported. It has been shown that bFGF and EGF synergize with TGF-β to 'superinduce' TIMP-1 production from human fibroblasts (Edwards et al., 1987). Retinoic acid is strikingly similar to TGF-β in its actions on collagenase and TIMP-1 secretion from fibroblasts (Brinckerhoff et al., 1980, Brinckerhoff et al., 1982, Bauer et al., 1982, Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991a, Edwards et al., 1987, Overall et al., 1989, Wright et al., 1991b). It can therefore be postulated that retinoic acid may also synergize with bFGF and EGF to stimulate the production of TIMP-1 from fibroblasts. This project therefore examines the effect of these growth factors in combination with retinoic acid on TIMP-1 secretion from fibroblasts.

Preliminary investigations in the Rheumatology Research Unit have indicated that retinoic acid and mononuclear cell factor synergistically stimulate the production of TIMP-1 from fibroblasts. Mononuclear cell factor is a mixture of various cytokines and growth factors (such as PDGF and TGF-β) (Heldin et al., 1993) and in order to further our understanding of TIMP-1 gene regulation it is important to pinpoint which
factor or factors are causing the effect. This question is addressed by investigating the effect of retinoic acid in combination with PDGF or TGF-β on TIMP-1 protein production from fibroblasts.

A further reason for investigating the retinoids is that these compounds have been used with some success in animal models of rheumatoid arthritis. It has been shown that adjuvant arthritis and streptococcal cell wall-induced arthritis are suppressed by retinoid treatment although collagen-induced arthritis is exacerbated (Trentham and Brinckerhoff, 1982, Brinckerhoff et al., 1983, Brinckerhoff et al., 1985). The therapeutic value of retinoids may be related to the inhibitory effect of these compounds on collagenase production from fibroblasts (Brinckerhoff et al., 1980, Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991b) combined with the stimulatory effect on TIMP-1 production from the same cells (Clark et al., 1987, Wright et al., 1991b). However, the potentially destructive effect of retinoids on cartilage discussed earlier in this chapter must be taken into account in any strategy involving the use of retinoid-based drugs to treat this disease.

One advantage of using retinoids is that a large number of natural and synthetic retinoids already exists and some of these compounds are already used in other clinical situations such as acute promyelocytic leukaemia (Meng-er et al., 1988, Castaigne et al., 1990, Warrell et al., 1991) and dermatological disorders (Ehmann and Voorhess, 1982). This means that information on side-effects and toxicity is already available. The structure-activity relationship of many retinoids has also been studied (e.g. Newton et al., 1980) and it has shown that it is possible to create synthetic compounds in which the beneficial effects are enhanced and undesirable side-effects are diminished. In particular, recent work has identified conformationally restricted retinoids which selectively inhibit AP-1 activity but do not activate gene transcription (Fanjul et al., 1994). Since retinoic acid is believed to inhibit collagenase by inhibiting AP-1 activity, this suggests that it is possible to design retinoids which selectively suppress collagenase production from connective tissue cells. Indeed, a recent report has shown that certain synthetic retinoids can inhibit transcription from
the stromelysin AP-1 motif via RAR-α without also inducing gene transcription through this receptor (Nagpal et al., 1995).

The aim of this project is therefore to increase our understanding of the effects of retinoids on collagenase and TIMP-1 secretion from connective tissue cells; with particular emphasis on the manner in which retinoic acid influences TIMP-1 gene expression when combined with polypeptide growth factors. The insights which these investigations provide into the regulation of TIMP-1 gene expression in connective tissue cells and possible therapeutic applications of this information are discussed.
CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1. PREPARATION OF FIBROBLAST CULTURES

2.1.1. Skin fibroblasts

The culture conditions were based on the method of Dayer et al., (1976). Skin tissue was obtained from the site of incision of routine abdominal surgery or from foreskin tissue. Skin tissue was digested for 3 to 4 hours in serum-free Dulbecco's modification of Eagle's medium (DMEM) supplemented with 3.7g/l sodium bicarbonate containing 4mg/ml of bacterial collagenase, followed by a further digestion in 0.25% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) for 30 minutes to 1 hour. The tissue fragments were then dispersed by gentle pipetting with a pasteur pipette. The cell suspension was centrifuged for 5 minutes at a relative centrifugal force of 80 (80g) at room temperature (21°C) and washed 3 times in DMEM containing 10% heat inactivated foetal calf serum (FCS). The pellet was resuspended in DMEM containing 10% FCS and cell numbers and viability determined using a haemocytometer. The cells were plated out at a concentration of 2-3 x 10^6 cells per 75cm² tissue culture flask in DMEM containing 3.7g/l sodium bicarbonate, 10% FCS, 50μg/ml gentamicin and 2.5μg/ml amphotericin.

2.1.2. Synovial fibroblasts

Synovial tissue was obtained from patients with rheumatoid or osteoarthritis undergoing synovectomy or joint replacement operations. Fat and fibrous connective tissue were carefully dissected away and the remaining synovium finely chopped. The synovium fragments were then digested and cultured in 75cm² flasks as for the skin fibroblasts.
2.1.3. Tendon fibroblasts

Adult human biceps tendon was removed aseptically from fresh cadavers within a short time of death (<12 hours). After washing 3 times in Hanks' balanced salt solution (HBSS), all fat and loose tissue surrounding the tendon was dissected away and small fragments were cut from it. Fragments were then placed in groups in 50mm tissue culture grade petri dishes and each group was covered with a 10mm diameter circular sterile glass coverslip held in place with a spot of sterile silicone grease. 3ml of DMEM supplemented with 3.7g/l sodium bicarbonate, 10% FCS and antibiotics was then added to each dish and the cultures were incubated at 37°C in a humidified atmosphere. Once adequate cell migration and replication had occurred, the coverslips were inverted using fine sterile forceps and the media and explants were carefully removed. The fibroblasts were washed in HBSS and removed from the dish and coverslips by incubation in 0.25% trypsin and 0.02% EDTA in calcium and magnesium-free HBSS. The cells were transferred to 25cm² plastic culture flasks and maintained in monolayer in DMEM supplemented with 3.7g/l sodium bicarbonate, 10% FCS and antibiotics.

Tissue fragments from the surface of the tendon were cultured separately from those within it as a previous report has suggested that the superficial cells are most active in tendon repair (Rank et al., 1980).

2.2. GENERAL MAINTENANCE AND PASSAGING OF FIBROBLAST CULTURES

The skin, synovial and tendon fibroblasts were maintained in 75cm² flasks and fed once a week with DMEM supplemented with either 20mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) or 3.7g/l sodium bicarbonate and 1mM sodium pyruvate, 10% FCS, 50μg/ml gentamicin or penicillin/streptomycin (100IU/ml/100μg/ml) and 2.5μg/ml amphotericin or 20U/ml nystatin suspension.
When the fibroblasts had grown to confluence, the cells were passaged by incubation in 0.25% trypsin, 0.02% EDTA in calcium and magnesium-free HBSS for 5 minutes at 37°C. The resulting cell suspension was harvested into 1x volume of DMEM containing 10% FCS in order to inhibit trypsin activity. This was followed by centrifugation (5 minutes, 80g, room temperature) and resuspension in DMEM containing 10% FCS. Cell number and viability were determined using a haemocytometer.

The cells were then replated into 75cm² flasks or into 24- or 48-well plates for the cell assay system.

2.3. CRYOPRESERVATION OF FIBROBLASTS

Stocks of all cell lines were preserved under liquid nitrogen in cryomedium consisting of either 90% FCS and 10% dimethylsulphoxide (DMSO) or DMEM supplemented with 10% FCS and 10% DMSO at a density of 1 x 10⁶ cells per ml.

2.4. PREPARATION OF ACID-TREATED FOETAL CALF SERUM

5M hydrochloric acid was added to 100ml of FCS until a pH of 3.0 was reached. This solution was then incubated at room temperature for 90 minutes after which the serum was returned to pH 7.4 by the addition of 5M sodium hydroxide. The resulting acid-treated FCS (ATFCS) was filtered through a 0.45μm and 0.2μm sterile filter and stored in 5ml aliquots at -20°C. The acid treatment denatures any endogenous α₅-macroglobulin present in the serum.

2.5. PREPARATION OF RETINOIC ACID AND GROWTH FACTORS

All-trans-retinoic acid was dissolved in DMSO at a concentration of 0.128M and stored at -20°C in 50μl aliquots until required. Each of these aliquots was used to make a further set of aliquots at a concentration of 10⁻³M in DMSO and these were stored in 250μl aliquots at -20°C until required for use in the cell assay system. All-
trans-retinoic acid was diluted to a concentration of 10^{-5} M in DMEM containing 1% ATFCS and filter-sterilized using a 0.2μm sterile filter for use in experiments.

Lyophilized human recombinant basic FGF or human recombinant EGF was reconstituted in sterile tissue culture grade phosphate-buffered saline (PBS) to a concentration of 100μg/ml and growth factor which was not required for immediate use was frozen at -70°C in 5.5μl aliquots. For use in the cell assay system, a dilution series of the growth factor was prepared in DMEM containing 1%ATFCS.

Human recombinant PDGF-BB at a concentration of 100μg/ml in 1M acetic acid (as supplied by the manufacturer) was thawed and used to prepare a dilution series in DMEM containing 1% ATFCS for use in the cell assay system. The vial of 100μg/ml stock solution was rinsed with 1M acetic acid containing 0.25% bovine serum albumin to ensure complete recovery of the growth factor. The contents of one vial were used immediately in the cell assay system as it was found that another freeze-thaw cycle resulted in significant loss of biological activity.

Human purified TGF-β1 at a concentration of 20μg/ml in 30% acetonitrile/0.10% trifluoroacetic acid (as supplied by the manufacturer) was thawed and used to prepare a dilution series in DMEM containing 1% ATFCS for use in the cell assay system. The contents of 1 vial were used immediately in the cell assay system as it was found that another freeze-thaw cycle resulted in significant loss of biological activity.

Human recombinant IL-1β was diluted to a concentration of 1μg/ml in DMEM containing 1% ATFCS and stored in 20μl aliquots at -70°C until required for use in the cell assay system.

Lyophilised neutralising polyclonal antibody to human bFGF was reconstituted in DMEM containing 1% ATFCS to give a concentration of 1mg/ml and this solution was either frozen at -20°C in appropriate aliquots or further diluted with DMEM containing 1% ATFCS to a concentration of 500μg/ml for use in the cell assay system.

Lyophilised neutralising polyclonal antibody to human EGF or PDGF-BB was reconstituted with sterile PBS to give a concentration of 1mg/ml and this stock
solution was either frozen at -20°C in appropriate aliquots or diluted in DMEM containing 1% ATFCS to the final working strength for use in the cell assay system. Lyophilised neutralising polyclonal antibody to human TGF-β1 was reconstituted in distilled water to give a solution of 1mg/ml and this solution was either frozen at -20°C in appropriate aliquots or diluted with DMEM containing 1% ATFCS to a final concentration of 13μg/ml for use in the cell assay system.

2.6. CELL ASSAY SYSTEM

The effects of retinoic acid and growth factors on the fibroblasts were measured in the following basic assay system. Skin, synovial or tendon fibroblasts were passaged and resuspended at a concentration of 5 x 10^4 cells per ml in DMEM containing 10% FCS. The cells were seeded into either a 24-well plate at 1ml per well or a 48-well plate at 0.5ml per well giving a cell density of 2.5 x 10^4 cells per cm². The plates were incubated overnight at 37°C to enable attachment of the cells to the plastic. The cell monolayers were then washed with HBSS three times and 1ml (24-well plates) or 0.5ml (48-well plates) of DMEM containing 1% ATFCS was added to each well. The plates were then incubated at 37°C for 48 hours. This 48 hour incubation in low serum allows the levels of TIMP-1 and collagenase produced by the cells to fall below the threshold for stimulation, thus allowing the effect of stimulatory agents to be subsequently examined. The use of ATFCS instead of untreated serum is discussed below. Fresh medium (DMEM with 1% ATFCS) containing the test reagents was then added to the wells and the plates were incubated for a further 72 hours after which the cell supernates were harvested. The cell monolayers were then washed twice with HBSS and stored at -20°C until their protein or DNA content was determined. The cell supernates were made 0.02% with respect to sodium azide and were stored for a maximum of two weeks at 4°C until assayed as a batch for TIMP-1 and interstitial collagenase.

The effect of each test reagent or combination of test reagents was examined in triplicate in all experiments. Each type of experiment was carried out at least 3 times
using 3 different skin, synovial or tendon cell lines for the 3 experiments. In many cases, additional experiments were performed either by testing further cell lines or by testing the same cell line more than once. The cells were used for experiments at low to moderate passage numbers (passage 2 to passage 15).

Levels of TIMP-1 and collagenase in the cell supernates were measured using enzyme-linked immunosorbent assays (ELISAs) as described below. The use of ELISAs instead of enzyme assays for the measurement of these parameters has a number of advantages. When collagenase is measured by enzyme assay, it must first be activated since it is secreted as the inactive zymogen from cells (e.g. Murphy et al., 1985a). If TIMP-1 or TIMP-2 are also present in the same sample (as is usually the case in cell culture supernates), then collagenase-TIMP-1 or collagenase-TIMP-2 complexes are formed and only the excess of collagenase over inhibitors is effectively measured. Metalloproteinase inhibitors can also be measured using an enzyme assay; in this case the ability of the sample to inhibit collagen degradation is measured. The drawback of such an assay is that it cannot distinguish between different metalloproteinase inhibitors (e.g. between TIMP-1 and TIMP-2).

ATFCS rather than untreated serum was used so that it was possible to use the cell supernates from experiments to measure levels of stromelysin and gelatinase activity using assays for enzyme activity. Immunoassays for stromelysin and gelatinase have not yet been developed at the Rheumatology Research Unit, hence enzyme assays are the only choice in this case. Enzyme assays are not possible in the presence of α2-macroglobulin because activation of the enzymes results in the formation of irreversible complexes with α2-macroglobulin as described in chapter 1. Acid treatment of the serum destroys α2-macroglobulin and therefore makes it possible to use the cell supernates for measurement of stromelysin and gelatinase. In fact, the results shown in this thesis do not include any data from such assays. However, it is possible to measure these parameters in future investigations with the cell supernates from the experiments because ATFCS rather than untreated serum has been used.
2.7. MEASUREMENT OF INTERSTITIAL COLLAGENASE AND TIMP-1 BY ELISAS

2.7.1. Free TIMP-1 ELISA

TIMP-1 was measured using a double-antibody sandwich ELISA which has been previously developed in the Rheumatology Research Unit (Clark et al., 1991). The assay measures free TIMP-1 (Clark et al., 1991) and TIMP-1 in complex with gelatinase or stromelysin (unpublished observations) but does not measure TIMP-1-collagenase complex (Clark et al., 1991).

96-well Maxisorp plates were coated overnight at 4°C with 100μl per well of 4μg/ml RRU-T1 (a monoclonal antibody to human TIMP-1) in ELISA PBS (0.14M sodium chloride, 2.7mM potassium chloride, 8mM di-sodium hydrogen orthophosphate, 1.5mM potassium dihydrogen orthophosphate, 3mM sodium azide or 0.25mM thimerosal). The plates were then blocked for at least an hour at room temperature with 150μl per well of 10mg/ml bovine serum albumin in PBS with 3mM sodium azide. The plates were washed 3 times in wash buffer (PBS with 0.25mM thimerosal + 0.05% Tween 20) and the samples and standards were added at 100μl per well in duplicate to the plates. A standard curve was prepared by diluting purified human TIMP-1 in protein diluent (PBS with 0.25mM thimerosal + 0.05% Tween + 0.5mg/ml bovine serum albumin) to give a range of concentrations from 5 to 50ng/ml. The samples were applied to the plates either neat or appropriately diluted in protein diluent. The plates were incubated at room temperature for 2 hours after which they were washed 3 times in wash buffer. 100μl per well of 200ng/ml biotinylated RRU-T2 (a monoclonal antibody to human TIMP-1) in protein diluent was then added to the plates followed by incubation at room temperature for another 2 hours. The plates were washed again 3 times in wash buffer and then incubated at room temperature for 30 minutes in 100μl per well of 1/5000 fold dilution of streptavidin-horse-radish peroxidase (HRP) in protein diluent. The plates were then washed 3 times in wash buffer and incubated with 100μl per well of o-phenylenediamine and 0.03% sodium
perborate, pH 5.0 for 5 minutes at room temperature in the dark. The reaction was stopped with 50μl per well of 2M sulphuric acid and the absorbance at 492nm was measured. The concentration of TIMP-1 in the samples was determined from the standard curve using the computer software MS-DOS Titersoft.

2.7.2. Total TIMP-1 ELISA

TIMP-1 was also measured using an alternative double-antibody sandwich ELISA which has been developed more recently at the Rheumatology Research Unit (Plumpton et al., 1995). This assay measures free TIMP-1 and TIMP-1 in complex with collagenase, stromelysin or gelatinase. For the purpose of the experiments performed in this thesis, the total TIMP-1 ELISA is preferable to the previously developed assay (Clark et al., 1991) because it measures the total TIMP-1 content of the cell supernates, whereas the previous assay does not measure TIMP-1-collagenase complex. The experiments described in chapters 3 and 4 using tendon fibroblasts were carried out using the total TIMP-1 ELISA but those using skin or synovial fibroblasts were performed with the first ELISA (Clark et al., 1991) because at this time the total TIMP-1 assay was still being developed. The data in chapters 5-8 is from skin and synovial fibroblasts and it was decided to use the first assay for this work so that it would be consistent with the preliminary experiments in chapters 3 and 4. In actual fact, there should be very little difference between the results from the first and subsequent ELISA because the cells produce collagenase only as the inactive zymogen (e.g. Murphy et al., 1985a) therefore precluding the formation of TIMP-1-collagenase complexes.

Maxisorp 96-well plates were coated overnight at 4°C with 100μl per well of 5μg/ml of RRU-T5 (a monoclonal antibody to human TIMP-1). The plates were then blocked and washed as for the free TIMP-1 ELISA. Samples (either neat or appropriately diluted in protein diluent) and standards were added to the plates as for the free TIMP-1 ELISA. The plates were incubated at room temperature for 2 hours, washed 3 times with wash buffer and then incubated with 100μl per well of 800ng/ml of biotinylated
RRU-T1 in protein diluent for a further 2 hours at room temperature. The plates were washed again and incubated for 30 minutes at room temperature with 100μl per well of 1/1000 dilution of streptavidin-HRP in protein diluent. The plates were then developed with o-phenylenediamine and sodium perborate and the reaction stopped as for the free TIMP-1 ELISA. The absorbance was read at 492nm and the quantity of TIMP-1 in the samples calculated using the standard curve as for the free TIMP-1 assay.

2.7.3. Collagenase ELISA.

Interstitial collagenase was measured in a double-antibody sandwich ELISA which has been previously developed at the Rheumatology Research Unit (Clark et al., 1992). The assay measures free enzyme (both latent and active), as well as all collagenase-containing complexes except that with α₁-macroglobulin (Clark et al., 1992).

Maxisorp 96-well plates were coated overnight at 4°C with RRU-CL1 (a monoclonal antibody to human interstitial collagenase) at 1μg/ml in ELISA PBS. The plates were blocked and then washed 3 times as for the free TIMP-1 ELISA. Samples (either neat or appropriately diluted in protein diluent) and standards were added to the plates as for the free TIMP-1 ELISA. The standard curve was prepared by diluting purified human interstitial procollagenase in protein diluent to give a range of concentrations from 2ng/ml to 100ng/ml. The plates were incubated for 2 hours at room temperature, washed 3 times and 100μl per well of 8μg/ml of a biotinylated polyclonal antibody to collagenase in protein diluent was added to the plates. After another 2 hour incubation at room temperature, the plates were washed again and incubated with streptavidin-HRP followed by o-phenylenediamine and sodium perborate as for the free TIMP-1 ELISA. The reaction was stopped and the absorbance measured as for the free TIMP-1 ELISA and the quantity of collagenase in the samples was calculated using the standard curve.
2.8. LOWRY ASSAY FOR PROTEIN

The determination of the protein content of the cell monolayers was based on the modification of the Lowry protein assay (Lowry, 1951) by Patterson, (1979). The assay was performed using a kit in which the alkaline copper solution in Lowry's original protocol (Lowry solution C) is replaced with a modified reagent with a much greater stability and shelf life.

0.5ml (24-well plates) or 0.25ml (48-well plates) of 0.1% sodium dodecyl sulphate (SDS) was added to each well and the plates were incubated at room temperature for 30 minutes with constant shaking. A standard curve was prepared using bovine serum albumin diluted in 0.1% SDS at a range of concentrations from 2μg/ml to 500μg/ml. 200μl of samples and standards were transferred to 9.5mm x 11.3mm x 44.2mm polystyrene test tubes and 1ml of modified Lowry solution C was added to each tube followed by immediate vortexing of the tubes. The tubes were then incubated at room temperature for exactly 10 minutes. 100μl of Folin and Ciocalteu's reagent which had been freshly diluted 1:1 with distilled water was then added to each tube followed by immediate vortexing of the tubes. The tubes were incubated for a further 30 minutes at room temperature and the absorbance of the standards and samples was then measured at 750nm. The concentration of protein in the samples was calculated using the standard curve.

The levels of TIMP-1 and collagenase measured by ELISA in the cell supernates were corrected with respect to the quantity of cell protein in the monolayer measured by the Lowry assay such that the results are expressed as ng of TIMP-1 or ng of collagenase per μg of cell protein. This procedure ensures that any stimulation or inhibition of cell growth induced by the test reagents and any error in seeding the cells at the same density in each well is taken into account.

2.9. DNA ASSAY USING HOECHST 33258

In most of the experiments in chapter 4, the DNA content of the cell monolayer was measured using Hoechst 33258 instead of a protein measurement using the modified
Lowry assay. This assay was attempted rather than continuing with the Lowry assay because measurement of cell DNA gives a more reliable indication of stimulation or inhibition of cell growth induced by the test reagents than a protein assay. An assay for protein also measures stimulation or inhibition of extracellular matrix synthesis by the test reagents. The procedure used was based on the assay developed previously by West et al., (1985).

1 ml (24-well plates) or 0.5 ml (48-well plates) of 10 mM EDTA (pH 12.3 with sodium hydroxide) was added to each well and the plates were incubated at 37°C for 20 minutes with constant shaking. The plates were then cooled on ice and the solution was mixed with a pasteur pipette to ensure homogeneity. A range of standards was constructed using a stock solution of 2 mg/ml calf thymus DNA in 10 mM EDTA, pH 12.3 which was frozen in appropriate aliquots at -20°C. The exact concentration of this stock solution was checked by measuring the absorbance at 260 nm of stock solution diluted to 100 μg/ml with 10 mM EDTA, pH 12.3. The stock solution was then diluted with 10 mM EDTA, pH 12.3 to give standards ranging from 0.05 μg/ml to 4 μg/ml calf thymus DNA.

1050 μl of each standard in duplicate and 450 μl of each sample plus 600 μl of 10 mM EDTA, pH 12.3, also in duplicate were transferred to 9.5 mm x 11.3 mm x 44.2 mm polystyrene tubes. 75 μl of potassium dihydrogen orthophosphate was then added to each tube in order to adjust the pH to 7.0 and the tubes were vortexed. Immediately before use, a stock solution of Hoechst 33258 at 200 μg/ml in distilled water which was kept in the dark at 4°C, was diluted to 200 ng/ml using 100 mM sodium chloride, 10 mM Tris[hydroxymethyl] aminomethane (Tris), pH 7.0 as the diluent. 1125 μl of the diluted Hoechst 33258 was then added to each tube followed by immediate vortexing. The fluorescence of the samples and standards was then measured with excitation at 350 nm and emission at 455 nm. The quantity of DNA in the cell monolayers was calculated using the standard curve.

The levels of TIMP-1 and collagenase measured by ELISA in the cell supernates were corrected with respect to the quantity of cell DNA in the monolayer measured using
Hoechst 33258 such that the results are expressed as ng of TIMP-1 or ng of collagenase per µg of cell DNA.

In order to compare the suitability of DNA measurement as opposed to protein measurement, an experiment was set up using the human synovial cell line SY 33 (passage 5) to compare the effects of PDGF-BB on protein and DNA levels in the cell monolayer. Cells were seeded out and prepared for stimulation according to the cell assay system described above. The cells were then stimulated with PDGF-BB at 1, 10 and 100 ng/ml for 72 hours. Control cells to which no PDGF-BB was added were also included. The culture medium was then removed, the cell monolayers were washed 3 times with HBSS and assayed for either cell protein using the Lowry assay or cell DNA using the Hoechst assay. Six replicate culture wells were used for each test condition and 3 were used to assay protein while the other 3 were used to measure DNA. The protocols do not allow protein and DNA to be measured in the same sample.

The results of this experiment are shown in figure 2.1. The levels of both protein and DNA in the cell monolayer are stimulated by PDGF-BB but DNA levels are not stimulated to the same extent as protein levels. This suggests that part of the stimulation of cellular protein in response to PDGF-BB is due to increased extracellular matrix synthesis rather than increased cell number. This in turn indicates that the Lowry assay is open to misinterpretation in the experimental system used and that the measurement of DNA is preferable.

However, the use of the DNA assay was eventually abandoned because it was found that unacceptable levels of variability occurred between replicate samples suggesting that the measurements were unreliable. The Lowry assay was used for the remainder of the data in the thesis as it is clearly better to use an assay which gives accurate readings even with the attendant drawbacks of measuring protein instead of DNA.
Figure 2.1.
The effect of PDGF-BB on the levels of total cellular protein and DNA in the human synovial cell line SY 33 (passage 5)
2.10. STATISTICAL ANALYSIS

Statistical analysis was performed either by one way analysis of variance or by multiple regression. In experiments in which the effect of a single test reagent was investigated, statistical analysis was by one way analysis of variance while in experiments in which a synergistic interaction between 2 test reagents was observed, statistical treatment was by multiple regression. The levels of significance are indicated as *, where p is less than 0.05, ** where p is less than 0.01 and *** where p is less than 0.001.

2.11. MATERIALS

Amphotericin, DMSO, bovine serum albumin, calf thymus DNA, all-trans-retinoic acid, o-phenylenediamine, nystatin suspension, tissue culture PBS and sodium perborate were from Sigma Chemical Company Ltd., Poole, Dorset, U.K., heat-inactivated foetal calf serum was from Advanced Protein Products Ltd., Brierly Hill, West Midlands, U.K., DMEM and 5 and 10ml sterile plastic pipettes were from ICN Biomedicals Ltd., Thane, Oxon, U.K., DMEM, gentamicin, penicillin/streptomycin, trypsin-EDTA, HBSS, calcium and magnesium-free HBSS, glutamine, 96-well Maxisorp plates, cryovials, sodium bicarbonate and sodium pyruvate were from Gibco BRL Life Technologies Ltd., Paisley, Scotland, U.K., 24-well culture plates, 48-well culture plates, 25cm² and 75cm² plastic flasks and 25ml sterile plastic pipettes were from COSTAR U.K. Ltd., High Wycombe, Bucks., U.K., bacterial collagenase was from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A., petri dishes, 16 x 100mm test tubes and universals were from Bibby Sterilin, Staffodshire, U.K., IL-1β was a generous gift from Glaxo Laboratories, Greenford, Middlesex, U.K., human recombinant EGF, PDGF-BB and human purified TGF-β were from Genzyme Diagnostics, West Malling, Kent, U.K., human recombinant bFGF was from Universal Biologicals Ltd., London, U.K., polyclonal neutralising antibodies to bFGF, EGF and PDGF-BB were from R. & D. Systems Europe, Abingdon, Oxon, U.K., polyclonal neutralising antibody to TGF-β was from Collaborative Biomedical
Products, Bedford, M.A., U.S.A., streptavidin-HRP was from Dako, High Wycombe, Bucks., U.K., modified Lowry protein assay kit was from Pierce and Warriner, Chester, U.K., bijoux and glass pasteur pipettes were from L.I.P. Ltd., Shipley, W. Yorks., U.K., polystyrene tubes were from Denley Instruments Ltd., Luckham Division, Burgess Hill, West Sussex, U.K., 0.2μm and 0.45μm sterile filters were from Sartorius AG, Göttingen, Germany, disposable scalpels were from Swann-Morton, Sheffield, U.K., and Hoechst 33258 was from Calbiochem, California, U.S.A.
CHAPTER THREE

THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH bFGF OR EGF ON TIMP-1 AND COLLAGENASE PROTEIN SECRETION FROM HUMAN SKIN, SYNOVIAL AND TENDON FIBROBLASTS.

3.1. INTRODUCTION

This chapter describes an investigation into the effect of the naturally-occurring retinoid, all-trans-retinoic acid when combined with the polypeptide growth factors bFGF or EGF on the production of TIMP-1 and collagenase protein from human skin, synovial and tendon fibroblasts.

Previous work has shown that retinoic acid down-regulates the production of interstitial collagenase protein from human skin and synovial fibroblasts (Brinckerhoff et al., 1980, Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991b) while simultaneously increasing the production of TIMP-1 protein and mRNA from these cells (Clark et al., 1987, Wright et al., 1991b). These parameters are also modulated by bFGF which has been shown to stimulate the production of mRNA and protein for both collagenase and TIMP-1 in human foetal lung fibroblasts (Edwards et al., 1987). Similarly, EGF also stimulates the production of mRNA and protein for collagenase and TIMP-1 in human foetal lung fibroblasts and the production of collagenase protein in human foreskin fibroblasts (Edwards et al., 1987, Chua et al., 1985).

Although these previous reports have investigated the effect of single growth factors or cytokines on the expression of collagenase and TIMP-1, very little attention has been given to possible interactions between 2 or more such factors. One report has shown that bFGF and EGF synergize with TGF-β to 'superinduce' TIMP-1 mRNA and protein from human foetal lung fibroblasts (Edwards et al., 1987), suggesting that important interactions do occur between factors when they are present simultaneously. Retinoic acid is strikingly similar to TGF-β in its actions on collagenase and TIMP-1...
secretion from fibroblasts (Brinckerhoff et al., 1980, Brinckerhoff et al., 1982, Bauer et al., 1982, Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991a, Edwards et al., 1987, Overall et al., 1989, Wright et al., 1991b). This suggests that retinoic acid may also synergize with bFGF and EGF to stimulate TIMP-1 production from fibroblasts. The upregulation of TIMP-1 production by cells is one possible means by which pathological connective tissue breakdown can be controlled as outlined in chapter 1. However, the prerequisite for this therapeutic goal is a detailed understanding of the manner in which TIMP-1 gene expression and protein production are regulated in connective tissue cells. These investigations into possible interactions between retinoic acid and polypeptide growth factors on TIMP-1 protein production are therefore pertinent to the achievement of this aim.

This work is published in Archives of Biochemistry and Biophysics and a copy of the paper is attached at the end of the thesis.
3.2. METHODS AND RESULTS

3.2.1. Cytotoxicity of all-trans-retinoic acid

The cytotoxicity of all-trans-retinoic acid in human fibroblasts was first assessed in order to ensure that the concentrations used in subsequent experiments were not toxic to the cells. This was monitored by comparing the quantity of lactate dehydrogenase (LDH) released into the culture medium from skin fibroblasts treated with retinoic acid to that released by cells in culture medium only. The effect of retinoic acid at concentrations of 10 mM-10 M on LDH release was investigated in triplicate in 3 human skin cell lines using the cell assay system described in chapter 2. After 72 hours, the cell supernates were harvested and assayed for LDH activity using a commercial kit supplied by Promega. The kit utilizes the conversion of a tetrazolium salt into a red formazan product by LDH, the absorbance of which can be measured at 492 nm. The method is based on that described by Nachlas et al. (1960). The cell supernates were stored at 4°C for a maximum of 7 days prior to assay. 50μl of culture medium from each test condition was pipetted in duplicate into 96-well plastic plates followed by the addition of 50μl of the substrate mixture supplied by the kit to each well. The plate was then incubated in the dark for 30 minutes. The reaction was stopped by the addition of 50μl per well of stop solution and the absorbance was measured at 492 nm. A positive control was included in each assay in duplicate which consisted of a 1/2000 fold dilution of LDH supplied by the kit. In order to assess the level of background absorbance caused by phenol red and LDH present in the culture medium, every assay also included unconditioned culture medium samples in duplicate. Statistical analysis was by one way analysis of variance. Surprisingly, in 2 out of the 3 cell lines investigated (hsf 13 and hsf 15), no cytotoxicity was seen even at the highest concentration of retinoic acid used (10 M). However, in the third cell line (hsf 9), LDH release rose significantly above that measured in unstimulated cells using 5 x 10 M retinoic acid and a further increase was seen in cells treated with 10 M retinoic acid. This is shown in figure 3.1. It was
Figure 3.1. (top)
Production of LDH activity by the human skin cell line hsf 9 (passage 3) following treatment with all-trans-retinoic acid.

Figure 3.2. (bottom)
Production of TIMP-1 protein by the human skin cell line hsf 9 (passage 3) following treatment with all-trans-retinoic acid.
therefore concluded that the highest concentration of retinoic acid which could be safely used in experiments without causing cytotoxicity was $10^{-5}$ M. This data was also applied to experiments with synovial and tendon fibroblasts.

### 3.2.2. The effect of all-trans-retinoic acid alone on TIMP-1 protein production in human skin fibroblasts.

Initially, the effect of retinoic acid only on TIMP-1 protein production from skin fibroblasts was investigated in order to find out which concentration gives the maximal induction of TIMP-1 protein. This concentration of retinoic acid could then be used in further experiments with bFGF or EGF additionally present, with the growth factor at a range of different concentrations. Previous work has shown that the maximal induction of TIMP-1 protein in human fibroblasts from various tissue origins occurs with either $10^{-6}$ M or $10^{-5}$ M all-trans-retinoic acid (Clark et al., 1987, Wright et al., 1991b).

The effect of all-trans-retinoic acid only ($10^{-6}$M-$10^{-4}$M) on TIMP-1 protein production was investigated in triplicate in 3 human skin cell lines using the cell assay system described in chapter 2. After 72 hours, the cell supernates were harvested and assayed for TIMP-1 by ELISA as described in chapter 2. The protein content of the cell monolayers was measured as described in chapter 2 and the levels of TIMP-1 were corrected with respect to the cell protein such that the results are expressed as ng of TIMP-1 per μg of cell protein. Statistical analysis was by one way analysis of variance.

In 2 of the cell lines investigated (hsf 13 and hsf 15), it was found that maximal induction of TIMP-1 protein was achieved at $10^{-4}$ M all-trans-retinoic acid; with lower concentrations having a smaller effect. In these cell lines, $10^{-4}$ M retinoic acid was not cytotoxic as described above. However, in the third cell line (hsf 9), the maximum induction of TIMP-1 protein occurred at a concentration of $10^{-5}$ M and higher levels caused a dramatic reduction in the output of TIMP-1 due to cytotoxicity at these higher concentrations. Figures 3.2. illustrates this.
10^{-5}\text{M} \text{ retinoic acid therefore appears to give the best induction of TIMP-1 protein without causing toxicity in any of the cell lines. Hence it was decided to use this concentration for further experiments investigating the effect of retinoic acid in combination with bFGF and EGF on TIMP-1 protein production.}

3.2.3. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human skin fibroblasts

The effect of 10^{-5}\text{M retinoic acid in combination with 1, 10 and 100ng/ml of bFGF or EGF on TIMP-1 protein production in 3 human skin cell lines was investigated using the cell assay system described in chapter 2. Each cell line was investigated twice. The effect of the following test conditions was examined in triplicate in each experiment. The concentration range used for the growth factors was based on previous investigations in which the effect of bFGF and EGF on TIMP-1 and collagenase secretion from human fibroblasts or rabbit periosteal tissue has been investigated (Chua et al., 1985, Edwards et al., 1987, van der Zee et al., 1993). In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.}

1. control - DMEM + 1\% ATFCS only
2. recombinant bFGF or recombinant EGF at concentrations of 1, 10 and 100ng/ml
3. all-trans-retinoic acid at 10^{-5}\text{M}
4. all-trans-retinoic acid at 10^{-5}\text{M} + recombinant bFGF or EGF at concentrations of 1, 10 and 100ng/ml.

After 72 hours, the production of TIMP-1 protein by the cells under these various conditions was assessed as described above in section 3.2.2. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.
In 10 out of 12 experiments, $10^4$M retinoic acid alone caused a significant increase in TIMP-1 production above that of the control cells. EGF also significantly stimulated TIMP-1 production in 4 out of 6 experiments but bFGF stimulated TIMP-1 production in only 1 experiment out of 6. When $10^4$M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either bFGF or EGF, a dose-dependent synergistic increase in TIMP-1 production was seen in all experiments although the magnitude of the response varied in different experiments. Figures 3.3. and 3.4. show the results of a representative experiment using $10^4$M retinoic acid applied in combination with 1, 10 and 100ng/ml of either bFGF or EGF respectively. Tables 3.1. and 3.2. show the results of all experiments performed.

3.2.4. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human synovial fibroblasts

The effect of $10^4$M retinoic acid in combination with 1, 10 and 100ng/ml of bFGF or EGF on TIMP-1 production in 3 human synovial fibroblast cell lines was also investigated. These experiments were performed in an identical manner to those described in section 3.2.3., the only difference being that synovial fibroblasts were used in place of skin fibroblasts. It is useful to investigate the effect of these agents in more than 1 type of human fibroblast in order to establish whether the effects seen with the skin fibroblasts are typical of all fibroblasts or whether they are specific to cells derived from the skin. The synovial fibroblasts were also investigated because of their obvious relevance to joint disease.

TIMP-1 was significantly stimulated in all experiments by $10^4$M retinoic acid alone. In addition, both bFGF and EGF alone had a significant effect on stimulating TIMP-1 production in all experiments. When $10^4$M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either bFGF or EGF, a dose-dependent synergistic increase in TIMP-1 was observed in all experiments although the magnitude of the response varied in different experiments. The results of 2 representative experiments using bFGF or EGF respectively are shown in figures 3.5. and 3.6. The results from
Figures 3.3. (top) and 3.4. (bottom)

Production of TIMP-1 protein by the human skin cell line hsf 12 (passage 12) following treatment with bFGF (figure 3.3.) or EGF (figure 3.4.) alone and in the additional presence of retinoic acid.
Table 3.1. The production of TIMP-1 protein by human skin fibroblasts following treatment with bFGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
### Table 3.2.
The production of TIMP-1 protein by human skin fibroblasts following treatment with EGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and EGF are as follows: ♦ p<0.05, ♦♦ p<0.01, ♦♦♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.98 +/- 0.15</td>
<td>3.76 +/- 0.05</td>
<td>8.30 +/- 0.03</td>
<td>23.37 +/- 2.05</td>
<td>11.75 +/- 0.20</td>
<td>3.60 +/- 0.06</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M</td>
<td>24.81 +/- 1.03 ***</td>
<td>10.31 +/- 0.35 ***</td>
<td>21.37 +/- 0.89 ***</td>
<td>28.69 +/- 2.18</td>
<td>15.11 +/- 0.82 **</td>
<td>7.53 +/- 0.25 ***</td>
</tr>
<tr>
<td>EGF 1ng/ml</td>
<td>8.31 +/- 0.19 ***</td>
<td>not done</td>
<td>12.71 +/- 0.37 ***</td>
<td>16.89 +/- 0.97</td>
<td>11.63 +/- 0.24</td>
<td>5.51 +/- 0.08 ***</td>
</tr>
<tr>
<td>EGF 10ng/ml</td>
<td>7.48 +/- 0.29 ***</td>
<td>4.59 +/- 0.09 ***</td>
<td>12.86 +/- 0.33 ***</td>
<td>23.67 +/- 1.18</td>
<td>12.32 +/- 0.79</td>
<td>5.32 +/- 0.22 ***</td>
</tr>
<tr>
<td>EGF 100ng/ml</td>
<td>6.93 +/- 0.11 ***</td>
<td>5.29 +/- 0.04 ***</td>
<td>11.71 +/- 0.37 ***</td>
<td>22.10 +/- 0.97</td>
<td>11.76 +/- 0.51</td>
<td>not done</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 1ng/ml</td>
<td>43.95 +/- 1.29 **</td>
<td>not done</td>
<td>36.25 +/- 1.70 ***</td>
<td>29.41 +/- 1.19</td>
<td>24.32 +/- 0.57 ***</td>
<td>26.34 +/- 1.05 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 10ng/ml</td>
<td>52.64 +/- 4.57 **</td>
<td>24.00 +/- 0.79 ***</td>
<td>34.29 +/- 0.57 ***</td>
<td>38.75 +/- 1.93 **</td>
<td>26.99 +/- 0.41 ***</td>
<td>24.15 +/- 0.76 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 100ng/ml</td>
<td>40.87 +/- 1.31 ***</td>
<td>23.27 +/- 0.71 ***</td>
<td>36.90 +/- 0.86 ***</td>
<td>41.05 +/- 1.76 ***</td>
<td>22.49 +/- 0.48 ***</td>
<td>not done</td>
</tr>
</tbody>
</table>
Figure 3.5. (top)
Production of TIMP-1 protein by the human synovial cell line SY 7 (passage 3) following treatment with bFGF alone and in the additional presence of retinoic acid.

Figure 3.6. (bottom)
Production of TIMP-1 protein by the human synovial cell line SY 4 (passage 6) following treatment with EGF alone and in the additional presence of retinoic acid.
Table 3.3. The production of TIMP-1 protein by human synovial fibroblasts following treatment with bFGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per µg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>SY 4 passage 6</td>
<td>SY 35 passage 8</td>
<td>SY 33 passage 4</td>
</tr>
<tr>
<td>Control</td>
<td>13.80 +/- 0.46</td>
<td>18.04 +/- 0.64</td>
<td>11.17 +/- 0.37</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M</td>
<td>22.38 +/- 1.02***</td>
<td>21.89 +/- 1.44*</td>
<td>19.61 +/- 0.64***</td>
</tr>
<tr>
<td>EGF 1ng/ml</td>
<td>28.21 +/- 1.43***</td>
<td>26.80 +/- 1.33***</td>
<td>17.09 +/- 0.38***</td>
</tr>
<tr>
<td>EGF 10ng/ml</td>
<td>31.15 +/- 0.91***</td>
<td>25.58 +/- 0.76***</td>
<td>17.48 +/- 0.26***</td>
</tr>
<tr>
<td>EGF 100ng/ml</td>
<td>31.36 +/- 1.29***</td>
<td>24.34 +/- 0.48***</td>
<td>16.63 +/- 0.40***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 1ng/ml</td>
<td>67.02 +/- 3.26***</td>
<td>36.76 +/- 1.07**</td>
<td>38.63 +/- 1.30***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 10ng/ml</td>
<td>79.36 +/- 1.72***</td>
<td>39.42 +/- 1.25***</td>
<td>38.75 +/- 1.33***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5M + EGF 100ng/ml</td>
<td>73.77 +/- 2.06***</td>
<td>43.92 +/- 2.32***</td>
<td>33.83 +/- 0.65***</td>
</tr>
</tbody>
</table>

Table 3.4. The production of TIMP-1 protein by human synovial fibroblasts following treatment with EGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per µg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and EGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
all experiments are shown in tables 3.3. and 3.4.

The results show that synovial fibroblasts respond to retinoic acid in combination with bFGF or EGF with a synergistic induction of TIMP-1 in a similar manner to skin fibroblasts. However, the magnitude of the responses is generally smaller in synovial fibroblasts than in skin fibroblasts.

3.2.5. The effect of all-trans-retinoic acid in combination with bFGF or EGF on TIMP-1 protein production in human tendon fibroblasts

The effect of 10⁻⁴M retinoic acid in combination with 1, 10 and 100ng/ml of bFGF or EGF on TIMP-1 production in 3 human tendon fibroblast cell lines was investigated. These experiments were performed in an identical manner to those described in section 3.2.3., the only difference being that tendon fibroblasts were used in place of skin fibroblasts.

TIMP-1 was significantly stimulated in all experiments by 10⁻⁴M retinoic acid alone. In addition, both bFGF and EGF alone had a significant effect on stimulating TIMP-1 production in all experiments. When 10⁻⁴M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either bFGF or EGF, a dose-dependent synergistic increase in TIMP-1 was observed in all experiments although the magnitude of the response varied in different experiments. The results of 2 representative experiments using bFGF or EGF respectively are shown in figures 3.7. and 3.8. The results from all experiments are shown in tables 3.5. and 3.6. The results show that tendon fibroblasts respond to retinoic acid in combination with bFGF or EGF with a synergistic induction of TIMP-1 in a similar manner to skin fibroblasts.

3.2.6. The effect of specific neutralising antibodies to bFGF or EGF on the synergistic induction of TIMP-1 in response to retinoic acid and bFGF or EGF

The effect on TIMP-1 protein production of 10⁻⁴M retinoic acid in combination with bFGF or EGF in the additional presence of neutralising antibodies to the growth factors was investigated using 3 synovial cell lines (bFGF and retinoic acid) or 3 skin
Production of TIMP-1 protein by the human tendon cell line HTB 14 (passage 6) following treatment with bFGF alone and in the additional presence of retinoic acid.

**Figure 3.7. (top)**

Production of TIMP-1 protein by the human tendon cell line HTB 27 (passage 4) following treatment with EGF alone and in the additional presence of retinoic acid.

**Figure 3.8. (bottom)**
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>HTB 14 passage 6</td>
<td>HTB 6 passage 4</td>
<td>HTB 27 passage 5</td>
</tr>
<tr>
<td>Control</td>
<td>10.10 +/- 0.67</td>
<td>20.04 +/- 0.64</td>
<td>7.53 +/- 0.18</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>19.24 +/- 1.89 **</td>
<td>30.29 +/- 1.17 ***</td>
<td>10.49 +/- 0.31 ***</td>
</tr>
<tr>
<td>bFGF 1ng/ml</td>
<td>14.09 +/- 0.76 **</td>
<td>30.26 +/- 0.93 ***</td>
<td>9.90 +/- 0.25 ***</td>
</tr>
<tr>
<td>bFGF 10ng/ml</td>
<td>22.59 +/- 1.23 ***</td>
<td>40.15 +/- 1.61 ***</td>
<td>14.91 +/- 1.13 ***</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>18.29 +/- 0.74 ***</td>
<td>40.24 +/- 1.48 ***</td>
<td>13.65 +/- 0.65 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 1ng/ml</td>
<td>36.77 +/- 2.51 ***</td>
<td>46.86 +/- 1.50 **</td>
<td>22.87 +/- 0.81 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 10ng/ml</td>
<td>73.40 +/- 0.90 ***</td>
<td>85.22 +/- 2.67 ***</td>
<td>29.97 +/- 1.28 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 100ng/ml</td>
<td>83.95 +/- 6.06 ***</td>
<td>119.24 +/- 10.83 ***</td>
<td>31.89 +/- 0.86 ***</td>
</tr>
</tbody>
</table>

Table 3.5. The production of TIMP-1 protein by human tendon fibroblasts following treatment with bFGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>HTB 6 passage 6</td>
<td>HTB 14 passage 6</td>
<td>HTB 27 passage 4</td>
</tr>
<tr>
<td>Control</td>
<td>8.96 +/- 0.60</td>
<td>12.07 +/- 0.44</td>
<td>4.28 +/- 0.36</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁵ M</td>
<td>13.41 +/- 0.93 **</td>
<td>20.61 +/- 0.63 ***</td>
<td>9.85 +/- 0.32 ***</td>
</tr>
<tr>
<td>EGF 1ng/ml</td>
<td>13.51 +/- 0.43 ***</td>
<td>17.22 +/- 0.64 ***</td>
<td>6.65 +/- 0.46 **</td>
</tr>
<tr>
<td>EGF 10ng/ml</td>
<td>12.86 +/- 0.86 **</td>
<td>18.46 +/- 0.22 ***</td>
<td>7.04 +/- 0.24 ***</td>
</tr>
<tr>
<td>EGF 100ng/ml</td>
<td>9.00 +/- 1.33</td>
<td>15.45 +/- 0.70 **</td>
<td>6.22 +/- 0.29 **</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁵ M + EGF 1ng/ml</td>
<td>25.45 +/- 1.34 ***</td>
<td>44.45 +/- 1.74 ***</td>
<td>30.58 +/- 0.42 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁵ M + EGF 10ng/ml</td>
<td>23.33 +/- 1.23 **</td>
<td>43.85 +/- 1.96 ***</td>
<td>26.75 +/- 1.42 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁵ M + EGF 100ng/ml</td>
<td>21.89 +/- 2.92</td>
<td>47.36 +/- 6.24 **</td>
<td>26.16 +/- 0.38 ***</td>
</tr>
</tbody>
</table>

Table 3.6. The production of TIMP-1 protein by human tendon fibroblasts following treatment with EGF either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and EGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
cell lines (EGF and retinoic acid). This was in order to demonstrate that these effects resulted from the presence of the growth factors and not from an experimental artefact such as bacterial endotoxin.

The effect of the following test conditions was examined in triplicate using the cell assay system described in chapter 2. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously. The antibodies were obtained from a commercial source and control sera were therefore not available. The concentration of antibody used in each case was based on data supplied by the manufacturer from which the amount of antibody required to give full neutralization of the concentration of growth factor used was calculated.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF at 100ng/ml or recombinant EGF at 10ng/ml
3. all-trans-retinoic acid at 10^{-5}M
4. all-trans-retinoic acid at 10^{-5}M + recombinant bFGF at 100ng/ml or recombinant EGF at 10ng/ml
5. all-trans-retinoic acid at 10^{-5}M + recombinant bFGF at 100ng/ml + polyclonal neutralising antibody to bFGF at 500μg/ml
6. control + polyclonal neutralising antibody to EGF at 20μg/ml
   recombinant EGF at 10ng/ml + polyclonal neutralising antibody to EGF at 20μg/ml
   all-trans-retinoic acid at 10^{-5}M + polyclonal neutralising antibody to EGF at 20μg/ml
   all-trans-retinoic acid at 10^{-5}M + recombinant EGF at 10ng/ml + polyclonal neutralising antibody to EGF at 20μg/ml.

After 72 hours, the production of TIMP-1 protein by the cells under these various conditions was assessed as described above in section 3.2.2.
In all experiments, 10^{-5} M retinoic acid and 100 ng/ml of bFGF both caused a small increase in TIMP-1 production when applied alone to synovial fibroblasts and a much greater synergistic increase in TIMP-1 when applied together. When 10^{-5} M retinoic acid and 100 ng/ml of bFGF were applied to human synovial fibroblasts in the additional presence of 500 µg/ml of the polyclonal neutralising antibody to bFGF, the synergistic induction of TIMP-1 was completely abolished in all experiments. Figure 3.9 shows a representative experiment.

Similarly, 10^{-5} M retinoic acid and 10 ng/ml of EGF each caused a small increase in TIMP-1 production when applied alone to skin fibroblasts and a much greater synergistic increase in TIMP-1 when applied together. When 10^{-5} M retinoic acid and 10 ng/ml of EGF were applied to skin fibroblasts in the additional presence of 20 µg/ml of the polyclonal neutralising antibody to EGF, the synergistic induction of TIMP-1 was completely abolished in all experiments. Figure 3.10 shows a representative experiment.

These experiments therefore indicate that the observed synergistic induction of TIMP-1 protein in response to retinoic acid combined with bFGF or EGF is caused specifically by the growth factors.

### 3.2.7. The effect of all-trans-retinoic acid in combination with bFGF or EGF on collagenase production in human skin, synovial and tendon fibroblasts

The results above indicate that retinoic acid in combination with bFGF or EGF causes a 'superinduction' of TIMP-1 from skin, synovial and tendon fibroblasts. Such an effect would be expected to have an inhibitory effect on connective tissue breakdown by these cells. However, if these agents in combination also induce matrix metalloproteinase production then the effect on connective tissue breakdown may be stimulatory. The dismantling of collagen by collagenases is believed to be the rate-limiting step in irreversible connective tissue breakdown, as outlined in chapter 1 of this thesis. These enzymes therefore play a key role in extracellular matrix turnover. The effect of 10^{-5} M retinoic acid in combination with 1, 10 and 100 ng/ml of bFGF or
Figures 3.9. (top) and 3.10. (bottom)

Effect of specific neutralizing antibodies to either bFGF (figure 3.9.) or EGF (figure 3.10.) on the synergistic induction of TIMP-1 protein from the human synovial cell line SY 33 (passage 6) treated with retinoic acid and bFGF (figure 3.9.) or the human skin cell line hsf 15 (passage 2) treated with retinoic acid and EGF (figure 3.10.).
EGF on collagenase protein production in human skin, synovial and tendon fibroblasts was therefore investigated. The harvested culture medium from the experiments described in sections 3.2.3., 3.2.4. and 3.2.5. was measured for levels of interstitial collagenase using the specific ELISA described in chapter 2. The levels of collagenase were corrected with respect to cell protein such that results are expressed as ng of collagenase per μg of cell protein. Statistical analysis was by one way analysis of variance.

Both bFGF and EGF when added alone at 1, 10 and 100ng/ml stimulated collagenase in all experiments with skin fibroblasts. Collagenase production was decreased compared to the control levels when 10⁻⁵M retinoic acid was added alone in all except 1 experiment with these cells. When 10⁻⁵M retinoic acid was added in combination with 1, 10 and 100ng/ml of bFGF or EGF to skin fibroblasts, retinoic acid inhibited bFGF-stimulated collagenase and EGF-stimulated collagenase in all experiments. Figures 3.11. and 3.12. show representative experiments using bFGF and EGF respectively and tables 3.7. and 3.8. show the results of all experiments.

Similar results were obtained with synovial fibroblasts (data not shown) although in the case of EGF, results were obtained from only 2 experiments out of 6 as the collagenase levels were below detection in the rest of the experiments. In the case of the tendon fibroblasts (data not shown), retinoic acid inhibited bFGF-stimulated collagenase in 2 out of 3 experiments. In these cells, EGF-stimulated collagenase was inhibited by retinoic acid in 2 experiments and in the third experiment, the levels of collagenase were below detection.
Production of collagenase protein by the human skin cell line hsf 13 (passage 13) following treatment with bFGF alone and in the additional presence of retinoic acid.

Production of collagenase protein by the human skin cell line hsf 15 (passage 5) following treatment with EGF alone and in the additional presence of retinoic acid.
Table 3.7. The production of collagenase protein by human skin fibroblasts following treatment with bFGF either alone or in the additional presence of retinoic acid. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of bFGF alone are as follows: ♦ ♦ ♦ p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 13</td>
<td>hsf 15</td>
<td>hsf 15</td>
<td>hsf 12</td>
</tr>
<tr>
<td></td>
<td>passage 11</td>
<td>passage 5</td>
<td>passage 6</td>
<td>passage 12</td>
</tr>
<tr>
<td>Control</td>
<td>1.60 +/-</td>
<td>1.71 +/-</td>
<td>4.94 +/-</td>
<td>0.38 +/-</td>
</tr>
<tr>
<td></td>
<td>0.57</td>
<td>0.00</td>
<td>0.26</td>
<td>0.008</td>
</tr>
<tr>
<td>Retinoic Acid 10^-M</td>
<td>0.21 +/-</td>
<td>0.12 +/-</td>
<td>1.28 +/-</td>
<td>0.16 +/-</td>
</tr>
<tr>
<td></td>
<td>0.01 ***</td>
<td>0.04 ***</td>
<td>0.05 ***</td>
<td>0.004 ***</td>
</tr>
<tr>
<td>EGF 1ng/ml</td>
<td>not done</td>
<td>6.08 +/-</td>
<td>3.70 +/-</td>
<td>6.45 +/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19 ***</td>
<td>0.28</td>
<td>0.04 ***</td>
</tr>
<tr>
<td>EGF 10ng/ml</td>
<td>8.19 +/-</td>
<td>6.39 +/-</td>
<td>12.23 +/-</td>
<td>5.76 +/-</td>
</tr>
<tr>
<td></td>
<td>0.74 ***</td>
<td>0.12 ***</td>
<td>1.00 ***</td>
<td>0.05 ***</td>
</tr>
<tr>
<td>EGF 100ng/ml</td>
<td>7.15 +/-</td>
<td>5.31 +/-</td>
<td>16.25 +/-</td>
<td>not done</td>
</tr>
<tr>
<td></td>
<td>0.08 ***</td>
<td>0.07 ***</td>
<td>0.80 ***</td>
<td></td>
</tr>
<tr>
<td>Retinoic Acid 10^-M + EGF 1ng/ml</td>
<td>0.48 +/-</td>
<td>0.48 +/-</td>
<td>1.56 +/-</td>
<td>0.35 +/-</td>
</tr>
<tr>
<td></td>
<td>0.04 ***</td>
<td>0.13 ***</td>
<td>0.13 ***</td>
<td>0.01 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-M + EGF 10ng/ml</td>
<td>0.34 +/-</td>
<td>0.34 +/-</td>
<td>3.18 +/-</td>
<td>0.34 +/-</td>
</tr>
<tr>
<td></td>
<td>0.02 ***</td>
<td>0.02 ***</td>
<td>0.23 ***</td>
<td>0.03 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-M + EGF 100ng/ml</td>
<td>0.42 +/-</td>
<td>0.47 +/-</td>
<td>3.22 +/-</td>
<td>not done</td>
</tr>
<tr>
<td></td>
<td>0.02 ***</td>
<td>0.01 ***</td>
<td>0.16 ***</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8. The production of collagenase protein by human skin fibroblasts following treatment with EGF either alone or in the additional presence of retinoic acid. The results are expressed as collagenase in ng per µg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of EGF alone are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
3.3. DISCUSSION

The results described in this chapter confirm previous investigations into the effect of retinoic acid, bFGF and EGF alone on TIMP-1 and collagenase secretion from human fibroblasts and describe for the first time the effect of the agents in combination on these proteins. The production of TIMP-1 and collagenase by human skin and synovial fibroblasts has previously been shown to be modulated by retinoids (Brinckerhoff et al., 1980, Bauer et al., 1983, Clark et al., 1987, Wright et al., 1991b). The data reported in this chapter are in close correlation with these findings since they show that TIMP-1 secretion is induced in skin, synovial and tendon fibroblasts in response to all-trans-retinoic acid while collagenase secretion is inhibited. The finding that bFGF and EGF stimulate collagenase production from skin, synovial and tendon fibroblasts is also in agreement with previous work using human foetal lung fibroblasts or foreskin fibroblasts in which similar effects have been found (Edwards et al., 1987, Chua et al., 1985).

In contrast to the previous report of Edwards et al. (1987) using foetal lung fibroblasts, there was no increase in TIMP-1 production by human skin fibroblasts in response to bFGF although TIMP-1 production was stimulated in human synovial and tendon fibroblasts by this growth factor. These discrepancies may indicate intrinsic differences in the response of different types of fibroblastic cells to bFGF or inconsistencies due to differences in the age of the subjects from whom the cells were derived. Alternatively, differences may result from variations in experimental protocol between this study and that of Edwards et al. (1987). The results in this chapter also show that skin, synovial and tendon fibroblasts respond to EGF with increased TIMP-1 production which correlates closely with the previous report by Edwards et al. (1987) using foetal lung fibroblasts.

Although the effect of retinoic acid, bFGF or EGF alone on fibroblasts has previously been described, this is the first study into the effect of retinoic acid in combination with these growth factors on TIMP-1 and collagenase production in fibroblasts. This investigation has shown for the first time that retinoic acid interacts synergistically
with both bFGF and EGF to stimulate the production of TIMP-1 protein from skin, synovial and tendon fibroblasts. It was postulated that retinoic acid might resemble TGF-β in its ability to synergize with bFGF and EGF to stimulate TIMP-1. The findings of this chapter show that this is indeed the case and serve to underline further the similarities between these 2 factors.

The synergistic effects are dose-dependent across a range of growth factor concentrations and are greater than the additive effect by up to 4 fold. The experiments using specific neutralising antibodies to the growth factors show that the synergistic induction of TIMP-1 is caused specifically by the growth factors rather than by an experimental artefact such as contaminating bacterial endotoxin. In addition, the concentration of retinoic acid used in these experiments is not toxic to the cells, as shown by the cytotoxicity data, although it is considerably greater than normal physiological concentrations of retinoids in serum. Retinol is normally present in serum at approximately $2 \times 10^{-6}$M bound to RBP and retinoic acid is additionally present at approximately $1 \times 10^{-8}$M bound to albumin (Goodman, 1984, Wolf, 1984, Goodman & Blaner, 1984).

The overall pattern of the synergistic induction of TIMP-1 was similar for all the cell lines investigated but the synovial fibroblasts generally gave a weaker response to the growth factors and retinoic acid than the skin and tendon fibroblasts. This discrepancy may be caused by differences in the tissue origin of the fibroblasts or the disease status of the tissue since the skin and tendon cells are from healthy tissue while the synovial cells are from arthritic synovium. It is possible that fibroblasts derived from diseased tissue are defective in their ability to produce TIMP-1 and that this abnormality contributes to the observed pathological connective tissue breakdown. The fact that 3 different types of human fibroblast all respond to retinoic acid in combination with bFGF or EGF to give a synergistic stimulation of TIMP-1 suggests that these responses are general phenomena of human fibroblasts rather than a specific response of cells of a certain tissue.
The mechanism by which retinoic acid and bFGF or EGF synergize to stimulate the production of TIMP-1 from fibroblasts is intriguing and is addressed in detail in later chapters of the thesis. A number of different theories can be put forward to explain these effects. One possibility is that the cells are rendered more sensitive to the effects of either retinoic acid or the growth factors due to the induction of receptors for one factor by the other. A previous report has shown that retinoic acid enhances the binding of $^{125}$I-labelled EGF to fibroblastic and epidermal mouse cell lines (Jetten, 1980). Other reports have shown that retinoic acid can also enhance the binding of EGF to normal rat kidney cells (Roberts et al., 1984). It is therefore possible that retinoic acid also increases the number of EGF receptors on human fibroblasts and that this increase is coupled to an increase in TIMP-1 production, particularly as the data also show that EGF alone can stimulate the production of TIMP-1 in these cells. In further support of this theory, a report has shown that retinoic acid potentiates the mitogenic effect of EGF in human skin fibroblasts (Harper and Savage, 1980). It is possible that the effects of bFGF and retinoic acid on skin, synovial and tendon fibroblasts could also be explained by a similar hypothesis - i.e. retinoic acid acts to increase the number of receptors for bFGF on fibroblasts. However, the drawback of this theory is that it fails to explain why bFGF- and EGF-stimulated collagenase is abrogated rather than upregulated in the presence of retinoic acid.

Another possibility is that the growth factors may induce the production of receptors for retinoic acid which could cause an increased sensitivity of the cells to retinoic acid. This in turn could result in the augmented TIMP-1 production and abrogated collagenase secretion which the data shows. The possibility that the synergistic mechanisms involve the induction of receptors for 1 agent by the other is considered in detail in chapter 6 of the thesis. Alternatively, the observed synergistic increases in TIMP-1 may not be due to an effect on any of the receptors involved but instead are due to synergistic increases in transcription from the TIMP-1 gene.

The effect of retinoic acid when applied in combination with bFGF or EGF to fibroblasts on collagenase secretion was also investigated. It was found that retinoic
acid blocked the induction of collagenase from skin, synovial and tendon fibroblasts in response to the growth factors such that growth factor-stimulated collagenase was either totally or almost totally inhibited. Retinoic acid is thus able to modify acutely the response of skin, synovial and tendon fibroblasts to bFGF and EGF. When either of these growth factors is applied alone, the cells are stimulated to produce collagenase and to a lesser extent TIMP-1 and such a situation may encourage extracellular matrix breakdown. When the growth factors are applied in the presence of retinoic acid, extracellular matrix synthesis is favoured since retinoic acid downregulates the production of collagenase by these growth factors and also causes an increase in TIMP-1 which is greater than that produced by the additive effect of the agents.

The data presented in this chapter therefore provide evidence that cross-talk between retinoids and growth factors does occur. Furthermore, this cross-talk may result in an inhibition of connective tissue breakdown and thereby contribute to the ability of retinoids to suppress disease activity in animal models of rheumatoid arthritis. However, the findings of this investigation are puzzling in the light of other work showing that retinoic acid induces proteoglycan loss from cartilage (e.g. Fell and Mellanby, 1952) since they suggest that retinoic acid is an agent which prevents connective tissue breakdown. This discrepancy may be due to the different cell types involved (chondrocytes instead of fibroblasts) or to the fact that retinoids are potent stimulators of plasminogen activator production which has been suggested to play a key role in cartilage resorption via plasmin production (Meats et al., 1985). Another possibility is that retinoids have a biphasic effect such that lower concentrations have a stimulatory effect on metalloproteinase production. There is some evidence to suggest that this is the case for human skin and synovial fibroblast collagenase (Clark et al., 1987, Wright et al., 1991b). However, most of the work which has been done on the effect of retinoids on proteoglycan loss from cartilage has in fact utilized high concentrations of retinoids (10^-5 M to 10^-4 M). These concentrations have been shown to be effective in inducing proteoglycan and in some cases collagen release from
cartilage derived from a number of species (Fell and Mellanby, 1952, Dingle et al., 1966, Dingle et al., 1975, Dingle and Dingle, 1980, Jubb and Fell, 1980, Hembry et al., 1982, Caputo et al., 1987, Campbell and Handley, 1987, Buttle et al., 1993). A further possibility is that the breakdown of cartilage collagen in response to retinoids is mediated by the induction of MMP-13 (collagenase-3). In contrast to MMP-1 where all previous reports show repression in response to retinoic acid, it has been shown that MMP-13 is induced by retinoids in rodent chondrocytes and bone cells (Ballock et al., 1994, Heath et al., 1990, Connolly et al., 1994). It is therefore possible that the effect of retinoic acid on cartilage collagen can be explained by the induction of this enzyme in chondrocytes. Retinoic acid may also stimulate the production of 'aggrecanase', the putative enzyme believed to be responsible for the breakdown of cartilage proteoglycan.
CHAPTER FOUR

THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH PDGF-BB OR TGF-β ON TIMP-1 AND COLLAGENASE PROTEIN SECRETION FROM HUMAN SKIN, SYNOVIAL AND TENDON FIBROBLASTS.

4.1. INTRODUCTION

The results presented in chapter 3 showed that cross-talk occurs between all-trans-retinoic acid and 2 polypeptide growth factors, namely bFGF and EGF in the effect of these agents on collagenase and TIMP-1 secretion from human fibroblasts. This chapter examines the interaction of all-trans-retinoic acid with 2 further growth factors on TIMP-1 and collagenase production from these cells.

Preliminary investigations in the Rheumatology Research Unit have indicated that retinoic acid and mononuclear cell factor synergistically stimulate the production of TIMP-1 protein from human fibroblasts (unpublished observations). Mononuclear cell factor is a human blood monocyte-derived cytokine mixture that stimulates synovial fibroblasts to produce collagenase and prostaglandin E₂ (Dayer et al., 1979). The activity of mononuclear cell factor has been shown to be primarily due to IL-1 (Mizel et al., 1981, Dayer et al., 1986) but it also contains a number of other cytokines and growth factors. These may include growth factors such as PDGF which is produced by monocytes (Heldin et al., 1993) and TGF-β. It is therefore important to pinpoint which factor or factors are causing this synergistic effect in the presence of retinoic acid. This question has been addressed by investigating the effect of retinoic acid in combination with PDGF or TGF-β on TIMP-1 protein induction from fibroblasts.

A further reason for investigating PDGF is that this growth factor has many similarities with bFGF and EGF in its effects on mesenchymal cells and in its intracellular mechanism of action. bFGF, EGF and PDGF are all potent mitogens for
mesenchymal cells (Gospodarowicz et al., 1987, Carpenter and Zendegui, 1986, Heldin et al., 1985) and are all known to stimulate the production of collagenase and TIMP-1 protein from human fibroblasts (Chua et al., 1985, Bauer et al., 1985, Edwards et al., 1987, Circolo et al., 1991). In addition, these 3 growth factors are all believed to elicit their effects on cells via receptor tyrosine kinases and subsequently via a similar pool of intracellular substrates. The expression of proteins such as c-fos are also stimulated by all three growth factors in fibroblasts (Müller et al., 1984, Kruijer et al., 1984). Given these similarities between bFGF, EGF and PDGF, it is reasonable to hypothesize that PDGF might also resemble the other 2 growth factors in their ability to cause a synergistic increase in TIMP-1 in the presence of retinoic acid.

The aim of this chapter is therefore to extend the findings of chapter 3 by investigating whether or not retinoic acid is able to interact synergistically with these 2 additional growth factors to 'superinduce' TIMP-1 from human fibroblasts.

This work is published in the Journal of Cellular Physiology and a copy of the paper is attached at the end of the thesis.
4.2. METHODS AND RESULTS

4.2.1. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on TIMP-1 protein production in human skin fibroblasts

The effect of 10^5 M retinoic acid in combination with 1, 10 and 100 ng/ml of PDGF-BB or TGF-β on TIMP-1 protein production in 3 human skin cell lines was investigated using the cell assay system described in chapter 2. PDGF-BB was used rather than either of the other 2 isoforms (PDGF-AA and PDGF-AB) since only this form will bind to all types of receptor dimer on cells (Hart and Bowen-Pope, 1990). The effect of the following test conditions was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant PDGF-BB or purified TGF-β at concentrations of 1, 10 and 100 ng/ml
3. all-trans-retinoic acid at 10^5 M
4. all-trans-retinoic acid at 10^5 M + recombinant PDGF-BB or purified TGF-β at concentrations of 1, 10 and 100 ng/ml.

After 72 hours, the cell supernates were harvested and assayed for TIMP-1 by ELISA as described in chapter 2. The DNA content of the cell monolayers was measured as described in chapter 2 and the levels of TIMP-1 were corrected with respect to the cell DNA such that the results are expressed as ng of TIMP-1 per µg of cell DNA. In some experiments, the protein content of the cell monolayer was measured instead of the DNA content and the results are expressed as ng of TIMP-1 per µg of cell protein. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.

In 5 out of 8 experiments, 10^5 M retinoic acid caused a significant increase in TIMP-1 production above that of control cells. PDGF-BB and TGF-β alone had no significant
effect on TIMP-1 production in most experiments; a significant stimulation was seen in only 1 experiment out of 4 in each case. When $10^{-8}$M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either PDGF-BB or TGF-β, a dose-dependent synergistic increase in TIMP-1 production was seen in all experiments although there was some variation in the magnitude of the response. Figures 4.1. and 4.2. show the results of a representative experiment using $10^{-8}$M retinoic acid applied in combination with 1, 10 and 100ng/ml of either PDGF-BB or TGF-β respectively. Tables 4.1. and 4.2. show the results of all experiments performed.

4.2.2. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on TIMP-1 protein production in human synovial fibroblasts

The effect of $10^{-8}$M retinoic acid in combination with 1, 10 and 100ng/ml of PDGF-BB or TGF-β on TIMP-1 production in 3 human synovial fibroblast cell lines was also investigated. These experiments were performed in an identical manner to those described in section 4.2.1., the only difference being that synovial fibroblasts were used in place of skin fibroblasts. TIMP-1 was significantly stimulated in 5 out of 6 experiments by $10^{-8}$M retinoic acid alone. PDGF-BB and TGF-β alone stimulated TIMP-1 production in some experiments (2 out of 3 and 1 out of 3 respectively). When $10^{-8}$M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either PDGF-BB or TGF-β, a dose-dependent synergistic increase in TIMP-1 was observed in all experiments although the magnitude of the response varied in different experiments.

The results of 2 representative experiments using PDGF-BB or TGF-β respectively are shown in figures 4.3. and 4.4. respectively. The results from all experiments are shown in tables 4.3. and 4.4.

4.2.3. The effect of all-trans-retinoic acid in combination with PDGF-BB and TGF-β on TIMP-1 protein production in human tendon fibroblasts

The effect of $10^{-8}$M retinoic acid in combination with 1, 10 and 100ng/ml of PDGF-
Production of TIMP-1 protein by the human skin cell line hsf 13 (passage 10) following treatment with PDGF-BB alone and in the additional presence of retinoic acid.

Figure 4.1. (top)
Production of TIMP-1 protein by the human skin cell line hsf 13 (passage 10) following treatment with PDGF-BB alone and in the additional presence of retinoic acid.

Figure 4.2. (bottom)
Production of TIMP-1 protein by the human skin cell line hsf 12 (passage 10) following treatment with TGF-β alone and in the additional presence of retinoic acid.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1aga</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
<td>hsf 15 passage 7</td>
<td>hsf 13 passage 8</td>
<td>hsf 13 passage 10</td>
<td>hsf 12 passage 11</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>88.37 +/- 4.90</td>
<td>425.00 +/- 93.56</td>
<td>58.60 +/- 2.82</td>
<td>58.60 +/- 1.76</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M</td>
<td>95.97 +/- 1.08</td>
<td>700.00 +/- 40.41 *</td>
<td>91.49 +/- 1.05 ***</td>
<td>56.16 +/- 1.24</td>
</tr>
<tr>
<td>PDGF-BB 1ng/ml</td>
<td>68.17 +/- 0.99</td>
<td>256.67 +/- 11.74</td>
<td>53.38 +/- 2.58</td>
<td>55.96 +/- 2.31</td>
</tr>
<tr>
<td>PDGF-BB 10ng/ml</td>
<td>82.80 +/- 0.81</td>
<td>293.33 +/- 25.12</td>
<td>45.19 +/- 1.39</td>
<td>53.40 +/- 3.17</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml</td>
<td>110.63 +/- 2.92 **</td>
<td>236.67 +/- 23.89</td>
<td>44.74 +/- 1.47</td>
<td>52.54 +/- 5.61</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M + PDGF-BB 1ng/ml</td>
<td>86.83 +/- 1.93</td>
<td>1003.33 +/- 84.43 **</td>
<td>166.75 +/- 3.20 ***</td>
<td>102.20 +/- 4.01 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M + PDGF-BB 10ng/ml</td>
<td>208.77 +/- 7.98 ***</td>
<td>1021.67 +/- 91.90 **</td>
<td>249.77 +/- 4.62 ***</td>
<td>151.30 +/- 10.26 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M + PDGF-BB 100ng/ml</td>
<td>173.47 +/- 3.10 ***</td>
<td>1310 +/- 22.94 ***</td>
<td>371.50 +/- 12.68 ***</td>
<td>219.58 +/- 12.06 ***</td>
</tr>
</tbody>
</table>

**Table 4.1.** The production of TIMP-1 protein by human skin fibroblasts following treatment with PDGF-BB either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell DNA. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and PDGF-BB are as follows: • p<0.05, • p<0.01, •• p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 12 passage 10</td>
<td>hsf 13 passage 9</td>
<td>hsf 13 passage 7</td>
<td>hsf 15 passage 2</td>
</tr>
<tr>
<td>Control</td>
<td>68.76 +/- 1.61</td>
<td>124.17 +/- 5.78</td>
<td>2.00 +/- 0.11</td>
<td>65.13 +/- 3.42</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M</td>
<td>67.60 +/- 1.89</td>
<td>192.00 +/- 6.64 ***</td>
<td>3.64 +/- 0.11 ***</td>
<td>186.07 +/- 6.36 ***</td>
</tr>
<tr>
<td>TGF-β 1ng/ml</td>
<td>54.64 +/- 3.96</td>
<td>103.85 +/- 6.44</td>
<td>2.36 +/- 0.09</td>
<td>68.97 +/- 1.22</td>
</tr>
<tr>
<td>TGF-β 10ng/ml</td>
<td>47.33 +/- 1.93</td>
<td>119.61 +/- 15.99</td>
<td>2.07 +/- 0.14</td>
<td>78.92 +/- 2.74 *</td>
</tr>
<tr>
<td>TGF-β 100ng/ml</td>
<td>56.21 +/- 6.31</td>
<td>78.36 +/- 4.44</td>
<td>2.12 +/- 0.02</td>
<td>88.51 +/- 0.95 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-4}M + TGF-β 1ng/ml</td>
<td>147.98 +/- 13.51 ***</td>
<td>175.17 +/- 2.31</td>
<td>6.00 +/- 0.23 ***</td>
<td>295.80 +/- 14.60 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-4}M + TGF-β 10ng/ml</td>
<td>227.33 +/- 4.94 ***</td>
<td>331.67 +/- 15.33 ***</td>
<td>8.13 +/- 0.56 ***</td>
<td>319.17 +/- 31.79 **</td>
</tr>
<tr>
<td>Retinoic Acid 10^{-5}M + TGF-β 100ng/ml</td>
<td>266.15 +/- 15.34 ***</td>
<td>443.33 +/- 24.44 ***</td>
<td>7.83 +/- 0.63 ***</td>
<td>415.83 +/- 17.06 ***</td>
</tr>
</tbody>
</table>

Table 4.2. The production of TIMP-1 protein by human skin fibroblasts following treatment with TGF-β either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell DNA except in the case of hsf 13 passage 7 in which the results are expressed as ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and TGF-β are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
Figure 4.3. (top)
Production of TIMP-1 protein by the human synovial cell line SY 7 (passage 5) following treatment with PDGF-BB alone and in the additional presence of retinoic acid.

Figure 4.4. (bottom)
Production of TIMP-1 protein by the human synovial cell line SY 7 (passage 4) following treatment with TGF-β alone and in the additional presence of retinoic acid.
Table 4.3. The production of TIMP-1 protein by human synovial fibroblasts following treatment with PDGF-BB either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell DNA for SY 31 passage 5 and ng per μg of cell protein for the other 2 experiments. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and PDGF-BB are as follows: ♦ p<0.05, » ♦ p<0.01, ♦ ♦ ♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>SY 7</td>
<td>SY 33</td>
<td>SY 31</td>
</tr>
<tr>
<td></td>
<td>passage 5</td>
<td>passage 4</td>
<td>passage 5</td>
</tr>
<tr>
<td>Control</td>
<td>8.82 +/- 0.63</td>
<td>4.98 +/- 0.075</td>
<td>161.31 +/- 9.75</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>10.78 +/- 0.22 *</td>
<td>10.38 +/- 0.33 ***</td>
<td>326.53 +/- 42.63 **</td>
</tr>
<tr>
<td>PDGF-BB 1ng/ml</td>
<td>9.78 +/- 0.37</td>
<td>5.23 +/- 0.14</td>
<td>130.15 +/- 4.59</td>
</tr>
<tr>
<td>PDGF-BB 10ng/ml</td>
<td>7.89 +/- 0.64</td>
<td>5.72 +/- 0.34</td>
<td>118.61 +/- 6.87</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml</td>
<td>12.21 +/- 0.75 **</td>
<td>6.34 +/- 0.22 ***</td>
<td>117.85 +/- 1.39</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 1ng/ml</td>
<td>13.78 +/- 0.25 ***</td>
<td>11.17 +/- 0.32</td>
<td>568.27 +/- 64.85 **</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 10ng/ml</td>
<td>15.02 +/- 0.24 ***</td>
<td>12.76 +/- 0.22 ***</td>
<td>521.26 +/- 14.98 **</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 100ng/ml</td>
<td>22.47 +/- 0.81 ***</td>
<td>15.40 +/- 0.22 ***</td>
<td>667.59 +/- 136.58 ♦</td>
</tr>
<tr>
<td>Experiment</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Cell Line</td>
<td>SY 7 passage 4</td>
<td>SY 4 passage 5</td>
<td>SY 35 passage 9</td>
</tr>
<tr>
<td>Control</td>
<td>201.37 +/- 29.44</td>
<td>7.09 +/- 0.25</td>
<td>15.94 +/- 0.60</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>435.70 +/- 8.89 ***</td>
<td>14.03 +/- 0.53 ***</td>
<td>16.74 +/- 0.48</td>
</tr>
<tr>
<td>TGF-β 1ng/ml</td>
<td>172.41 +/- 10.02</td>
<td>6.59 +/- 0.18</td>
<td>not done</td>
</tr>
<tr>
<td>TGF-β 10ng/ml</td>
<td>177.77 +/- 5.94</td>
<td>5.33 +/- 0.087</td>
<td>not done</td>
</tr>
<tr>
<td>TGF-β 100ng/ml</td>
<td>154.97 +/- 10.58</td>
<td>5.64 +/- 0.16</td>
<td>18.94 +/- 0.39 **</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 1ng/ml</td>
<td>513.57 +/- 31.58 *</td>
<td>17.62 +/- 0.98 **</td>
<td>not done</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 10ng/ml</td>
<td>577.59 +/- 33.13 **</td>
<td>20.43 +/- 0.29 ***</td>
<td>not done</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 100ng/ml</td>
<td>669.35 +/- 51.64 **</td>
<td>20.87 +/- 0.51 ***</td>
<td>34.26 +/- 2.80 ***</td>
</tr>
</tbody>
</table>

Table 4.4. The production of TIMP-1 protein by human synovial fibroblasts following treatment with TGF-β either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein except in the case of SY 7 passage 4 in which the results are expressed as ng per μg of cell DNA. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and TGF-β are as follows: • p<0.05, •• p<0.01, ••• p<0.001.
BB or TGF-β on TIMP-1 production in 3 human tendon fibroblast cell lines was investigated. These experiments were performed in an identical manner to those described in section 4.2.1., the only difference being that tendon fibroblasts were used in place of skin fibroblasts.

TIMP-1 was significantly stimulated in all experiments by 10⁻⁵M retinoic acid alone. PDGF-BB and TGF-β alone stimulated TIMP-1 production in only a minority of experiments (1 out of 3 experiments in each case). When 10⁻⁵M retinoic acid was applied in combination with 1, 10 and 100ng/ml of either PDGF-BB or TGF-β, a dose-dependent synergistic increase in TIMP-1 was observed in most experiments although the magnitude of the response varied in different experiments. The results of 2 representative experiments using PDGF-BB or TGF-β respectively are shown in figures 4.5. and 4.6. respectively. The results from all experiments are shown in tables 4.5. and 4.6.

4.2.4. The effect of specific neutralising antibodies to PDGF-BB or TGF-β on the synergistic induction of TIMP-1 in response to retinoic acid and PDGF-BB or TGF-β

The effect on TIMP-1 protein production of 10⁻⁵M retinoic acid in combination with PDGF-BB or TGF-β in the additional presence of neutralising antibodies to the growth factors was investigated using 3 human skin cell lines. This was in order to demonstrate that these effects resulted from the presence of the growth factors and not from an experimental artefact such as bacterial endotoxin.

These experiments were set up using the cell assay system described in chapter 2. The effect of the following test conditions was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously. The antibodies were obtained from a commercial source and control sera were therefore not available. The concentration of antibody used in each case was based on data supplied by the manufacturer from which the amount of antibody required to give full neutralization...
Figures 4.5. (top) and 4.6. (bottom)

Production of TIMP-1 protein by the human tendon cell line HTB 14 (passage 7) following treatment with PDGF-BB (figure 4.5.) or TGF-β (figure 4.6.) alone and in the additional presence of retinoic acid.
Table 4.5. The production of TIMP-1 protein by human tendon fibroblasts following treatment with PDGF-BB either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and PDGF-BB are as follows: • p<0.05, •• p<0.01, ••• p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>HTB 6</td>
<td>HTB 14</td>
<td>HTB 27</td>
</tr>
<tr>
<td></td>
<td>passage 5</td>
<td>passage 7</td>
<td>passage 3</td>
</tr>
<tr>
<td>Control</td>
<td>18.05 +/-</td>
<td>10.71 +/-</td>
<td>4.82 +/-</td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>0.89</td>
<td>0.09</td>
</tr>
<tr>
<td>Retinoic</td>
<td>21.97 +/-</td>
<td>24.13 +/-</td>
<td>16.07 +/-</td>
</tr>
<tr>
<td>Acid 10^-5M</td>
<td>0.55 ***</td>
<td>0.74 ***</td>
<td>1.05 ***</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>16.59 +/-</td>
<td>12.66 +/-</td>
<td>11.10 +/-</td>
</tr>
<tr>
<td>1ng/ml</td>
<td>0.82</td>
<td>0.50</td>
<td>0.32 ***</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>15.76 +/-</td>
<td>12.44 +/-</td>
<td>13.21 +/-</td>
</tr>
<tr>
<td>10ng/ml</td>
<td>0.60</td>
<td>0.58</td>
<td>1.12 ***</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>13.30 +/-</td>
<td>12.02 +/-</td>
<td>16.04 +/-</td>
</tr>
<tr>
<td>100ng/ml</td>
<td>0.65</td>
<td>0.37</td>
<td>0.39 ***</td>
</tr>
<tr>
<td>Retinoic</td>
<td>28.84 +/-</td>
<td>28.50 +/-</td>
<td>21.96 +/-</td>
</tr>
<tr>
<td>Acid 10^-5M</td>
<td>0.77 ***</td>
<td>0.45 ***</td>
<td>0.90</td>
</tr>
<tr>
<td>+ PDGF-BB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinoic</td>
<td>21.84 +/-</td>
<td>35.11 +/-</td>
<td>21.82 +/-</td>
</tr>
<tr>
<td>Acid 10^-5M</td>
<td>0.75</td>
<td>1.92 ***</td>
<td>0.60</td>
</tr>
<tr>
<td>+ PDGF-BB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinoic</td>
<td>29.02 +/-</td>
<td>52.38 +/-</td>
<td>27.02 +/-</td>
</tr>
<tr>
<td>Acid 10^-5M</td>
<td>1.39 ***</td>
<td>3.34 ***</td>
<td>0.36</td>
</tr>
<tr>
<td>+ PDGF-BB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6. The production of TIMP-1 protein by human tendon fibroblasts following treatment with TGF-β either alone or in the additional presence of retinoic acid. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and TGF-β are as follows: ♦ p<0.05, ♦♦ p<0.01, ♦♦♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>HTB 6 passage 6</td>
<td>HTB 27 passage 4</td>
<td>HTB 14 passage 7</td>
</tr>
<tr>
<td>Control</td>
<td>6.30 +/- 0.43</td>
<td>4.96 +/- 0.21</td>
<td>8.91 +/- 0.58</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻³M</td>
<td>11.70 +/- 0.24 ***</td>
<td>10.40 +/- 0.55 ***</td>
<td>20.27 +/- 0.59 ***</td>
</tr>
<tr>
<td>TGF-β 1ng/ml</td>
<td>6.34 +/- 0.52</td>
<td>4.09 +/- 0.12</td>
<td>10.83 +/- 0.43 *</td>
</tr>
<tr>
<td>TGF-β 10ng/ml</td>
<td>7.99 +/- 0.37 *</td>
<td>4.38 +/- 0.13</td>
<td>13.11 +/- 0.27 ***</td>
</tr>
<tr>
<td>TGF-β 100ng/ml</td>
<td>7.46 +/- 0.26</td>
<td>4.63 +/- 0.17</td>
<td>11.54 +/- 0.78 *</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻³M + TGF-β 1ng/ml</td>
<td>21.99 +/- 1.43 ***</td>
<td>13.74 +/- 0.47 ***</td>
<td>27.26 +/- 1.26 **</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻³M + TGF-β 10ng/ml</td>
<td>24.32 +/- 0.61 ***</td>
<td>17.41 +/- 0.25 ***</td>
<td>38.82 +/- 1.22 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻³M + TGF-β 100ng/ml</td>
<td>25.22 +/- 0.63 ***</td>
<td>13.19 +/- 0.61 **</td>
<td>41.59 +/- 1.47 ***</td>
</tr>
</tbody>
</table>
of the concentration of growth factor used was calculated.

1. control - DMEM + 1% ATFCS only
2. recombinant PDGF-BB at 100ng/ml or purified TGF-β at 10ng/ml
3. all-trans-retinoic acid at 10^5 M
4. all-trans-retinoic acid at 10^5 M + recombinant PDGF-BB at 100ng/ml or purified TGF-β at 10ng/ml
5. control + polyclonal neutralising antibody to PDGF-BB at 25µg/ml
   recombinant PDGF-BB at 100ng/ml + polyclonal neutralising antibody to PDGF-BB at 25µg/ml
   all-trans-retinoic acid at 10^5 M + polyclonal neutralising antibody to PDGF-BB at 25µg/ml
   all-trans-retinoic acid at 10^5 M + recombinant PDGF-BB at 100ng/ml + polyclonal neutralising antibody to PDGF-BB at 25µg/ml
6. control + polyclonal neutralising antibody to TGF-β at 13µg/ml
   purified TGF-β at 10ng/ml + polyclonal neutralising antibody to TGF-β at 13µg/ml
   all-trans-retinoic acid at 10^5 M + polyclonal neutralising antibody to TGF-β at 13µg/ml
   all-trans-retinoic acid at 10^5 M + purified TGF-β at 10ng/ml + polyclonal neutralising antibody to TGF-β at 13µg/ml

After 72 hours, the production of TIMP-1 protein by the cells under these various conditions was assessed as described above in section 4.2.1.

In all experiments, 10^5 M retinoic acid and 10ng/ml TGF-β applied alone both caused a significant increase in TIMP-1 production from the cells and a synergistic induction of TIMP-1 when applied together. PDGF-BB when applied alone caused an increase in TIMP-1 in only 1 experiment out of 3 but interacted synergistically with 10^5 M retinoic acid to stimulate TIMP-1 when both were present in all experiments. When 10^5 M retinoic acid and 100ng/ml of PDGF-BB were applied to human skin fibroblasts
in the additional presence of 25μg/ml of the polyclonal neutralising antibody to PDGF-BB, the synergistic induction of TIMP-1 was completely abolished in all experiments. Figure 4.7. shows a representative experiment. Similarly, when 10^5 M retinoic acid and 10ng/ml of TGF-β were applied to skin fibroblasts in the additional presence of 13μg/ml of the polyclonal neutralising antibody to TGF-β, the synergistic induction of TIMP-1 was completely abolished in all experiments. Figure 4.8. shows a representative experiment.

4.2.5. The effect of all-trans-retinoic acid in combination with PDGF-BB or TGF-β on collagenase production in human skin, synovial and tendon fibroblasts

The effect of 10^5 M retinoic acid in combination with 1, 10 and 100ng/ml of PDGF-BB or TGF-β on collagenase protein production in 3 human skin cell lines, 3 synovial cell lines and 3 tendon fibroblast cell lines was investigated. The harvested culture medium from the experiments described in sections 4.2.1., 4.2.2. and 4.2.3. was measured for levels of interstitial collagenase using the specific ELISA described in chapter 2. The levels of collagenase were corrected with respect to the cell protein or cell DNA such that results are expressed as ng of collagenase per μg of cell protein or cell DNA. Statistical analysis was by one way analysis of variance.

It was found that PDGF-BB significantly stimulated collagenase at 10 and 100ng/ml in all experiments with skin fibroblasts, but at 0.1-1ng/ml, collagenase production was significantly inhibited. It therefore appears that PDGF-BB has a biphasic effect on collagenase production from human skin fibroblasts. Figure 4.9. shows a representative experiment and table 4.7. shows the results from all experiments.

10^5 M retinoic acid was found to have a significant inhibitory effect on collagenase production in all experiments when compared to control levels. When 10^5 M retinoic acid was applied in combination with 1, 10 and 100ng/ml of PDGF-BB, it was found that retinoic acid downregulated PDGF-BB-stimulated collagenase in all experiments (see figure 4.9. and table 4.7.).
Figures 4.7. (top) Effect of specific neutralizing antibodies to PDGF-BB on the synergistic induction of TIMP-1 protein from the human skin cell line hsf 12 (passage 13) treated with retinoic acid and PDGF-BB.

Figure 4.8. (bottom) Effect of specific neutralizing antibodies to TGF-β on the synergistic induction of TIMP-1 protein from the human skin cell line hsf 15 (passage 3) treated with retinoic acid and TGF-β.
Figure 4.9. (top)
Production of collagenase protein by the human skin cell line hsf 15 (passage 7) following treatment with PDGF-BB alone and in the additional presence of retinoic acid.

Figure 4.10. (bottom)
Production of collagenase protein by the human skin cell line hsf 12 (passage 10) following treatment with TGF-β alone and in the additional presence of retinoic acid.
Table 4.7. The production of collagenase protein by human skin fibroblasts following treatment with PDGF-BB either alone or in the additional presence of retinoic acid. The results are expressed as collagenase in ng per µg of cell DNA. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of PDGF-BB alone are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 13</td>
<td>hsf 12</td>
<td>hsf 15</td>
</tr>
<tr>
<td></td>
<td>passage 10</td>
<td>passage 11</td>
<td>passage 7</td>
</tr>
<tr>
<td>Control</td>
<td>26.09 +/- 4.33</td>
<td>40.64 +/- 2.06</td>
<td>53.63 +/- 7.21</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>6.45 +/- 0.33 **</td>
<td>3.87 +/- 0.126 ***</td>
<td>14.30 +/- 0.26 ***</td>
</tr>
<tr>
<td>PDGF-BB 0.1ng/ml</td>
<td>not done</td>
<td>not done</td>
<td>18.17 +/- 1.09 **</td>
</tr>
<tr>
<td>PDGF-BB 1ng/ml</td>
<td>14.10 +/- 0.73 *</td>
<td>26.61 +/- 2.49 **</td>
<td>39.10 +/- 2.00</td>
</tr>
<tr>
<td>PDGF-BB 10ng/ml</td>
<td>137.72 +/- 7.73 ***</td>
<td>95.19 +/- 10.17 ***</td>
<td>146.7 +/- 5.09 ***</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml</td>
<td>219.5 +/- 14.56 ***</td>
<td>122.92 +/- 11.61 ***</td>
<td>260.5 +/- 9.76 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 1ng/ml</td>
<td>7.35 +/- 0.67 ***</td>
<td>5.59 +/- 0.62 ***</td>
<td>21.03 +/- 5.05 **</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 10ng/ml</td>
<td>17.99 +/- 1.77 ***</td>
<td>5.17 +/- 0.41 ***</td>
<td>49.00 +/- 4.96 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 100ng/ml</td>
<td>42.45 +/- 4.82 ***</td>
<td>8.27 +/- 0.50 ***</td>
<td>100.37 +/- 4.69 ***</td>
</tr>
</tbody>
</table>

Table 4.7. The production of collagenase protein by human skin fibroblasts following treatment with PDGF-BB either alone or in the additional presence of retinoic acid. The results are expressed as collagenase in ng per µg of cell DNA. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of PDGF-BB alone are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 15 passage 2</td>
<td>hsf 12 passage 10</td>
<td>hsf 13 passage 8</td>
<td>hsf 15 passage 8</td>
<td>hsf 15 passage 7</td>
</tr>
<tr>
<td>Control</td>
<td>31.62 +/- 1.40</td>
<td>73.55 +/- 3.18</td>
<td>1.02 +/- 0.07</td>
<td>328.17 +/- 20.36</td>
<td>9.45 +/- 0.41</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>4.82 +/- 0.44 ***</td>
<td>49.61 +/- 1.93 ***</td>
<td>0.59 +/- 0.04 ***</td>
<td>123.33 +/- 11.94 ***</td>
<td>5.22 +/- 0.07 ***</td>
</tr>
<tr>
<td>TGF-β 0.1ng/ml</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>6.47 +/- 0.28 ***</td>
</tr>
<tr>
<td>TGF-β 1ng/ml</td>
<td>17.72 +/- 0.99 ***</td>
<td>56.11 +/- 4.51 *</td>
<td>not done</td>
<td>181.67 +/- 14.52 ***</td>
<td>6.31 +/- 0.25 ***</td>
</tr>
<tr>
<td>TGF-β 10ng/ml</td>
<td>19.20 +/- 1.72 ***</td>
<td>39.56 +/- 1.15 ***</td>
<td>not done</td>
<td>159.33 +/- 12.12 ***</td>
<td>6.85 +/- 0.33 ***</td>
</tr>
<tr>
<td>TGF-β 100ng/ml</td>
<td>26.22 +/- 0.67 **</td>
<td>34.84 +/- 3.90 ***</td>
<td>0.61 +/- 0.03 ***</td>
<td>158.17 +/- 9.10 ***</td>
<td>not done</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 0.1ng/ml</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>4.80 +/- 0.04 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 1ng/ml</td>
<td>4.48 +/- 0.23</td>
<td>41.42 +/- 8.64</td>
<td>not done</td>
<td>96.67 +/- 5.71</td>
<td>4.07 +/- 0.19 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 10ng/ml</td>
<td>3.74 +/- 0.26</td>
<td>36.50 +/- 4.55</td>
<td>not done</td>
<td>95.33 +/- 8.52</td>
<td>4.10 +/- 0.20 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + TGF-β 100ng/ml</td>
<td>4.24 +/- 0.30</td>
<td>29.46 +/- 2.69</td>
<td>0.60 +/- 0.02</td>
<td>76.77 +/- 15.23 *</td>
<td>not done</td>
</tr>
</tbody>
</table>

Table 4.8. The production of collagenase protein by human skin fibroblasts following treatment with TGF-β either alone or in the additional presence of retinoic acid. The results are expressed as collagenase in ng per µg of cell DNA except in the cases of hsf 13 passage 8 and hsf 15 passage 7 in which the results are expressed as ng per µg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of retinoic acid alone are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
TGF-β downregulated collagenase production at 1, 10 and 100ng/ml in all experiments when compared to the control levels of collagenase. When $10^{-5}$M retinoic acid was applied to the cells in the presence of 1, 10 and 100ng/ml TGF-β, no further downregulation of collagenase was seen in addition to that caused by the agents alone in 3 out of 5 experiments. However, in 2 further experiments, TGF-β caused some further downregulation of collagenase in addition to that caused by $10^{-5}$M retinoic acid but the effect was not as great as the additive effect of the agents applied alone. Figure 4.10. shows the results of 1 of the 5 experiments and table 4.8. shows the results of all experiments.

In synovial cells (data not shown), PDGF-BB stimulated collagenase in 2 out of 3 experiments in a dose-dependent manner at 10 and 100ng/ml. 1ng/ml of PDGF-BB either had no effect on collagenase secretion or the level of collagenase secreted under these conditions was not detectable. Retinoic acid at $10^{-5}$M downregulated the production of collagenase from 2 out of 3 experiments; the effect of retinoic acid could not be established in the 3rd experiment as the control levels of collagenase were below detection. When retinoic acid at $10^{-5}$M was applied in the additional presence of PDGF-BB at 1, 10 and 100ng/ml it was found that PDGF-BB-stimulated collagenase was downregulated. In tendon fibroblasts (data not shown), PDGF-BB at 1, 10 and 100ng/ml stimulated collagenase in 2 out of 3 experiments; the levels of collagenase were below detection in the third experiment. No inhibitory effect of PDGF-BB was seen at 1ng/ml. Retinoic acid at $10^{-5}$M applied in the additional presence of PDGF-BB downregulated PDGF-BB-stimulated collagenase. Retinoic acid alone also inhibited control levels of collagenase although this was only demonstrated in a minority of experiments due to control levels of collagenase being mainly below detection.

The effect of TGF-β alone and of TGF-β in combination with $10^{-5}$M retinoic acid on collagenase production from synovial and tendon fibroblasts could not be ascertained as the levels of collagenase produced in these experiments were below detection.
4.3. DISCUSSION

Previous investigations into the effect of TGF-β on TIMP-1 production have shown an induction of TIMP-1 in response to this factor in human gingival fibroblasts (Overall et al., 1989). In contrast, the results in this study show that TIMP-1 is not induced in human skin, synovial or tendon fibroblasts by TGF-β alone in most experiments. Another study using human foetal lung fibroblasts (Edwards et al., 1987) showed that TGF-β alone had no significant effect on TIMP-1. Also, a previous investigation by Wright et al. (1991a) showed that TGF-β sometimes induced TIMP-1 in skin and synovial fibroblasts but in other cases, there was no effect unless IL-1β is additionally present. The effect of TGF-β on TIMP-1 production in fibroblasts was therefore probably dependent upon the type of fibroblast used and upon additional factors determined by the culture conditions which are present in only some of the experiments.

It was also found that PDGF-BB stimulated TIMP-1 secretion in some experiments with synovial cell lines but only in a minority of experiments with the skin and tendon fibroblast cell lines. A previous report has shown that PDGF is able to stimulate the secretion of TIMP-1 from human skin fibroblasts (Circolo et al., 1991). The lack of response from the cells in this study suggests that it is dependent on additional factors (e.g. growth factors produced by the cells) which are not present in these experiments.

Although the effects of PDGF-BB, TGF-β and retinoic acid alone on human fibroblasts have already been studied, this is the first investigation of their effects when they are applied in combination. The results of the previous chapter demonstrated that bFGF and EGF interact in a synergistic manner with all-trans-retinoic acid to stimulate the production of TIMP-1 from human skin, synovial and tendon fibroblasts. It was postulated on the basis of the similarities between these growth factors and PDGF-BB with respect to their effects on fibroblasts, that PDGF-BB might also be able to synergize with all-trans-retinoic acid. The data shown in this chapter have indicated that this is indeed the case and serve to underline further the similarities between these 3 growth factors in their actions upon fibroblasts. The
effect of TGF-β in combination with retinoic acid on TIMP-1 production in human fibroblasts has also been investigated showing that a synergistic interaction occurs between retinoic acid and TGF-β which is similar to that seen with the other growth factors investigated. The results identify PDGF-BB and TGF-β as 2 possible factors in mononuclear cell factor which are responsible for causing a synergistic induction of TIMP-1 in response to mononuclear cell factor and retinoic acid (Rheumatology Research Unit, unpublished results). The data show that the effects are dose-dependent across a range of concentrations of PDGF-BB and TGF-β. In addition, it has been demonstrated that specific neutralising antibodies to the growth factors abolish the synergistic induction of TIMP-1 demonstrating that the effects are specifically caused by the growth factors.

The mechanism by which these synergistic effects are mediated requires further study, and a number of different theories are possible. It is of interest that retinoic acid has been previously reported to increase the number of receptors for TGF-β in HL-60 human promyelocytic cells (Falk et al., 1990). Such data suggests that retinoic acid and TGF-β might synergistically increase TIMP-1 production through an effect on the number of TGF-β receptors on cells although such a phenomena has not yet been described for fibroblasts. It is possible that the synergistic response of fibroblasts to retinoic acid and PDGF-BB is also mediated via a similar mechanism in which retinoic acid enhances the binding of PDGF-BB to its receptors. Another feasible mechanism is the induction of retinoic acid receptors by the growth factors as discussed in chapter 3.

Alternatively, the effect may occur at the level of expression of the TIMP-1 gene. Overall et al., (1989, 1991) have shown that treatment of human gingival fibroblasts with TGF-β results in an increase in TIMP-1 mRNA as a result of increased TIMP-1 gene transcription. Furthermore, Campbell et al., (1991) have found that the response to TGF-β can be directed by an AP-1 binding site in the murine TIMP-1 gene. The synergistic effect on TIMP-1 production when retinoic acid and TGF-β are both present may result from synergistic interactions between activated retinoic acid
receptors and a transcription factor responsible for mediating the effects of TGF-β on expression of the gene. This transcription factor may consist of a *fos/jun* heterodimer since TGF-β is known to increase the mRNA levels for these proteins in cells (Liboi et al., 1988, Pertovaara et al., 1989, Li et al., 1990) and these proteins bind as a heterodimer to the AP-1 site (Halazonetis et al., 1988).

Investigations into regulation of the collagenase gene by retinoic acid show that retinoic acid receptors are able to interact with the collagenase AP-1 site by an indirect mechanism involving binding to proteins such as *c-jun* (Schüle et al., 1991, Pan et al., 1992, Pan and Brinckerhoff, 1994). This results in downregulation of collagenase gene transcription in response to retinoic acid. In the case of the TIMP-1 gene, similar interactions could take place between retinoic acid receptors and proteins which bind to the AP-1 site. However, in this case, these interactions may lead to positive regulation of the gene due to the presence of additional proteins which do not bind to the collagenase AP-1 site. The existence of such proteins has been demonstrated using the murine TIMP-1 gene (Edwards et al., 1992).

The synergistic effect of retinoic acid and PDGF-BB on TIMP-1 secretion may also be mediated via a similar mechanism. PDGF-BB stimulation of cells causes the induction of mRNA for *c-fos* and *c-fos* protein (Müller et al., 1984, Kruijer et al., 1984). PDGF-BB-induced TIMP-1 production may be mediated by *c-fos* since previous work has shown that AP-1-binding sites are involved in the induction of TIMP-1 by serum from the murine TIMP-1 gene (Campbell et al., 1991, Edwards et al., 1992). The synergistic effect of retinoic acid and PDGF-BB on TIMP-1 production may result from interactions between activated retinoic acid receptors and *c-fos* or other transcription factors responsible for the effects of PDGF-BB. If the synergistic response does involve *c-fos*, then the fact that bFGF, TGF-β and to a lesser extent EGF also stimulate the production of *c-fos* by cells (Liboi et al., 1988, Müller et al., 1984) may account for the strikingly similar effect of these different growth factors on TIMP-1 secretion in the presence of retinoic acid.
The effect of TGF-β on collagenase secretion in human fibroblasts has been previously described (Overall et al., 1989, Wright et al., 1991a) and the results of this chapter showing an inhibition of collagenase production in response to this factor are in close correlation with these findings. It has also been shown previously that collagenase production is stimulated in human skin fibroblasts in response to PDGF (Bauer et al., 1985, Chua et al., 1985). The results shown in this chapter are in agreement with this data since they have shown a similar effect in human skin, synovial and tendon fibroblasts. However the data additionally indicates that PDGF has a biphasic effect, since 0.1–1ng/ml of PDGF-BB appears to inhibit collagenase production from skin cells. In order to substantiate this claim further, it would be useful to perform further experiments using a more detailed range of concentrations of PDGF-BB between 0 and 10ng/ml. The results of the previous studies can be explained by the fact that the concentrations of growth factor used were not sufficiently low for the inhibitory effect to be seen. Interestingly, a recent report has shown that in rat bone cells, PDGF-BB exerts an initial stimulatory effect on MMP-13 gene transcription and MMP-13 protein production followed by an inhibitory effect on these parameters at later time points (Varghese et al., 1996). This report along with the data shown in this chapter therefore indicate that PDGF can have both stimulatory and inhibitory effects on expression of the collagenases.

It is interesting to speculate upon the possible physiological role of this biphasic action on collagenase production. PDGF is thought to be an important mediator of wound healing (Ross and Vogel, 1978), a process which involves both the laying down of collagen and the remodelling of this collagen by the action of collagenases in order to provide a repair structure of the correct tensile strength (Grillo and Gross, 1967, Riley and Peacock, 1967, Eisen, 1969, Donoff et al., 1971). PDGF may play a critical role in controlling this series of events through modulating fibroblast collagenase secretion in a biphasic manner in which the balance between collagen synthesis and remodelling is determined by the local concentration of PDGF in the healing wound.
The effects of PDGF-BB and TGF-β in combination with retinoic acid on collagenase secretion from human skin, synovial and tendon fibroblasts were also investigated. It was found that retinoic acid downregulated PDGF-BB-stimulated collagenase in all types of fibroblast. Retinoic acid is therefore able to acutely modify the response of skin, synovial and tendon fibroblasts to PDGF-BB. When PDGF-BB is applied alone, the cells are stimulated to produce collagenase and to a lesser extent TIMP-1 and such a situation may encourage extracellular matrix breakdown. When PDGF-BB is applied in the presence of retinoic acid, extracellular matrix synthesis is favoured since retinoic acid downregulates the production of collagenase and also causes an increase in TIMP-1 which is greater than the additive effect of the agents. These findings once again do not correlate with the ability of retinoic acid to induce connective tissue breakdown in cartilage as discussed in chapter 3.

Surprisingly, retinoic acid and TGF-β did not act in an additive manner to inhibit the production of collagenase from human skin fibroblasts. A previous investigation has suggested that the induction of c-fos is involved in the inhibitory effect of TGF-β on collagenase (Kerr et al., 1990). Paradoxically, the repression of collagenase gene expression by retinoic acid has been shown to involve the inhibition of c-fos production (Lafyatis et al., 1990) although other mechanisms have also been put forward (Schüle et al., 1991, Pan et al., 1992, Pan and Brinckerhoff, 1994). Such data suggests that the effects of TGF-β and retinoic acid on collagenase gene expression involve opposite effects on the induction of c-fos. Such an argument could explain the data reported in this study showing that retinoic acid and TGF-β do not affect collagenase production in an additive manner.

In summary, therefore, this chapter has extended the observations of chapter 3 by demonstrating that retinoic acid is capable of interacting synergistically with 2 further growth factors, namely PDGF-BB and TGF-β to stimulate the production of TIMP-1 from human fibroblasts. The ability of bFGF, EGF and PDGF to cause this effect correlates with the similarities of these 3 growth factors in their effects on mesenchymal cells and their intracellular mechanism of action. These observations
suggest that the synergistic induction of TIMP-1 by bFGF, EGF and PDGF-BB in combination with retinoic acid may be caused by a common mechanism. In particular, all 3 of these growth factors induce the expression of c-fos in cells (Müller et al., 1984, Kruijer et al., 1984) a characteristic also shared by TGF-β (Liboi et al., 1988) which may explain why the latter growth factor also has the same effect. This common pathway of c-fos induction may be important in the synergistic induction of TIMP-1, since by analogy with the murine system, it is probable that c-fos and AP-1 sites are involved in the expression of the human TIMP-1 gene (Campbell et al., 1991, Edwards et al., 1992). The possible mechanisms by which these synergistic effects may be taking place are explored in detail in later chapters of this thesis.
CHAPTER FIVE

THE EFFECT OF INTERLEUKIN-1β ON THE SYNERGISTIC INDUCTION OF TIMP-1 BY ALL-TRANS-RETINOIC ACID AND POLYPEPTIDE GROWTH FACTORS.

5.1. INTRODUCTION

In chapters 3 and 4, it was shown that retinoic acid interacts synergistically with bFGF, EGF, PDGF-BB and TGF-β to 'superinduce' TIMP-1 protein production from human fibroblasts. These findings may explain previous, unpublished observations showing that a greater induction of TIMP-1 results from treatment of cells with mononuclear cell factor and retinoic acid together than with retinoic acid alone. Mononuclear cell factor is an undefined mixture of cytokines and growth factors and the effects seen with this agent may be attributable to the presence of polypeptide growth factors.

However, one of the main components of mononuclear cell factor is the pro-inflammatory cytokine, interleukin-1 (IL-1). IL-1 belongs to a gene family which is composed of 3 members which all bind to the same receptors (Dinarello, 1994). IL-1 α and IL-1β are receptor agonists while the third family member is an IL-1 receptor antagonist. In general, IL-1α and IL-1β induce the same biological responses, but in some cells differences occur between the two.

The effect of IL-1 on TIMP-1 protein production from fibroblasts has been previously investigated and conflicting data has been obtained. IL-1 stimulated TIMP-1 protein production from human foetal lung, synovial and skin fibroblasts (Murphy et al., 1985a, Ito et al., 1992) although in rabbit uterine cervical fibroblasts TIMP-1 protein production was suppressed by IL-1α (Ito et al., 1988). Another study showed no effect at all on TIMP-1 production from human synovial fibroblasts by IL-1 (MacNaul et al., 1990). The precise reasons for these discrepancies are not clear but perhaps they are due to slight differences in experimental conditions in different studies.
The aim of this chapter is to investigate the effect of retinoic acid in combination with IL-1β on TIMP-1 production from human fibroblasts. It is possible that IL-1β may synergize with retinoic acid to induce TIMP-1 production in a similar manner to the polypeptide growth factors investigated in chapters 3 and 4. In addition, the synergistic induction of TIMP-1 caused by retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β may be further enhanced by the additional presence of IL-1β.

IL-1 is also a potent inducer of fibroblast collagenase (Dayer et al., 1986, Postlethwaite et al., 1983). Previous findings have also shown that IL-1 and PDGF in combination synergistically increase the production of collagenase from human skin fibroblasts (Circolo et al., 1991). In addition, collagenase production can also be synergistically stimulated in chondrocytes using IL-1 and bFGF (Phadke, 1987) and in periosteal tissue using IL-1 and EGF (van der Zee et al., 1993). In this chapter, the effect of IL-1 in combination with bFGF and EGF on collagenase protein secretion from human skin and synovial fibroblasts is investigated. It is possible that IL-1 may synergize with bFGF or EGF to 'superinduce' the production of collagenase from human fibroblasts in a similar manner to the effects observed with chondrocytes and periosteal tissue.
5.2. METHODS AND RESULTS

5.2.1. The effect of all-trans-retinoic acid in combination with polypeptide growth factors on TIMP-1 protein production in human skin fibroblasts in the additional presence of IL-1β.

The effect of 10⁻⁶M retinoic acid in combination with 1, 10 and 100ng/ml of bFGF, EGF, PDGF-BB or TGF-β on TIMP-1 protein production in 3 human skin cell lines in the additional presence of 1ng/ml of IL-1β was investigated using the cell assay system described in chapter 2.

The effect of the following test reagents was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml
3. all-trans-retinoic acid at 10⁻⁶M
4. all-trans-retinoic acid at 10⁻⁶M + recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml
5. recombinant IL-1β at 1ng/ml
6. recombinant IL-1β at 1ng/ml + all-trans-retinoic acid at 10⁻⁶M.
7. recombinant IL-1β at 1ng/ml + recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml
8. recombinant IL-1β at 1ng/ml + all-trans-retinoic acid at 10⁻⁶M + recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml

The concentrations of growth factors used were based on the concentrations giving the greatest synergistic stimulation of TIMP-1 protein in the experiments in chapters 3 and 4.
After 72 hours, the cell supernates were harvested and assayed for TIMP-1 by ELISA as described in chapter 2. The protein content of the cell monolayers was measured as described in chapter 2 and the levels of TIMP-1 were corrected with respect to the cell protein such that the results are expressed as ng of TIMP-1 per μg of cell protein. In some experiments, the DNA content of the cell monolayer was measured instead of the protein content and the results are expressed as ng of TIMP-1 per μg of cell DNA. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.

In the majority of experiments, 10⁻⁴M retinoic acid alone caused a significant increase in TIMP-1 production above that of control cells. IL-1β applied alone at 1ng/ml produced disparate effects on TIMP-1 protein production - in 10 experiments TIMP-1 production was inhibited while in the other 8 experiments there was no effect on TIMP-1. When 1ng/ml IL-1β was applied in the additional presence of 10⁻⁴M retinoic acid, disparate results were again obtained. In 7 experiments, the addition of IL-1β had no effect on retinoic acid-stimulated TIMP-1, while in another 7 experiments, IL-1β caused further stimulation of TIMP-1. In the remaining 4 experiments, IL-1β inhibited retinoic acid-stimulated TIMP-1 production. These data are shown in table 5.1. It is therefore concluded from these experiments that IL-1β has no consistent effect on TIMP-1 protein production from human skin fibroblasts either when applied alone or in the additional presence of retinoic acid.

Next, the effect of IL-1β at 1ng/ml on the synergistic induction of TIMP-1 by retinoic acid and growth factors was investigated. When 1ng/ml IL-1β with 10⁻⁴M retinoic acid was applied in combination with 1, 10 and 100ng/ml of bFGF, a dose-dependent synergistic increase in TIMP-1 production was seen in all 3 of the cell lines investigated. The magnitude of the response varied in different experiments. These data were compared to the effect of retinoic acid in combination with bFGF without the additional presence of IL-1β. In 5 experiments, it was found that the degree of synergism was smaller when IL-1β was present compared to when it was not. Figure 5.1. shows an example of this effect. When the same experiments were done using
Table 5.1. The production of TIMP-1 protein by human skin fibroblasts following treatment with IL-1β either alone or in the additional presence of retinoic acid. The passage number of the cell line in each experiment is shown in brackets. The results are expressed as TIMP-1 in ng per µg of cell protein or in ng per µg of cell DNA; the latter experiments are marked ★. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of retinoic acid are as follows: • p<0.05, •• p<0.01, ••• p<0.001.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No Additions</th>
<th>IL-1β 1ng/ml</th>
<th>Retinoic Acid 10^-6M</th>
<th>Retinoic Acid 10^-6M + IL-1β 1ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 12 (10)</td>
<td>1.86 +/- 0.26</td>
<td>12.59 +/- 0.53</td>
<td>13.43 +/- 0.66 *</td>
<td>15.16 +/- 1.33</td>
</tr>
<tr>
<td>hsf 12 (10)</td>
<td>68.33 +/- 45.07</td>
<td>433.33 +/- 18.74 **</td>
<td>863.33 +/- 16.26 **</td>
<td>568.33 +/- 86.81 **</td>
</tr>
<tr>
<td>hsf 12 (11)</td>
<td>58.60 +/- 1.76</td>
<td>39.48 +/- 1.62 ***</td>
<td>56.16 +/- 1.24</td>
<td>112.29 +/- 9.41 ***</td>
</tr>
<tr>
<td>hsf 12 (12)</td>
<td>3.61 +/- 0.14</td>
<td>1.65 +/- 0.04 ***</td>
<td>7.53 +/- 0.25 ***</td>
<td>8.46 +/- 0.22</td>
</tr>
<tr>
<td>hsf 13 (6)</td>
<td>5.98 +/- 0.15</td>
<td>5.77 +/- 0.32</td>
<td>24.81 +/- 1.03 ***</td>
<td>16.90 +/- 0.53 ***</td>
</tr>
<tr>
<td>hsf 13 (7)</td>
<td>2.00 +/- 0.11</td>
<td>1.79 +/- 0.09</td>
<td>3.64 +/- 0.11 ***</td>
<td>3.61 +/- 0.16</td>
</tr>
<tr>
<td>hsf 13 (8)</td>
<td>6.48 +/- 0.13</td>
<td>3.66 +/- 0.10 ***</td>
<td>11.06 +/- 0.50 ***</td>
<td>7.60 +/- 0.25 ***</td>
</tr>
<tr>
<td>hsf 13 (8)</td>
<td>425 +/- 92.58</td>
<td>205 +/- 30.63 *</td>
<td>700.00 +/- 40.41 *</td>
<td>492.67 +/- 54.37 *</td>
</tr>
<tr>
<td>hsf 13 (10)</td>
<td>58.60 +/- 2.82</td>
<td>32.81 +/- 2.23 **</td>
<td>91.49 +/- 1.05 **</td>
<td>212.33 +/- 8.65 ***</td>
</tr>
<tr>
<td>hsf 13 (11)</td>
<td>3.76 +/- 0.05</td>
<td>3.02 +/- 0.05 ***</td>
<td>10.31 +/- 0.35 ***</td>
<td>14.27 +/- 0.38 ***</td>
</tr>
<tr>
<td>hsf 13 (12)</td>
<td>4.31 +/- 0.24</td>
<td>5.20 +/- 0.18</td>
<td>9.06 +/- 0.37 ***</td>
<td>12.00 +/- 0.36 ***</td>
</tr>
<tr>
<td>hsf 15 (2)</td>
<td>5.61 +/- 0.047</td>
<td>6.49 +/- 0.53</td>
<td>14.22 +/- 0.94 ***</td>
<td>25.74 +/- 5.52</td>
</tr>
<tr>
<td>hsf 15 (2)</td>
<td>65.13 +/- 3.42</td>
<td>74.85 +/- 4.25</td>
<td>186.07 +/- 6.36 ***</td>
<td>313.25 +/- 14.02 ***</td>
</tr>
<tr>
<td>hsf 15 (5)</td>
<td>8.30 +/- 0.03</td>
<td>6.80 +/- 0.21 ***</td>
<td>21.37 +/- 0.89 ***</td>
<td>24.64 +/- 0.59 *</td>
</tr>
<tr>
<td>hsf 15 (6)</td>
<td>24.71 +/- 1.46</td>
<td>21.53 +/- 0.90</td>
<td>30.27 +/- 1.22 ***</td>
<td>34.62 +/- 1.79</td>
</tr>
<tr>
<td>hsf 15 (8)</td>
<td>368.33 +/- 28.46</td>
<td>159.00 +/- 5.38 ***</td>
<td>487.67 +/- 27.99 *</td>
<td>402.83 +/- 14.01</td>
</tr>
<tr>
<td>hsf 15 (9)</td>
<td>837.5 +/- 23.97</td>
<td>839.17 +/- 14.83</td>
<td>1345.17 +/- 32.78 ***</td>
<td>2350.83 +/- 81.81 ***</td>
</tr>
</tbody>
</table>
Figures 5.1. (top) and 5.2. (bottom)

Production of TIMP-1 protein by the human skin cell line hsf12 (passage 10) following treatment with bFGF (figure 5.1.) or EGF (figure 5.2.) alone and in the additional presence of retinoic acid only or retinoic acid and IL-1β (see key).
EGF in the place of bFGF, the degree of synergism was smaller when IL-1β was present compared to when it was not in 4 out of 6 experiments. In 1 of these experiments, there was no synergistic response at all in the presence of IL-1β. Figure 5.2. shows the results of this latter experiment. In 1 of the remaining 2 experiments, the degree of synergism was the same in the absence and presence of IL-1β and in the other, the response in the presence of IL-1β was more potent than in its absence.

When 1ng/ml IL-1β and 10⁻⁴M retinoic acid were applied in combination with 1, 10 and 100ng/ml of PDGF-BB, a synergistic increase in TIMP-1 production was seen in all cell lines investigated although there was variation in the magnitude of the response. The cells were less responsive to the effect of 10⁻⁴M retinoic acid and PDGF-BB in the additional presence of IL-1β, since in 4 experiments, the degree of synergism was smaller or non-existent when IL-1β was present compared to when it was not. Figure 5.3. shows the results of 1 of these experiments in which no synergism at all is seen in the additional presence of IL-1β. When 1ng/ml IL-1β and 10⁻⁴M retinoic acid were applied in combination with 1, 10 and 100ng/ml of TGF-β, again, a synergistic increase in TIMP-1 production was seen in all 3 of the cell lines investigated. The effect of retinoic acid and TGF-β in the additional presence of 1ng/ml IL-1β was very similar to that observed when IL-1β was not present. The results did not appear to indicate that IL-1β consistently either enhanced or dampened the response. Figure 5.4. shows the results of 1 experiment in which IL-1β was seen to enhance the synergistic induction of TIMP-1.

It would appear from these experiments that in the case of bFGF, EGF and PDGF-BB, the synergistic induction of TIMP-1 is usually less powerful in the presence of IL-1β and that IL-1β therefore has a dampening effect on the mechanism. However, in many of these experiments, although the degree of synergism which contributes to the response is smaller, the actual levels of TIMP-1 protein produced are equal to or greater than that seen in the absence of IL-1β (e.g. figures 5.2. and 5.3.). Hence in these experiments, the explanation could be that a maximum level of TIMP-1 production has been reached above which further stimulation is not possible.
Production of TIMP-1 protein by the human skin cell line hsf 15 (passage 9) following treatment with PDGF-BB alone and in the additional presence of retinoic acid only or retinoic acid and IL-1β (see key).

Figure 5.3. (top)

Production of TIMP-1 protein by the human skin cell line hsf 12 (passage 10) following treatment with TGF-β alone and in the additional presence of retinoic acid only or retinoic acid and IL-1β (see key).
5.2.2. The effect of bFGF and EGF in combination with IL-1β on collagenase production from human skin and synovial fibroblasts

The effect of IL-1β in combination with 1, 10 and 100ng/ml of bFGF or EGF on collagenase protein production was investigated in 3 human skin cell lines and 3 human synovial cell lines using the cell assay system described in chapter 2.

The effect of the following test reagents was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF or EGF at concentrations of 1, 10 and 100ng/ml
3. recombinant IL-1β at 1ng/ml
7. recombinant IL-1β at 1ng/ml + recombinant bFGF or EGF at concentrations of 1, 10 and 100ng/ml

After 72 hours, the cell supernates were harvested and assayed for collagenase by ELISA as described in chapter 2. The protein content of the cell monolayers was measured as described in chapter 2 and the levels of collagenase were corrected with respect to the cell protein such that the results are expressed as ng of collagenase per µg of cell protein. Statistical analysis was by one way analysis of variance or multiple regression.

bFGF was found to stimulate the production of collagenase in all experiments with both skin and synovial fibroblasts. The effect was dose-dependent and maximal at 100ng/ml. Collagenase production was also stimulated in all experiments with skin fibroblasts by EGF; this response was also dose-dependent but the dose giving the maximum response varied widely between 1 and 100ng/ml in different experiments. The effect of EGF in synovial fibroblasts could not be assessed as the levels of collagenase were below detection.
The addition of 1ng/ml of IL-1β alone to both types of cells caused a significant stimulation of collagenase in all experiments. Collagenase levels were further increased by the additional presence of either bFGF or EGF at the above concentrations. In 4 out of 5 experiments with skin fibroblasts, and all 3 experiments with synovial fibroblasts, IL-1β and bFGF applied together caused an increase in collagenase which was greater than the additive effects of each applied alone. Figure 5.5. shows a typical example of this effect using skin fibroblasts and tables 5.2. and 5.3. show the results of all experiments. The synergistic increase was dose-dependent in all experiments and the maximum response occurred using 100ng/ml of growth factor in all experiments except 1. A similar synergistic increase in collagenase was also observed in 5 out of 6 experiments in skin fibroblasts using IL-1β and EGF together. In this case, the dose of growth factor giving the maximum response varied between 1 and 100ng/ml in different experiments. Figure 5.6. shows an example of this effect and table 5.4 shows the results of all experiments. EGF also caused a further increase in collagenase production above that caused by IL-1β in all 3 synovial cell lines. Whether this effect was synergistic or merely additive could not be established as the levels of collagenase in the absence of IL-1β were below detection (data not shown).
Figure 5.5. (top)
Production of collagenase protein by the human skin cell line hsf 15 (passage 2) following treatment with bFGF either alone or in the additional presence of IL-1β.

Figure 5.6. (bottom)
Production of collagenase protein by the human skin cell line hsf 15 (passage 5) following treatment with EGF alone or in the additional presence of IL-1β.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 13</td>
<td>hsf 13</td>
<td>hsf 15</td>
<td>hsf 15</td>
<td>hsf 12</td>
</tr>
<tr>
<td></td>
<td>passage 6</td>
<td>passage 13</td>
<td>passage 2</td>
<td>passage 6</td>
<td>passage 10</td>
</tr>
<tr>
<td>Control</td>
<td>5.15 +/-</td>
<td>0.49 +/-</td>
<td>1.15 +/-</td>
<td>7.48 +/-</td>
<td>16.96 +/-</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0.01</td>
<td>0.04</td>
<td>0.68</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-1β</td>
<td>28.88 +/-</td>
<td>3.40 +/-</td>
<td>6.93 +/-</td>
<td>12.41 +/-</td>
<td>31.56 +/-</td>
</tr>
<tr>
<td>1ng/ml</td>
<td>0.26 ***</td>
<td>0.18 ***</td>
<td>0.31 ***</td>
<td>0.54 ***</td>
<td>2.06 ***</td>
</tr>
<tr>
<td>bFGF</td>
<td>4.80 +/-</td>
<td>0.60 +/-</td>
<td>1.87 +/-</td>
<td>3.89 +/-</td>
<td>21.69 +/-</td>
</tr>
<tr>
<td>1ng/ml</td>
<td>0.16</td>
<td>0.034</td>
<td>0.19 **</td>
<td>0.26</td>
<td>0.48 **</td>
</tr>
<tr>
<td>bFGF</td>
<td>6.94 +/-</td>
<td>4.77 +/-</td>
<td>6.16 +/-</td>
<td>4.17 +/-</td>
<td>64.90 +/-</td>
</tr>
<tr>
<td>10ng/ml</td>
<td>0.27</td>
<td>0.08 ***</td>
<td>0.39 ***</td>
<td>0.25</td>
<td>2.32 ***</td>
</tr>
<tr>
<td>bFGF</td>
<td>28.19 +/-</td>
<td>6.52 +/-</td>
<td>8.82 +/-</td>
<td>20.25 +/-</td>
<td>50.53 +/-</td>
</tr>
<tr>
<td>100ng/ml</td>
<td>1.27 ***</td>
<td>0.31 ***</td>
<td>0.59 ***</td>
<td>1.86 ***</td>
<td>0.76 ***</td>
</tr>
<tr>
<td>IL-1β</td>
<td>27.93 +/-</td>
<td>5.73 +/-</td>
<td>11.98 +/-</td>
<td>14.43 +/-</td>
<td>51.44 +/-</td>
</tr>
<tr>
<td>1ng/ml +</td>
<td>1.20</td>
<td>0.30 ***</td>
<td>0.25 ***</td>
<td>0.42 *</td>
<td>2.19 ***</td>
</tr>
<tr>
<td>bFGF</td>
<td>1ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>32.71 +/-</td>
<td>9.38 +/-</td>
<td>21.42 +/-</td>
<td>25.59 +/-</td>
<td>105.71 +/-</td>
</tr>
<tr>
<td>1ng/ml +</td>
<td>0.28</td>
<td>0.21 ***</td>
<td>0.56 ***</td>
<td>0.52 ***</td>
<td>4.73 ***</td>
</tr>
<tr>
<td>bFGF</td>
<td>10ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>37.52 +/-</td>
<td>13.15 +/-</td>
<td>26.67 +/-</td>
<td>45.70 +/-</td>
<td>75.21 +/-</td>
</tr>
<tr>
<td>1ng/ml +</td>
<td>0.29</td>
<td>0.34 ***</td>
<td>1.17 ***</td>
<td>1.23 ***</td>
<td>3.49 *</td>
</tr>
<tr>
<td>bFGF</td>
<td>100ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. The production of collagenase protein by human skin fibroblasts following treatment with bFGF either alone or in the additional presence of IL-1β. The results are expressed as collagenase in ng per µg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of bFGF and IL-1β are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>SY 33 passage 8</td>
<td>SY 34 passage 3</td>
<td>SY 7 passage 3</td>
</tr>
<tr>
<td>Control</td>
<td>0.08 +/- 0.051</td>
<td>3.96 +/- 0.12</td>
<td>0.48 +/- 0.04</td>
</tr>
<tr>
<td>IL-1β 1ng/ml</td>
<td>2.97 +/- 0.20 ***</td>
<td>15.12 +/- 0.24 ***</td>
<td>25.24 +/- 1.50 ***</td>
</tr>
<tr>
<td>bFGF 1ng/ml</td>
<td>0.18 +/- 0.03</td>
<td>4.15 +/- 0.17</td>
<td>0.54 +/- 0.044</td>
</tr>
<tr>
<td>bFGF 10ng/ml</td>
<td>0.30 +/- 0.03 **</td>
<td>5.43 +/- 0.16 ***</td>
<td>0.78 +/- 0.030 ***</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>1.02 +/- 0.08 ***</td>
<td>10.47 +/- 0.14 ***</td>
<td>2.49 +/- 0.15 ***</td>
</tr>
<tr>
<td>IL-1β 1ng/ml + bFGF 1ng/ml</td>
<td>3.99 +/- 0.36 *</td>
<td>17.43 +/- 1.86</td>
<td>28.63 +/- 0.84</td>
</tr>
<tr>
<td>IL-1β 1ng/ml + bFGF 10ng/ml</td>
<td>6.45 +/- 0.49 ***</td>
<td>21.39 +/- 0.87 ***</td>
<td>24.31 +/- 2.78</td>
</tr>
<tr>
<td>IL-1β 1ng/ml + bFGF 100ng/ml</td>
<td>11.63 +/- 1.10 ***</td>
<td>37.21 +/- 5.30 **</td>
<td>32.43 +/- 1.08 *</td>
</tr>
</tbody>
</table>

Table 5.3. The production of collagenase protein by human synovial fibroblasts following treatment with bFGF either alone or in the additional presence of IL-1β. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of bFGF and IL-1β are as follows: ♦ p<0.05, ♦♦ p<0.01, ♦♦♦ p<0.001.
Table 5.4. The production of collagenase protein by human skin fibroblasts following treatment with EGF either alone or in the additional presence of IL-1β. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of EGF and IL-1β are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
5.3. DISCUSSION

Chapters 3 and 4 investigated the effect of retinoic acid in combination with various polypeptide growth factors on the production of interstitial collagenase and its natural inhibitor TIMP-1 from human fibroblasts. In this chapter, these investigations have been extended by examining the ability of the inflammatory cytokine IL-1β to enhance or modulate these effects.

The effect of IL-1β alone on TIMP-1 protein production from human skin fibroblasts was first investigated and IL-1β was found either to inhibit or to have no effect on this parameter. These inconsistent results are difficult to explain but may result from subtle differences in the conditions used in different experiments. The variability of the results does not appear to correlate with either the different cell lines or with the differences in passage number. Neither does it appear to depend on whether the results are expressed as ng of TIMP-1 per μg of protein or as ng of TIMP-1 per μg of DNA. One possible source of variation is the degree of confluency of the cells at the time of seeding out for experimentation. Although confluent cells were always used, this would have included cells which were just confluent (i.e. 1 monolayer covering the surface of the flask), hyper-confluent cells (2-3 layers thick) and other variations between these 2 extremes. Variation could also have arisen from the use of different batches of serum, culture medium or IL-1β during the time period in which the experiments were performed. As mentioned earlier, other workers have obtained conflicting data on the effect of IL-1 on TIMP-1 production from human fibroblasts (Murphy et al., 1985a, Ito et al., 1992, Ito et al., 1988, MacNaul et al., 1990) although consistent effects were seen within each study. On the basis of this work and the data presented in this chapter, it appears that IL-1 does not have any consistent effect on TIMP-1 protein production from human fibroblasts.

In addition, it was not possible to show any consistent effect of IL-1β on retinoic acid-induced TIMP-1 in this study. Again, the variability of this data is difficult to explain but may result from differences in experimental conditions as outlined above. These results therefore indicate that IL-1 is probably not responsible for the enhancement of
retinoic acid-induced TIMP-1 secretion seen with mononuclear cell factor. It is more likely to be due to the presence of polypeptide growth factors which synergize with retinoic acid to 'superinduce' TIMP-1 as demonstrated in chapters 3 and 4.

The effect of the additional presence of IL-1β on this synergistic induction of TIMP-1 was examined next. In the case of bFGF, EGF and PDGF-BB it was found that IL-1β caused a reduction in the level of synergistic interaction between retinoic acid and these growth factors in most experiments. However, no consistent effect was seen with IL-1β, retinoic acid and TGF-β. In many experiments, the levels of TIMP-1 produced by IL-1β, retinoic acid and bFGF, EGF or PDGF-BB in combination were equal to, or greater than that produced by retinoic acid and growth factor alone. This raises the possibility that IL-1β is not in fact interfering with the synergistic interactions but that a maximum level of TIMP-1 production has been attained in these cells which cannot be further stimulated. However, since other experiments showed that IL-1β both reduced the levels of TIMP-1 produced and reduced the degree of synergism seen, it is perhaps more likely that IL-1β does modulate the interactions occurring between retinoic acid and the growth factors.

What is (are) the mechanism(s) by which IL-1β causes these effects? One possibility is that IL-1β causes a reduction in the sensitivity of the cells to retinoic acid, growth factor or both, perhaps by modulating the expression of receptors for these factors. However, this explanation does not seem likely in the light of other data presented in this chapter. IL-1β has no consistent effect on retinoic acid-induced TIMP-1 production, suggesting that it does not have any consistent effect on altering the sensitivity of these cells to retinoic acid. In addition, IL-1β was found to synergize with bFGF and EGF to 'superinduce' skin fibroblast collagenase production, suggesting that IL-1β may render the cells more sensitive to the effect of the growth factors. Previous work has demonstrated a similar effect of IL-1 and PDGF on human skin fibroblast collagenase secretion (Circolo et al., 1991). Perhaps IL-1β instead interferes with cross-talk occurring between the the retinoid and growth factor signalling pathways. The fact that IL-1β interferes with the synergistic induction of
TIMP-1 by retinoic acid and bFGF, EGF or PDGF-BB, but not consistently with the response to retinoic acid and TGF-β may indicate similar mechanisms for the first 3 growth factors, but a distinct mechanism for TGF-β.

The mechanism by which IL-1β and bFGF or EGF synergize to stimulate fibroblast collagenase production is also of interest. The most straightforward explanation is that these responses occur as a result of modulation of receptor numbers or receptor affinity by one or both of the factors involved. It is feasible that either the growth factors could enhance the binding of IL-1 to the cell surface and/or that IL-1 increases the binding of the growth factors. A previous report has shown that IL-1 can inhibit the binding of EGF to its receptor in human fibroblasts by phosphorylation of the EGF receptor (Bird and Saklatvala, 1990); however this effect is very transient such that phosphorylation returns to baseline levels after 1 hour even in the continued presence of IL-1. This rapid, transient effect need not preclude an additional, slower and more sustained effect of IL-1 in increasing the binding of EGF to cells either via increased receptor affinity or increased receptor number. Indeed, previous reports have shown that under inflammatory conditions, the expression of EGF receptors is increased (Irwin et al., 1991, Nordlund et al., 1991). Alternatively, it may be that IL-1 and these growth factors synergize to stimulate collagenase production by modulating collagenase gene transcription or collagenase mRNA stability.

The data correlates well with previous findings showing that collagenase can also be synergistically stimulated in chondrocytes using IL-1 and bFGF (Phadke, 1987) and in periosteal tissue using IL-1 and EGF (van der Zee et al., 1993). It is also of interest that bFGF and EGF resemble PDGF in their ability to elicit this response in fibroblasts (Circolo et al., 1991, Smith et al., 1991, Harvey et al., 1993). Together, these findings suggest that inflammatory cytokines and polypeptide growth factors may cooperate in vivo to cause rapid connective tissue breakdown under inflammatory conditions.
CHAPTER SIX

TIME COURSE OF THE SYNERGISTIC INDUCTION OF TIMP-1 BY ALL-
TRANS-RETINOIC ACID AND GROWTH FACTORS AND THE EFFECT OF
SEQUENTIAL TREATMENT WITH THE AGENTS

6.1. INTRODUCTION

The data presented in chapters 3 and 4 demonstrate that all-trans-retinoic acid interacts in a synergistic manner with 4 polypeptide growth factors, bFGF, EGF, PDGF-BB and TGF-β to stimulate the production of TIMP-1 protein from skin, synovial and tendon fibroblasts. This chapter is concerned with investigating these responses of cells in greater detail in order to gain some insight into the possible mechanisms by which they are taking place.

There are a number of possible explanations for these synergistic responses. Firstly, the mechanisms may involve the induction of receptors for one agent by the other. Retinoic acid may induce the expression of growth factor receptors or alternatively the growth factors may induce the expression of retinoic acid receptors. It is also possible that both of these events may be contributing to the synergistic induction of TIMP-1 in response to the agents.

Secondly, the synergistic expression of TIMP-1 may be caused by the induction of a third factor by one or both of the agents. This third factor then interacts with either retinoic acid, or growth factor, or even both to cause a synergistic stimulation of TIMP-1 protein. A previous report has shown that retinoic acid can induce the expression of TGF-β in chick embryo chondrocytes although not in fibroblasts (Jakowlew et al., 1992). Another report has shown that retinoic acid induces the secretion of TGF-β2 but not TGF-β1 in normal rat kidney fibroblasts and human lung carcinoma cells (Danielpour et al., 1991). The synergistic stimulation of TIMP-1 in human fibroblasts could possibly be mediated by the induction of TGF-β by retinoic acid. The induced TGF-β may then interact synergistically with bFGF, EGF or
PDGF-BB to stimulate TIMP-1 production. Indeed, Edwards et al. (1987) have previously shown that bFGF and EGF interact synergistically with TGF-β to stimulate TIMP-1 expression in human foetal lung fibroblasts. If the synergistic induction of TIMP-1 protein in response to retinoic acid and bFGF or EGF is occurring through the induction of TGF-β, then low levels of TGF-β must be responsible for this. This is because retinoic acid and TGF-β (10-100ng/ml) alone without bFGF or EGF give a synergistic induction of TIMP-1 which is comparable to that seen with bFGF or EGF in combination with retinoic acid.

Thirdly, the synergistic responses may also be caused by effects on TIMP-1 gene transcription or on TIMP-1 mRNA stability. In particular, the effects may be caused by the interaction of activated retinoic acid receptors with nuclear proteins such as c-fos, causing a synergistic induction of TIMP-1 gene transcription as discussed in chapter 4. A fourth interesting possibility is that the responses occur by means of complex cross-talk between the retinoic acid and growth factor signalling pathways. A recent report has shown that the retinoic acid and cAMP signal transduction pathways may be coupled as a result of direct phosphorylation of retinoic acid receptors by protein kinase A (Huggenvik et al., 1993).

The purpose of the investigations in this and the following chapters is to test these different hypotheses and to show which, if any, are able to account for the observed responses. The data in these chapters are all obtained using human skin fibroblasts. These cells were chosen for further investigation of the synergistic mechanisms rather than synovial or tendon fibroblasts for 2 reasons. The primary reason is that skin fibroblasts appear to give a potent synergistic induction of TIMP-1 in response to the agents with the greatest consistency out of the 3 cell types. The other advantage of using these cells is that they generally grow faster than the other 2 types of fibroblast. Although it would be ideal to obtain data from all the cell types, this is very laborious and given the initial data, it is reasonable to predict that findings obtained using skin cells are applicable to synovial and tendon fibroblasts.
In this chapter, the time course of the synergistic induction of TIMP-1 protein in human skin fibroblasts by retinoic acid in combination with bFGF, EGF, PDGF-BB and TGF-β is investigated. This is in order to find out how much time after stimulation is required to give the maximal induction of TIMP-1 protein. This in turn gives some indication of whether this effect on TIMP-1 induction is a primary or secondary response of the cells to the agents.

The possible role of receptor induction in the synergistic mechanisms is investigated next. If the responses do involve the induction of receptors for 1 agent by the other, then it can be postulated that it is not necessary for both agents to be present together in order to see the response. If, for example, the mechanism involves the induction of growth factor receptors by retinoic acid, then treatment of the cells with retinoic acid alone followed by growth factor alone should still produce the response. Similarly, if the mechanism involves the induction of retinoic acid receptors by the growth factors, then treatment of the cells with growth factor alone followed by retinoic acid alone should cause a synergistic induction of TIMP-1. In addition, if the responses involve the induction of a third factor such as TGF-β, or the induction of nuclear proteins such as c-fos, then sequential treatment of the cells with the agents may produce a synergistic induction of TIMP-1. The experiments presented in this chapter therefore investigate these possibilities by examining the effect of sequential treatment of human skin fibroblasts with the agents to see whether this is able to cause a synergistic induction of TIMP-1 protein.
6.2. METHODS AND RESULTS

6.2.1. The effect of all-trans-retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β on TIMP-1 protein production in human skin fibroblasts at various time points after stimulation.

The effect of $10^{-5}$M retinoic acid in combination with bFGF, PDGF-BB or TGF-β at 100ng/ml or EGF at 10ng/ml on TIMP-1 protein production 6, 12, 24, 48 and 72 hours after stimulation was investigated. Three human skin cell lines were examined using the basic cell assay system. The concentrations of growth factors used were based on the concentrations giving the greatest synergistic stimulation of TIMP-1 protein in the preliminary experiments in chapters 3 and 4.

The effect of the following test conditions was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added simultaneously.

1. control - DMEM + 1% ATFCFS only
2. all-trans-retinoic acid at $10^{-5}$M
3. recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or EGF at 10ng/ml
4. all-trans-retinoic acid at $10^{-5}$M + recombinant bFGF, PDGF-BB or purified TGF-β at 100ng/ml or EGF at 10ng/ml.

After 6, 12, 24, 48 or 72 hours, the cell supernates were harvested and assayed for TIMP-1 by ELISA as described in chapter 2. The protein content of the cell monolayers was measured as described in chapter 2 and the levels of TIMP-1 were corrected with respect to the cell protein such that the results are expressed as ng of TIMP-1 per μg of cell protein. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.

In all experiments, TIMP-1 protein was induced significantly above control levels by retinoic acid at $10^{-5}$M applied alone. This effect appeared between 6 and 24 hours
after stimulation. The maximum induction of TIMP-1 by retinoic acid alone was seen between 24 and 72 hours after stimulation. Each of the growth factors tested also induced TIMP-1 protein above control levels when applied alone in all experiments. In all experiments, a synergistic induction of TIMP-1 protein was seen when retinoic acid was applied in combination with bFGF, EGF, PDGF-BB or TGF-β. The maximum synergistic induction of TIMP-1 by these agents was seen at 72 hours after stimulation except in 1 experiment with retinoic acid and TGF-β in which this was observed after 48 hours. The earliest time point at which a synergistic induction of TIMP-1 occurred varied between 6 and 24 hours.

In 2 experiments using the human skin cell line hsf 15 treated with retinoic acid and bFGF or retinoic acid and EGF, a 96 hour time point was also included. In both of these experiments it was found that the synergistic induction of TIMP-1 by the agents was decreased at 96 hours compared to the effect seen at 72 hours. This suggests that 72 hours is the optimal time period of stimulation after which the induction of TIMP-1 starts to return to baseline. A representative experiment showing the effect of retinoic acid alone, growth factor alone and both together on the induction of TIMP-1 protein at increasing time after stimulation is shown for each of the growth factors in figures 6.1.-6.4.

6.2.2. The effect of sequential treatment of human skin fibroblasts with all-trans-retinoic acid and bFGF, EGF, PDGF-BB or TGF-β.

In the first set of experiments in this section, the effect of retinoic acid treatment for 72 hours followed by treatment with growth factor for a further 72 hours was compared with the effect of both reagents together for 72 hours. Similarly, the effect of growth factor for 72 hours followed by retinoic acid for 72 hours was compared to the effect of both reagents together for 72 hours. 3 human skin cell lines were investigated which were seeded out and prepared for stimulation using the basic cell assay system. After the first 72 hour period of stimulation, all the wells were washed 3 times with HBSS prior to the addition of the test reagents for the second 72 hour
Figures 6.1. (top) and 6.2. (bottom).

Production of TIMP-1 protein at increasing time after stimulation by the human skin cell line hsf 13 (passage 9) following treatment with retinoic acid alone, bFGF alone and retinoic acid and bFGF combined (figure 6.1.) and by the human skin cell line hsf 12 (passage 13) following treatment with retinoic acid alone, EGF alone and retinoic acid and EGF combined (figure 6.2.).
Figures 6.3. (top) and 6.4. (bottom).

Production of TIMP-1 protein at increasing time after stimulation by the human skin cell line hsf 12 (passage 13) following treatment with retinoic acid alone, PDGF-BB alone and retinoic acid and PDGF-BB combined (figure 6.3.) and by the human skin cell line hsf 15 (passage 9) following treatment with retinoic acid alone, TGF-β alone and retinoic acid and TGF-β combined (figure 6.4.).
stimulation. The test conditions were as follows:

1. Control - DMEM + 1%ATFCS only for 72 hours followed by fresh DMEM + 1%ATFCS for a further 72 hours.
2. DMEM + 1%ATFCS only for 72 hours followed by 10⁻⁶M all-trans-retinoic acid for 72 hours.
3. DMEM + 1%ATFCS only for 72 hours followed by bFGF, PDGF-BB or TGF-β at 100ng/ml or EGF at 10ng/ml for 72 hours.
4. DMEM + 1%ATFCS only for 72 hours followed by 10⁻⁶M all-trans-retinoic acid in combination with bFGF, PDGF-BB or TGF-β at 100ng/ml or EGF at 10ng/ml for 72 hours.
5. 10⁻⁶M all-trans-retinoic acid for 72 hours followed by bFGF, PDGF-BB or TGF-β at 100ng/ml or EGF at 10ng/ml for 72 hours.
6. bFGF, PDGF-BB or TGF-β at 100ng/ml or EGF at 10ng/ml for 72 hours followed by 10⁻⁶M all-trans-retinoic acid for 72 hours.

The cell supernates were then harvested and assayed for TIMP-1 by ELISA as described in chapter 2. The protein content of the cell monolayers was measured as described in chapter 2 and the levels of TIMP-1 were corrected with respect to the cell protein such that the results are expressed as ng of TIMP-1 per µg of cell protein. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.

In all experiments, 10⁻⁶M retinoic acid alone, 100ng/ml bFGF alone and 10ng/ml EGF alone significantly stimulated TIMP-1 protein production from the cells. PDGF-BB added alone at 100ng/ml induced a significant increase in TIMP-1 protein production in 2 experiments out of 3 but TGF-β alone at 100ng/ml did not induce TIMP-1 protein production in any experiments. When added in combination, retinoic acid and bFGF, EGF, PDGF-BB or TGF-β caused a synergistic induction of TIMP-1 protein in all experiments. However, retinoic acid treatment for 72 hours followed by bFGF, EGF
PDGF-BB or TGF-β for 72 hours did not result in any synergistic induction of TIMP-1. In the case of bFGF, EGF or PDGF-BB, growth factor treatment for 72 hours followed by 10^{-5} M retinoic acid did not cause a synergistic induction of TIMP-1 either. However, in the case of TGF-β, a synergistic induction of TIMP-1 was observed when the cells were treated first with TGF-β at 100 ng/ml for 72 hours followed by retinoic acid at 10^{-5} M for 72 hours. Representative experiments are shown in figures 6.5, 6.6., 6.7. and 6.8., and the results of all experiments are shown in tables 6.1., 6.2. and 6.3.

It was observed that the synergistic induction of TIMP-1 protein by both agents together in these experiments was not as potent as that seen in the earlier experiments in chapters 3 and 4. In the case of TGF-β and PDGF-BB, the 3 experiments shown are from a total of 7 or 8 in which the rest were unsuccessful because no synergistic induction of TIMP-1 occurred (data not shown). The only difference in the experiments shown in this section and the experiments in chapters 3 and 4 is that the cells are incubated for an additional 72 hours in DMEM + 1% ATFCS prior to stimulation. In the experiments shown in this section, the induction of TIMP-1 by retinoic acid alone tends to be more potent than that seen in the earlier experiments. This suggests that prolonged incubation in medium containing a low level of serum may sensitize the cells to retinoic acid leading to a maximum or near maximum effect on TIMP-1 in response to retinoic acid alone. This in turn may cause the cells to become refractory to further stimulation by retinoic acid in combination with growth factors, thus explaining the weaker or non-existent synergistic effects observed.

The results suggest that in the case of bFGF, EGF and PDGF-BB, it is necessary for retinoic acid and the growth factor to be present together to cause a synergistic induction of TIMP-1. The data also suggests that the mechanisms do not involve the induction of receptors for 1 agent by the other or the induction of a third factor by 1 agent which interacts with the second agent. However, it is also possible that a transient induction of receptors or of a third factor may occur which returns to baseline within 72 hours.
Figures 6.5. (top) and 6.6. (bottom).

The effect of sequential treatment of cells with retinoic acid and bFGF in the human skin cell line hsf 15 (passage 2) (figure 6.5.) or retinoic acid and EGF in the human skin cell line hsf 13 (passage 15) (figure 6.6.). The cells were treated for 72 hours with the first agent and for 72 hours with the second agent.
Figures 6.7. (top) and 6.8. (bottom).
The effect of sequential treatment of cells with retinoic acid and PDGF-BB (figure 6.7.) or retinoic acid and TGF-β (figure 6.8.) in the human skin cell line hsf 12 (passage 12). The cells were treated for 72 hours with the first agent and for 72 hours with the second agent.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 12 passage 11</td>
<td>hsf 15 passage 2</td>
<td>hsf 13 passage 15</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then DMEM + 1% ATFCS for 72 hours</td>
<td>4.89 +/- 0.21</td>
<td>10.95 +/- 0.34</td>
<td>5.38 +/- 0.09</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then retinoic acid 10^{-5}M for 72 hours</td>
<td>14.28 +/- 1.49 ***</td>
<td>28.68 +/- 1.27 ***</td>
<td>17.62 +/- 0.37 ***</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then bFGF 100ng/ml for 72 hours</td>
<td>6.40 +/- 0.22 ***</td>
<td>18.40 +/- 0.65 ***</td>
<td>7.24 +/- 0.12 ***</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then retinoic acid 10^{-5}M + bFGF 100ng/ml for 72 hours</td>
<td>20.74 +/- 0.45 **</td>
<td>66.85 +/- 3.25 **</td>
<td>31.59 +/- 0.82 ***</td>
</tr>
<tr>
<td>Retinoic acid 10^{-5}M for 72 hours then bFGF 100ng/ml for 72 hours</td>
<td>15.64 +/- 0.59</td>
<td>34.96 +/- 1.68</td>
<td>16.08 +/- 0.46</td>
</tr>
<tr>
<td>bFGF 100ng/ml for 72 hours then retinoic acid 10^{-5}M for 72 hours</td>
<td>14.53 +/- 0.39</td>
<td>23.21 +/- 0.46</td>
<td>15.39 +/- 0.32</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then EGF 10ng/ml for 72 hours</td>
<td>6.57 +/- 0.20 ***</td>
<td>16.37 +/- 0.61 ***</td>
<td>8.52 +/- 0.09 ***</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours then retinoic acid 10^{-5}M + EGF 10ng/ml for 72 hours</td>
<td>22.49 +/- 0.42 ***</td>
<td>48.82 +/- 1.35 ***</td>
<td>30.80 +/- 0.51 ***</td>
</tr>
<tr>
<td>Retinoic acid 10^{-5}M for 72 hours then EGF 10ng/ml for 72 hours</td>
<td>15.37 +/- 0.40</td>
<td>28.72 +/- 0.62</td>
<td>14.93 +/- 0.35</td>
</tr>
<tr>
<td>EGF 10ng/ml for 72 hours then retinoic acid 10^{-5}M for 72 hours</td>
<td>14.17 +/- 0.40</td>
<td>25.05 +/- 0.58</td>
<td>14.83 +/- 0.51</td>
</tr>
</tbody>
</table>

Table 6.1. The production of TIMP-1 protein by human skin fibroblasts resulting from sequential treatment of the cells with retinoic acid and bFGF or EGF. The effect of stimulation with retinoic acid followed by bFGF or EGF and the effect of stimulation with bFGF or EGF followed by retinoic acid is compared to the effect of stimulation with both factors together. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF or EGF are as follows: • p<0.05, •• p<0.01, ••• p<0.001.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
</table>
| Cell Line  | hsf 12  
passage 10 | hsf 12  
passage 12 | hsf 13  
passage 14 |
| DMEM + 1% ATFCS  
for 72 hours then  
DMEM + 1% ATFCS  
for 72 hours | 5.17 +/- 0.08 | 4.08 +/- 0.15 | 143.20 +/- 5.83 |
| DMEM + 1% ATFCS  
for 72 hours then  
retinoic acid 10^(-5)M for 72 hours | 19.60 +/- 0.77 *** | 19.51 +/- 1.60 *** | 449.65 +/- 11.58 *** |
| DMEM + 1% ATFCS  
for 72 hours then  
PDGF-BB 100ng/ml for 72 hours | 4.95 +/- 0.14 | 6.53 +/- 0.20 *** | 174.32 +/- 2.80 *** |
| DMEM + 1% ATFCS  
for 72 hours then  
retinoic acid 10^(-5)M  
+ PDGF-BB 100ng/ml for 72 hours | 25.50 +/- 1.39 ** | 37.69 +/- 1.83 *** | 793.03 +/- 38.61 *** |
| Retinoic acid 10^(-5)M for 72 hours then PDGF-BB 100ng/ml for 72 hours | 11.43 +/- 0.54 | 16.35 +/- 0.65 | 407.62 +/- 5.50 |
| PDGF-BB 100ng/ml for 72 hours then retinoic acid 10^(-5)M for 72 hours | 18.55 +/- 0.91 | 18.80 +/- 0.44 | 487.20 +/- 12.77 |

Table 6.2. The production of TIMP-1 protein by human skin fibroblasts resulting from sequential treatment of the cells with retinoic acid and PDGF-BB. The effect of stimulation with retinoic acid followed by PDGF-BB and the effect of stimulation with PDGF-BB followed by retinoic acid is compared to the effect of stimulation with both factors together. The results are expressed as TIMP-1 in ng per µg of cell protein except for hsf 13 (passage 14) where the results are expressed as ng/ml of TIMP-1 protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and PDGF-BB are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.
Table 6.3. The production of TIMP-1 protein by human skin fibroblasts resulting from sequential treatment of the cells with retinoic acid and TGF-β. The effect of stimulation with retinoic acid followed by TGF-β and the effect of stimulation with TGF-β followed by retinoic acid is compared to the effect of stimulation with both factors together. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and TGF-β are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 15</td>
<td>hsf 12</td>
<td>hsf 9</td>
</tr>
<tr>
<td></td>
<td>passage 4</td>
<td>passage 12</td>
<td>passage 7</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>13.86 +/- 0.60</td>
<td>4.08 +/- 0.15</td>
<td>16.44 +/- 0.62</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>36.06 +/- 0.97 ***</td>
<td>19.51 +/- 1.60 ***</td>
<td>44.27 +/- 1.43 ***</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>12.91 +/- 0.65</td>
<td>4.48 +/- 0.10</td>
<td>16.85 +/- 1.36</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>46.43 +/- 0.98 ***</td>
<td>38.53 +/- 1.19 ***</td>
<td>56.93 +/- 1.83 ***</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>27.30 +/- 1.01</td>
<td>14.39 +/- 0.45</td>
<td>25.03 +/- 0.82</td>
</tr>
<tr>
<td>DMEM + 1% ATFCS for 72 hours</td>
<td>53.19 +/- 2.42 ***</td>
<td>31.81 +/- 1.17 ***</td>
<td>55.17 +/- 1.44 ***</td>
</tr>
</tbody>
</table>
This question was addressed in the case of bFGF and EGF by repeating the sequential treatment of the cells, but this time, the cells were incubated with the first agent for several different periods of time ranging from 6 hours to 48 hours. This was then followed by treatment with the second agent for 72 hours. The effect of these sequential treatments was compared to the effect of incubating the cells with both factors together for 72 hours. 3 experiments were carried out using 3 different human skin cell lines. Similar experiments were also attempted using PDGF-BB and retinoic acid, but these were unsuccessful because the cells failed to give a synergistic induction of TIMP-1 protein when both agents were applied together. This lack of effect may be due to sensitisation of the cells to retinoic acid during the first incubation, leading to a maximum effect on TIMP-1 in response to retinoic acid alone as has been discussed. These experiments were performed exactly as those described in the earlier part of this section except that the test reagents were applied to the cells as follows:

1. Control - DMEM + 1%ATFCS only for 6, 12, 24 or 48 hours followed by fresh DMEM + 1%ATFCS for a further 72 hours.
2. DMEM + 1%ATFCS only for 6, 12, 24 or 48 hours followed by 10⁻⁵M all-trans-retinoic acid for 72 hours.
3. DMEM + 1%ATFCS only for 6, 12, 24 or 48 hours followed by bFGF at 100ng/ml or EGF at 10ng/ml for 72 hours.
4. DMEM + 1%ATFCS only for 6, 12, 24 or 48 hours followed by 10⁻⁵M all-trans-retinoic acid in combination with bFGF at 100ng/ml or EGF at 10ng/ml for 72 hours.
5. 10⁻⁵M all-trans-retinoic acid for 6, 12, 24 or 48 hours followed by bFGF at 100ng/ml or EGF at 10ng/ml for 72 hours.
6. 10⁻⁵M all-trans-retinoic acid for 6, 12, 24 or 48 hours followed by DMEM + 1%ATFCS only for 72 hours.
7. bFGF at 100ng/ml or EGF at 10ng/ml for 6, 12, 24 or 48 hours followed by 10⁻⁵M all-trans-retinoic acid for 72 hours.
8. bFGF at 100ng/ml or EGF at 10ng/ml for 6, 12, 24 or 48 hours followed by DMEM + 1%ATFCS only for 72 hours.

In all experiments, treatment of the cells with retinoic acid alone at 10^−M for 72 hours caused a significant increase in TIMP-1 protein production while treatment with bFGF alone caused either no effect or a small stimulation of TIMP-1. When the cells were treated with retinoic acid and bFGF in combination for 72 hours, a synergistic increase in TIMP-1 protein production was seen in all experiments. Treatment of the cells with retinoic acid first for 6, 12, 24 or 48 hours followed by bFGF for 72 hours failed to produce a synergistic induction of TIMP-1 protein in all experiments. However, when the cells were treated first with bFGF for 6 or 12 hours followed by retinoic acid for 72 hours, a synergistic induction of TIMP-1 protein occurred in all experiments. When the period of stimulation with bFGF was increased to 24 hours, a synergistic response was seen in only 2 cell lines out of 3 (hsf 15 and hsf 10) and in both cases it was less potent than the effect observed with a 6 or 12 hour preincubation. When the cells were treated with bFGF for 48 hours followed by retinoic acid for 72 hours, there was no synergistic stimulation of TIMP-1 protein in any of the cell lines. Figures 6.9.-6.12. show the results obtained using the human skin cell line hsf 15 (passage 9) for each different preincubation time period.

In experiments in which 100ng/ml bFGF was replaced by 10ng/ml EGF, retinoic acid applied alone for 72 hours again stimulated TIMP-1 protein production in all experiments. Treatment with EGF alone for 72 hours did not stimulate TIMP-1 protein production in any experiments while the addition of retinoic acid in combination with EGF for 72 hours caused a synergistic stimulation of TIMP-1 protein in all experiments. When the cells were treated with retinoic acid first for 6, 12, 24 or 48 hours followed by EGF for a further 72 hours, there was no synergistic induction of TIMP-1 protein with any of the pre incubation time periods in any of the experiments. Similarly, when the cells were treated with EGF first followed by retinoic acid, there was again no synergistic induction of TIMP-1 protein with any
Figures 6.9. (top) and 6.10. (bottom).

The effect of sequential treatment of cells with retinoic acid and bFGF in the human skin cell line hsf 15 (passage 9) with a 6 hour incubation of the first agent and a 72 hour incubation with the second agent (figure 6.9.), or a 12 hour incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.10.).
Figures 6.11. (top) and 6.12. (bottom).
The effect of sequential treatment of cells with retinoic acid and bFGF in the human skin cell line hsf 15 (passage 9) with a 24 hour incubation of the first agent followed by a 72 hour incubation with the second agent (figure 6.11.) or a 48 hour incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.12.).
pre incubation period in any experiment. The results obtained using the human skin cell line hsf 13 (passage 3) for each different pre incubation time period are shown in figures 6.13.-6.16.

In the case of EGF, it appears that it is obligatory for both agents to be present together to cause a synergistic induction of TIMP-1 protein. By contrast however, a synergistic response can be obtained by a 6-24 hour pre stimulation with bFGF followed by retinoic acid for 72 hours. The most potent response is obtained with 6-12 hours of bFGF treatment; it declines at 12-24 hours and disappears altogether by 48 hours. In order to elucidate the effect of bFGF on the cells during this time period it would be useful to find out the minimum time of incubation which is required to give a synergistic induction of TIMP-1.

In order to answer this question, a further set of experiments were set up in which the cells were incubated with bFGF for a variety of time periods ranging from 10 minutes to 3 hours followed by retinoic acid treatment for 72 hours. In addition, the cells were also treated with retinoic acid first followed by bFGF. The effect of these sequential treatments was compared to the effect of incubating the cells with both factors together for 72 hours. 3 experiments were carried out using 3 different human skin cell lines and the protocol was the same as that described earlier. The following test conditions were examined:

1. Control - DMEM + 1%ATFCS only for 10 minutes, 30 minutes, 1 hour or 3 hours followed by fresh DMEM + 1%ATFCS for a further 72 hours
2. DMEM + 1%ATFCS only for 10 minutes, 30 minutes, 1 hour or 3 hours followed by 10⁻⁵M all-trans-retinoic acid for 72 hours
3. DMEM + 1%ATFCS only for 10 minutes, 30 minutes, 1 hour or 3 hours followed by bFGF at 100ng/ml for 72 hours
4. DMEM + 1%ATFCS only for 10 minutes, 30 minutes, 1 hour or 3 hours followed by 10⁻⁵M all-trans-retinoic acid in combination with bFGF at 100ng/ml for 72 hours

The effect of sequential treatment of cells with retinoic acid and EGF in the human skin cell line hsf 13 (passage 3) with a 6 hour incubation of the first agent followed by a 72 hour incubation with the second agent (figure 6.13.) or a 12 hour incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.14.).
Figures 6.15. (top) and 6.16. (bottom).

The effect of sequential treatment of cells with retinoic acid and EGF in the human skin cell line hsf 13 (passage 3) with a 24 hour incubation of the first agent followed by a 72 hour incubation with the second agent (figure 6.15.) or a 48 hour incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.16.).
6. 10^{-5} M all-trans-retinoic acid for 10 minutes, 30 minutes, 1 hour or 3 hours followed by DMEM + 1%ATFCS for 72 hours.

7. bFGF at 100ng/ml for 10 minutes, 30 minutes, 1 hour or 3 hours followed by 10^{-5} M all-trans-retinoic acid for 72 hours.

8. bFGF at 100ng/ml for 10 minutes, 30 minutes, 1 hour or 3 hours followed by DMEM + 1%ATFCS for 72 hours.

In all experiments, treatment of the cells with retinoic acid alone at 10^{-5} M for 72 hours caused a significant increase in TIMP-1 protein production. Treatment with bFGF alone caused a significant stimulation of TIMP-1 protein production in the experiments with hsf 10 and hsf 9 but not in the experiment with hsf 13. When the cells were treated with retinoic acid and bFGF in combination for 72 hours, a synergistic increase in TIMP-1 protein production was seen in all experiments. Treatment of the cells with retinoic acid first for 10 minutes, 30 minutes, 1 hour or 3 hours followed by bFGF for 72 hours failed to produce a synergistic induction of TIMP-1 protein in any of the experiments. However, when the cells were treated first with bFGF for any of these pre incubation times followed by retinoic acid for 72 hours, a synergistic induction of TIMP-1 protein occurred in all experiments. Figures 6.17.-6.20. show the results obtained using the cell line hsf 9 (passage 6) for each different pre incubation time period.

The results of these experiments indicate that exposure of the cells to bFGF for as little as 10 minutes is sufficient to give a synergistic induction of TIMP-1 when followed by 72 hours of retinoic acid treatment. The optimum pre incubation time with bFGF is 1 to 3 hours as these time points gave the greatest synergistic induction of TIMP-1 protein in all experiments performed. This optimum response was equivalent to the effect of treating the cells with both agents together for 72 hours. Such data suggest that bFGF is transiently stimulating the induction or the activity of a cellular protein which is responsible for causing the induction of TIMP-1. The precise kinetics of this event vary between different cell lines both in the length of
Figures 6.17. (top) and 6.18. (bottom).

The effect of sequential treatment of cells with retinoic acid and bFGF in the human skin cell line hsf 9 (passage 6) with a 10 minute incubation of the first agent followed by a 72 hour incubation with the second agent (figure 6.17.) or a 30 minute incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.18.).
Figures 6.19. (top) and 6.20. (bottom).

The effect of sequential treatment of cells with retinoic acid and bFGF in the human skin cell line hsf 9 (passage 6) with a 1 hour incubation of the first agent followed by a 72 hour incubation with the second agent (figure 6.19.) or a 3 hour incubation with the first agent followed by a 72 hour incubation with the second agent (figure 6.20.).
time required to give maximal stimulation and in the length of time before the stimulation starts to return to baseline. However, there seems to be little doubt that the stimulation of a transient event is occurring in these cells and that this event is responsible for the synergistic induction of TIMP-1.
6.3. DISCUSSION

The kinetics of the induction of TIMP-1 protein in response to retinoic acid in combination with bFGF, EGF, PDGF-BB and TGF-β was used as a starting point for attempting to understand the mechanism(s) by which these effects are taking place. With the exception of 1 experiment, the synergistic induction of TIMP-1 protein appeared to commence between 6 and 24 hours after stimulation and become maximal after 72 hours. Experiments in which the time of stimulation was increased to 96 hours suggest that 72 hours is the optimum time of exposure to the agents after which the responses begin to return to baseline. Clearly, the response of skin fibroblasts to retinoic acid and each of the 4 growth factors are slow rather than rapid effects. Such data suggest that the stimulation of TIMP-1 by these factors is a secondary rather than a primary response. Alternatively, increased stability of TIMP-1 mRNA may also account for the results as this would produce an elevated expression of TIMP-1 over an extended period following stimulation by the agents.

The induction of TIMP-1 protein by retinoic acid alone also appeared to be a quite slow effect which appeared between 6 and 24 hours and became maximal after 24-72 hours of stimulation. The time course of induction of TIMP-1 protein in human skin fibroblasts in response to retinoic acid alone has previously been investigated (Clark et al., 1987). An effect was seen after 8 hours of exposure to retinoic acid but not at 4 hours. The maximum effect occurred after 24 to 36 hours of stimulation with retinoic acid. These results are similar to those reported in this chapter, although my data overall shows a somewhat slower induction of TIMP-1. Such differences can probably be explained by slight differences in experimental protocol used in the 2 different studies. The data in this chapter showing the effect of growth factors alone on TIMP-1 induction were too variable to be able to draw any conclusions about the kinetics of these effects.

Other studies which have examined the effect of other agents on TIMP-1 protein induction from human fibroblasts have shown that in general, the induction of this inhibitor is a slow response and therefore probably a secondary effect on the cells. In
the case of bFGF or EGF applied alone or in combination with TGF-β to human foetal lung fibroblasts, induction of TIMP-1 protein is greater after 48 hours than after 24 hours (Edwards et al., 1987). The effects of concanavalin A and TPA on respectively repressing and inducing TIMP-1 protein production from human fibroblasts are also greater after 48 hours than after 24 hours (Overall and Sodek, 1990, Overall, 1994). In human gingival fibroblasts, the induction of TIMP-1 protein by TGF-β is a slow effect since it does not reach maximal levels until 48-72 hours after stimulation (Overall et al., 1989). TGF-β increases TIMP-1 gene transcription after 24 hours in these cells where as there is no effect after 7 hours. This contrasts with the induction of 72-000-Mr gelatinase by TGF-β in which an increase in transcription is seen after 7 hours and has returned almost to control levels after 24 hours (Overall et al., 1991).

The induction of TIMP-1 in human skin fibroblasts by PMA is a faster response since it is maximal after 8 hours of stimulation. However, this response is sustained at its maximal level for up to 72 hours after stimulation (Clark et al., 1985). The data shown in this chapter indicate that the synergistic induction of TIMP-1 by retinoic acid and growth factors is slow in onset and therefore fit in with what is already known about the regulation of this inhibitor.

Having established that the synergistic stimulation of TIMP-1 protein by retinoic acid and each of the 4 growth factors is slow and therefore probably a secondary effect, the question of possible primary effects of the agents on the cells was addressed next. The data shows that in the case TGF-β, treatment of the cells for 72 hours with this growth factor followed by retinoic acid for 72 hours gives a similar response to the effect of both agents together for 72 hours. However, in the case of bFGF, EGF and PDGF-BB, growth factor stimulation followed by retinoic acid treatment does not give any synergism. The results suggest that TGF-β induces an intracellular event which is responsible for causing the synergistic induction of TIMP-1 in the additional presence of retinoic acid. Such an effect may be rapidly initiated, but subsequently sustained at its maximum level for up to 72 hours; alternatively it may be slow in onset such that it does not peak until about 72 hours after stimulation. One possibility
is that TGF-β may be inducing the expression of retinoic acid receptors (RARs and RXRs) thus rendering the cells more sensitive to the effect of retinoic acid. This in turn could lead to a synergistic induction of TIMP-1. Retinoic acid is known to regulate the production of its own receptors in mesenchymal cells (Nugent et al., 1995 Pan and Brinckerhoff, 1994, Pan et al., 1995) and it is possible that TGF-β could potentiate this effect. However, it has also been shown that TGF-β in fact inhibits the effect of retinoic acid on inducing RAR-β in murine embryonic palate mesenchymal cells (Nugent et al., 1995). Such evidence suggests that TGF-β is not stimulating TIMP-1 production in the presence of retinoic acid through the induction of retinoic acid receptors. However, it is still possible that TGF-β may enhance the expression of other members of the RAR/RXR family besides RAR-β in cells. Furthermore, the effect of TGF-β on RAR-β in human skin fibroblasts may be different to that seen in murine embryonic palate cells.

The responsiveness of cells to retinoic acid is also influenced by the cellular retinoic acid-binding proteins CRABP and CRABP-II. It has been shown that TGF-β downregulates steady-state levels of CRABP mRNA but stimulates CRABP-II mRNA in murine embryonic palate mesenchymal cells (Nugent and Greene, 1994). The precise role of CRABP and CRABP-II is not understood. Some workers have suggested that CRABP sequesters retinoic acid in the cytoplasm and directs its metabolism to more polar compounds (Fiorella & Napoli 1991). This would reduce the amount of retinoic acid available to regulate gene expression in the nucleus (Boylan and Gudas, 1991). Hence it follows that a repression of CRABP would result in an enhanced response to retinoic acid. It is therefore possible that TGF-β may enhance the production of TIMP-1 protein in the presence of retinoic acid through effects on CRABP gene expression. However, others have suggested that the CRABPs have a role in delivering retinoic acid to the nuclear retinoic acid receptors (Ong, 1994). If this is the case, then the repression of either gene would dampen the response of cells to retinoic acid while a stimulatory effect would enhance this response.
An alternative explanation suggested in chapter 4 is that TGF-β may be inducing the production of a nuclear transcription factor which is able to interact synergistically with activated retinoic acid receptors to stimulate TIMP-1 gene transcription. Possible candidates for this transcription factor are the products of proto-oncogenes such as c-fos, c-jun, junB and c-myc since TGF-β is known to increase the mRNA levels for these proteins in cells (Leof et al., 1986, Liboi et al., 1988, Pertovaara et al., 1989, Spizz et al., 1987, Li et al., 1990). In rat fibroblasts, sustained expression of c-fos mRNA for up to at least 24 hours after stimulation has been observed in response to TGF-β; this was accompanied by an increase in c-fos protein for up to at least 7 hours after stimulation (Liboi et al., 1988). In murine NIH-3T3 fibroblasts, a similar effect was seen on induction of c-fos mRNA in response to TGF-β but no corresponding effect on protein was observed. The response of mouse embryo fibroblasts was different again since in this case a rapid, transient effect on c-fos mRNA was seen (Leof et al., 1986). Induction of c-myc mRNA by TGF-β was also evident for up to 24 hours after stimulation in mouse embryonic fibroblasts although the maximum response occurred 8-12 hours after stimulation (Leof et al., 1986). However, the induction of junB mRNA in these cells was a more transient effect which had nearly disappeared by 24 hours after stimulation (Pertovaara et al., 1989). The induction of junB in fibroblasts by TGF-β is therefore probably too transient to account for the synergistic induction of TIMP-1 in response to TGF-β and retinoic acid, unless the stability of junB protein allows it to persist for up to 72 hours after stimulation. In addition, the induction of junB mRNA may have different kinetics in human skin fibroblasts compared to mouse embryonic fibroblasts. However, c-fos and c-myc are possible candidates for involvement in the synergistic response in the light of previously published work, although further studies into the regulation of these genes in adult human skin fibroblasts by TGF-β are required.

In the case of EGF, the results shown in this study suggest that retinoic acid and EGF must be present simultaneously for a synergistic induction of TIMP-1 protein to occur. When the cells are treated with 1 agent for 72 hours followed by the other, no
synergistic stimulation occurs; furthermore when the incubation time with the first agent is reduced to as little as 6 hours there is still no synergistic response. However, the possibility that a briefer exposure to either retinoic acid or EGF followed by treatment with the second agent may have produced a response cannot be precluded. In chapter 3, it was suggested that the synergistic induction of TIMP-1 protein in response to retinoic acid and EGF could result from an increase in EGF binding sites on the cells in response to retinoic acid. A previous report has shown that retinoic acid enhances the binding of $^{125}\text{I}$-labelled EGF to fibroblastic mouse cell lines (Jetten, 1980). This report also showed that the enhancement of EGF binding increases, slowly becoming maximal after 48-72 hours of treatment with retinoic acid. Another report has shown that retinoic acid can also enhance the binding of EGF to normal rat kidney cells (Roberts et al., 1984). Again, this is a slow response which becomes maximal after approximately 60 hours of retinoic acid treatment. If a similar enhancement of EGF binding occurs in human skin fibroblasts then it cannot be related to the synergistic induction of TIMP-1 protein; otherwise TIMP-1 would have been synergistically stimulated in response to 48 or 72 hours of treatment with retinoic acid followed by EGF.

An important finding of this chapter is the discovery that bFGF induces a transient effect of some kind in the cells which is responsible for the synergistic induction of TIMP-1 in the additional presence of retinoic acid. It is intriguing to speculate on what this transient effect may be. The most likely possibility is that bFGF stimulates the synthesis of 1 or more proteins which are either part of, or able to interact with the retinoic acid signalling pathway. Possible candidates are the nuclear retinoic acid receptors (RARs and RXRs), the cellular retinoic acid-binding proteins (CRABP and CRABP-II) or proto-oncogene products such as c-fos and c-jun which can regulate TIMP-1 gene transcription through the TIMP-1 promoter AP-1 sites. Induction of RARs and/or RXRs would increase the sensitivity of the cells to retinoic acid, possibly resulting in a synergistic induction of TIMP-1 when retinoic acid is subsequently added. I have not found any evidence in the published literature which
indicates that bFGF can induce RARs or RXRs in cells and the possibility that this is occurring in human skin fibroblasts in response to bFGF is currently being investigated in the Rheumatology Research Unit. The effect of modulating intracellular levels of CRABP and CRABP-II is less easy to predict since the precise role of these proteins is not clearly understood as discussed above. A previous report has shown that bFGF causes a slight inhibitory effect on CRABP-II and a slight stimulatory effect on CRABP mRNA expression in murine embryonic palatal cells (Nugent and Greene, 1994).

The induction of proteins such as \textit{c-fos} and \textit{c-jun} may lead to synergistic effects on TIMP-1 gene transcription in the presence of retinoic acid as already mentioned. One possible mechanism for this is the interaction of activated retinoic acid receptors with these induced proteins at the TIMP-1 non-consensus AP-1 site. This has been demonstrated in the case of retinoic acid regulation of the collagenase gene (Schüle et al., 1991, Pan et al., 1992, Pan and Brinckerhoff, 1994). Such interactions result in downregulation of collagenase gene transcription in response to retinoic acid, but in the case of TIMP-1, the opposite effect could occur due to the presence of additional proteins which do not bind to the consensus collagenase AP-1 site (Edwards et al., 1992). bFGF may induce proteins which bind to the non-consensus TIMP-1 AP-1 site. Further modulation of TIMP-1 gene transcription could then take place via protein-protein interactions between activated retinoic acid receptors and these bFGF-induced proteins. This theory suggests that bFGF elicits its effects on the TIMP-1 promoter first followed by further modulation by retinoic acid. The data shown in this chapter support this hypothesis since they show that bFGF treatment followed by retinoic acid treatment (but not the other way round) produces a synergistic effect.

It is also possible that retinoic acid receptors bind directly to the TIMP-1 promoter. Examination of the human TIMP-1 promoter has revealed the existence of a putative RARE sequence (ACTTCCxxACTTGA) positioned at -367 to -380 (I.M. Clark and H.F. Bigg, unpublished observations) but it is not yet known whether this sequence
actually functions as a RARE. Synergistic interactions could perhaps take place between a RARE and the AP-1 sites.

Previous work has investigated the induction of transcription factors such as c-fos and c-myc by serum and by bFGF in rodent fibroblasts (Müller et al., 1984). The induction of c-fos mRNA by bFGF is extremely rapid being maximal after 30 minutes and returning to baseline by 2 hours. c-fos protein can also be detected in these cells after 1 hour of stimulation with bFGF. The induction of c-fos protein by bFGF is likely to return rapidly to baseline since c-fos protein levels stimulated by serum reach a maximum 1-2 hours after stimulation and are markedly reduced almost to baseline after 4 hours. Expression of c-myc mRNA in response to serum is a slower, more sustained event which reaches a maximum after 1 hour and then slowly declines to basal levels by 18 hours after stimulation. The initial induction of c-myc by bFGF over the first 4 hours is very similar to that induced by serum during the same time period. It can be postulated that the subsequent kinetics of c-myc mRNA induction by bFGF are also similar to that seen with serum - i.e. a gradual decline to baseline levels by 18 hours. c-myc protein can also be detected in these cells 1 hour after stimulation with serum or with bFGF. These findings suggest that the induction of c-fos by bFGF in fibroblasts is too transient to be responsible for the synergistic induction of TIMP-1 protein in response to subsequent addition of retinoic acid. In 2 experiments out of 3, the maximum induction of TIMP-1 protein was seen with as much as 12 hours of incubation with bFGF followed by retinoic acid. The induction of c-fos mRNA and protein by bFGF would be expected to return to baseline long before this time point. However the induction of c-myc could possibly play a part in the stimulation of TIMP-1 by bFGF and retinoic acid since this is a more sustained event. The optimum time for incubation with bFGF prior to retinoic acid treatment was found to be 1 to 3 hours and this correlates with a maximum induction of c-myc mRNA after 1 hour of stimulation. Although c-myc has not been reported to bind to a classical AP-1 sequence, it is possible that it may bind to the more promiscuous non-consensus AP-1 motif in the TIMP-1 promoter although this is purely speculative. Stimulation of
TIMP-1 gene transcription may then be driven by the mechanisms outlined above. Investigations are currently under way in the Rheumatology Research Unit to find out the time course of induction of c-fos, c-myc and c-jun mRNAs by bFGF in human skin fibroblasts.

The transient effect of bFGF on the cells is most likely to be an effect on the synthesis of 1 or more proteins. However, it is also possible that the activity of an existing protein is being modulated by a phosphorylation event stimulated by bFGF. Complex cross-talk can occur between the retinoic acid signalling pathway and other pathways which results in the phosphorylation of retinoic acid receptors. This in turn modifies the transcriptional activity of the retinoic acid receptors (Huggenvik et al., 1993). It is possible to distinguish between effects on de novo protein synthesis and the activation of existing proteins by the use of protein synthesis inhibitors such as cycloheximide. This is addressed in chapter 7 of the thesis.

Interestingly, treatment of the cells with retinoic acid first followed by bFGF was unable to produce any synergistic induction of TIMP-1, even when the incubation time with retinoic acid was as little as 10 minutes. This may be because retinoic acid induces a rapid effect on TIMP-1 gene transcription which has already returned to baseline by the time bFGF has induced intracellular effects. The addition of bFGF is therefore unable to further modulate this response. However, this theory seems unlikely in view of the slow kinetics of TIMP-1 induction which the data in this chapter shows.

Another possible explanation is that during the time period required for bFGF to induce intracellular events, all intracellular retinoic acid becomes bound either to nuclear retinoic acid receptors or is metabolized to other compounds. bFGF may upregulate one or more components of the retinoic acid signalling pathway such as RARs or RXRs, but this is unable to achieve a synergistic induction of TIMP-1 since there is no longer any free retinoic acid left to interact with these proteins. In the introduction to this chapter, it was suggested that in the case of bFGF or EGF, the synergistic induction of TIMP-1 protein might be occurring through the upregulation
of TGF-β by retinoic acid. This theory seems unlikely in view of the results showing that retinoic acid treatment followed by growth factor treatment cannot produce a synergistic induction of TIMP-1 at any of the time points investigated.

The fact that a very brief exposure of the cells to bFGF (10 minutes) is still able to produce a synergistic induction of TIMP-1 protein is a point of interest. In a previous report, it was shown that when bovine capillary endothelial cells are treated with bFGF for 10 or 30 minutes, washed extensively and then incubated in bFGF-free medium, plasminogen activator production was stimulated to the same extent as in cells exposed continuously to bFGF (Flaumenhaft et al., 1989). However, when steps were taken to remove bFGF from heparin-like binding sites in the extracellular matrix (but not from bFGF receptors) this long-term effect of a brief exposure to bFGF was abolished. It appeared that long-term effects of bFGF after a brief exposure to this growth factor were a consequence of bFGF binding to the extracellular matrix. The data shown in this chapter can perhaps be explained by a similar mechanism in which the cells continue to be exposed to extracellular-bound bFGF after its removal from the culture medium. However, it has also been demonstrated that phosphorylation of intracellular substrates by activated FGF receptors is maximal after 10 minutes of stimulation (Coughlin et al., 1988), suggesting that bFGF-stimulated signal transduction pathways are maximally stimulated following a brief exposure to growth factor. A brief exposure to bFGF may therefore irreversibly commit the cells to the long-term effects of this factor.

In conclusion, the data reported in this chapter begin to probe the mechanisms of the synergistic stimulation of TIMP-1 by retinoic acid and growth factors. The slow kinetics of TIMP-1 protein induction by retinoic acid and each of the 4 growth factors suggests that in all cases they are secondary rather than primary effects on the cells. Further experiments in which the effect of sequential treatment with the agents is examined reveal differences rather than similarities in the action of the 4 growth factors. Prolonged TGF-β treatment for 72 hours prior to the addition of retinoic acid was found to produce a synergistic response. It is suggested that the mechanism of
this response may involve the induction or repression of components of the retinoic acid signalling pathway, or the induction of transcription factors such as c-fos or c-myc. These possibilities require further investigation. bFGF was found to have a more short-lived effect on cells which could involve the transient induction of a new protein. The action of EGF on the cells showed a further variation since it appeared to be obligatory for both agents to be present together to cause a synergistic induction of TIMP-1. Out of the 4 growth factors investigated, bFGF and PDGF-BB show the greatest similarities with respect to their effects on mesenchymal cells. It would have been interesting to investigate whether a brief exposure of the cells to PDGF-BB followed by retinoic acid produced a synergistic induction of TIMP-1 in similarity to the data shown for bFGF.
CHAPTER SEVEN

THE EFFECT OF ALL-TRANS-RETINOIC ACID IN COMBINATION WITH bFGF, EGF, PDGF-BB OR TGF-β ON TIMP-1, TIMP-2 AND COLLAGENASE STEADY-STATE mRNA LEVELS IN HUMAN SKIN FIBROBLASTS

7.1. INTRODUCTION

In chapters 3 and 4 of this thesis it was demonstrated that retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β causes a potent synergistic induction of TIMP-1 protein from human fibroblasts. This chapter investigates the level at which this increase in TIMP-1 protein may be occurring.

The increased production of a protein from a cell can be regulated by the stimulating agent at one or more possible levels of intervention in the cellular machinery. Increased gene transcription leads to increased levels of mRNA resulting in enhanced levels of synthesis of the protein. Enhancement of the stability of mRNA in the cytoplasm also results in increased steady-state levels of mRNA leading to increased production of the protein. Less commonly, the processing of the primary RNA transcript and the efficiency with which processed mRNA is exported from the nucleus to the cytoplasm are further levels at which control may be exerted. The efficiency of translation of the mRNA into protein is also often utilized as a point of intervention. A final level of control is the ability of the cell to secrete newly-synthesized protein into the extracellular environment.

Previous investigations into the regulation of TIMP-1 production show that increased secretion of TIMP-1 protein is accompanied by increases in steady-state levels of mRNA for TIMP-1 inside the cells. This implies that control over TIMP-1 protein production is pretranslational and is occurring via increased gene transcription or increased mRNA stability. Retinoic acid stimulation of TIMP-1 protein in human skin fibroblasts is accompanied by increased levels of TIMP-1 mRNA (Clark et al.,
It has also been shown previously that bFGF and EGF when applied alone both cause an increase in steady-state levels of TIMP-1 mRNA and TIMP-1 protein production in human foetal lung fibroblasts (Edwards et al., 1987). The synergistic upregulation of TIMP-1 protein by bFGF or EGF in combination with TGF-β is also paralleled by increases in TIMP-1 mRNA levels. In human skin fibroblasts and human gingival fibroblasts PDGF and TGF-β respectively have both been shown to increase TIMP-1 protein production and TIMP-1 mRNA in these cells (Overall et al., 1989, Circolo et al., 1991). Oncostatin M also increases steady-state levels of mRNA for TIMP-1 and TIMP-1 protein production in human lung and synovial fibroblasts (Richards et al., 1993). In addition, TPA induces TIMP-1 protein and TIMP-1 mRNA in human gingival fibroblasts (Overall and Sodek, 1990).

In the case of agents which reduce the production of TIMP-1 from cells, the changes in the secretion of protein are again paralleled by changes in the levels of TIMP-1 mRNA. It has been shown that concanavalin A reduces both TIMP-1 protein production and TIMP-1 mRNA levels in human gingival fibroblasts (Overall and Sodek, 1990). In addition, retinoic acid reduces TIMP-1 mRNA levels in rat osteoblasts (Overall, 1994, Overall, 1995).

These previous investigations suggest that TIMP-1 gene expression is regulated mainly at a pretranslational level and involves changes in TIMP-1 mRNA levels. The synergistic induction of TIMP-1 protein by retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β is therefore also likely to be regulated in a similar manner. In this chapter, the effect of these agents on steady-state levels of TIMP-1 mRNA in human skin fibroblasts is therefore examined by Northern blotting.

The production of collagenase from cells also appears to be regulated mainly at a pretranslational level. IL-1, PDGF, TNF-α, EGF and bFGF have all been shown to increase both collagenase protein production and levels of collagenase mRNA in connective tissue cells (Stephenson et al., 1987, Bauer et al., 1985, Mitchell and Cheung, 1991, Edwards et al., 1987). Negative regulation of collagenase gene expression by TGF-β or retinoic acid also appears to be regulated by changes in levels...
of steady-state collagenase mRNA (Edwards et al., 1987, Overall et al., 1991, Clark et al., 1987). In this chapter, the effect of retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β on collagenase mRNA levels in human skin fibroblasts was measured in order to see whether the changes in collagenase protein production seen in chapters 3 and 4 are paralleled by changes in mRNA levels.

In contrast to TIMP-1 and collagenase which are highly stimulus-responsive, the expression of TIMP-2 appears to be largely constitutive and non-inducible (Stetler-Stevenson et al., 1990, Mackay et al., 1992, Leco et al., 1992). When TIMP-2 expression is influenced by stimuli, the response is often opposite to that of TIMP-1 (Stetler-Stevenson et al., 1990, Roeb et al., 1993, Roeb et al., 1995). However, in some cases, TIMP-1 and TIMP-2 are coordinately regulated in response to stimulating factors (Overall, 1994, Ulisse et al., 1994). In this chapter, the effect of retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β on TIMP-2 mRNA levels in human skin fibroblasts is investigated in order to compare the effect of these agents on TIMP-1 and TIMP-2 gene expression.

In chapter 6, it was demonstrated that a synergistic induction of TIMP-1 protein could occur in response to sequential treatment of the cells with bFGF followed by retinoic acid. This response was similar to that observed when the cells were treated with both factors together. It was postulated that bFGF was transiently increasing the production or activity of a cellular protein which was responsible for causing the synergistic induction of TIMP-1 in the additional presence of retinoic acid. The increase in production of such a protein would require de novo protein synthesis by the cells following treatment with bFGF and could therefore be blocked by a protein synthesis inhibitor such as cycloheximide. However, if bFGF was causing an increase in the activity of a protein by stimulating its phosphorylation or dephosphorylation, then this would not be interfered with by cycloheximide. In order to find out whether the synergistic induction of TIMP-1 by retinoic acid and bFGF requires new protein synthesis, the effect of cycloheximide on TIMP-1 mRNA induction by these factors was therefore investigated.
7.2. MATERIALS AND METHODS

7.2.1. General precautions to avoid contamination of RNA preparations with RNA-degrading enzymes

The most common problem encountered in the preparation of cellular RNA and its subsequent use in Northern blot hybridization studies is the contamination of samples with RNA-degrading enzymes (RNAases) resulting in the degradation of the RNA. In order to prevent this from arising, the following general precautions were taken. All manipulations and handling of reagents and equipment were done wearing gloves, as skin is a major source of contaminating RNAases. Glassware and spatulas were baked at 200°C overnight and tips, Sarstedt tubes and Eppendorf tubes were autoclaved. The following buffers were made with diethyl pyrocarbonate (DEPC)-treated distilled water: a 0.5M solution of disodium EDTA which was subsequently used for making all other EDTA-containing buffers, RNA gel electrophoresis loading buffer (0.25% bromophenol blue, 1mM EDTA, pH 8.0, 50% glycerol) and TE buffer (10mM Tris/hydrochloride, 10mM EDTA, pH 8.0). It was found to be adequate to use distilled water for all other buffers. DEPC-treated distilled water was prepared by adding 0.5ml of DEPC to 500ml of distilled water, followed by thorough mixing, overnight incubation at room temperature and autoclaving. Magnetic stirrers and lids for glassware were soaked in DEPC-treated distilled water overnight and then autoclaved. Finally, pH meter electrodes were rinsed with DEPC-treated distilled water before use.

7.2.2. Cell assay system

Human skin fibroblasts were passaged and resuspended at a concentration of $10^5$ cells per ml in DMEM containing 10% FCS. The cells were seeded into 100mm petri dishes at 28ml per dish giving a density of $5.0 \times 10^4$ cells per cm². The dishes were incubated overnight at 37°C to enable attachment of the cells to the plastic. The cell monolayers were washed 3 times with HBSS and 28ml of DMEM containing 1%
ATFCS was added to each dish. The plates were then incubated at 37°C for 48 hours. Fresh medium (DMEM with 1% ATFCS) containing the test reagents was then added to the dishes which were incubated at 37°C for various lengths of time which depended upon the particular experiment. 1 petri dish was used for each test condition examined.

The cell supernates were then discarded and the cell monolayers were washed twice with HBSS. The cells were harvested from the dishes in HBSS using cell scrapers and centrifuged for 5 minutes at 200g. The cell pellet from each dish was then used to prepare total cellular RNA as described below. Each experiment used 3 different human skin fibroblast cell lines with cells at low to moderate passage numbers (passage 3 to passage 12).

7.2.3. Preparation of total cellular RNA

Total cellular RNA was prepared from the cell pellet using a commercial kit (RNeasy™ supplied by Qiagen). This method relies on the binding of the RNA to a silica gel-based membrane under high salt conditions while DNA, protein and other contaminants pass straight through or are subsequently washed away. High quality RNA is then eluted from the membrane in distilled water.

Each cell pellet was lysed and homogenized under highly denaturing conditions to ensure inactivation of RNAases in 350μl of lysis buffer supplied by the kit. 350μl of 70% ethanol was added to each lysate followed by mixing and the samples were then applied to RNeasy spin columns. The columns were spun and washed several times using the buffers supplied by the kit (Once with 700μl of wash buffer RW1 and twice with 500μl of wash buffer RPE). Finally, RNA was eluted from each column in 45-100μl of DEPC-treated distilled water.

In most experiments, the RNA eluted under these conditions was too dilute to be used directly for gel electrophoresis. The RNA was therefore concentrated by precipitation and resuspension in a smaller volume. Precipitation was achieved by adding 2.5 volumes of 100% ethanol and 1/10 volume of 2M sodium acetate to each sample. The
samples were then mixed and incubated either overnight at -20°C or for 1 hour at -70°C. This was followed by centrifuging at 11600g for 15 minutes at 4°C. The supernates were discarded and the pellets were washed with ice-cold 75% ethanol followed by centrifuging for a further 10 minutes at 4°C and 11600g. The supernates were again removed and the pellets were briefly dried under vacuum, taking care to avoid overdrying. Each pellet was resuspended in 10μl of TE buffer and complete solution of the RNA was ensured by freezing and thawing the samples followed by a brief incubation (2 to 3 minutes) at 55°C.

The quantity of RNA was measured by monitoring the absorbance at 260nm of a 300-fold dilution of each sample. The purity of the preparations was assessed by measuring the ratio of the absorbance at 260nm to that at 280nm in which acceptable samples gave a ratio of at least 1.8. The RNA preparations were then stored at -20°C until used for gel electrophoresis.

### 7.2.4. RNA gel electrophoresis

A 1% agarose/formaldehyde gel was prepared by dissolving 1.16g of agarose in 101ml of distilled water followed by the addition of 3.5ml of 37-41% formaldehyde and 11.7ml of 10x running buffer (0.2M 3-(N-morpholino) propanesulphonic acid (MOPS), 0.05M sodium acetate, 0.01M EDTA).

Sufficient sample was removed from each RNA preparation to contain 5μg of RNA and this was made up to 9μl with 10mM EDTA. To this was added 4μl of 10x running buffer, 7μl of 37-41% formaldehyde and 20μl of Ultrapure formamide. The samples were mixed and incubated for 15 minutes at 55°C and subsequently placed immediately on ice. 10μl of loading buffer was then added to each sample followed by mixing. Samples containing a mixture of RNA standards of known size were also prepared for gel electrophoresis in an identical manner except that in this case, only 3μg of RNA was used.

In some experiments, 40μl of RNA sample was used (sufficient volume of RNA solution to give 5μg + 10mM EDTA up to 40μl) to which was added 10μl of the
following buffer: 0.16% bromophenol blue, 8mM EDTA, 0.1mg/ml ethidium bromide, 2.6% formaldehyde, 20% glycerol, 31% formamide, 80mM MOPS, 20mM sodium acetate. The advantage of using this procedure is that a larger volume of RNA solution can be used for electrophoresis which removes the need for precipitation and resuspension of the RNA.

Samples were then applied to the gel and the gel was run at 56-65 volts in 1x running buffer for 3 to 4 hours until the bromophenol blue had migrated about 10cm. The gel was stained with 1.5μg/ml ethidium bromide in distilled water for 15 minutes and then destained in distilled water for 5 minutes except in cases in which ethidium bromide had already been added to the samples. RNA was viewed under ultraviolet light, intact RNA being indicated by the presence of 2 sharp bands representing 28S and 18S ribosomal RNA (rRNA).

7.2.5. Blotting of RNA onto nylon membrane

4 pieces of filter paper and 1 piece of nylon membrane were cut to the same size as the gel and the gel was washed briefly in 1.5M sodium chloride, 0.15M sodium citrate (10x SSC). An additional piece of filter paper of the same width but approximately 4 times longer than the other pieces was cut for use as the wick. The filter paper and nylon membrane were soaked in 10x SSC until saturated. The wick was placed on top of 1 piece of filter paper followed by the gel, the nylon membrane and the 3 remaining pieces of filter paper, taking care to remove all air bubbles. 3 inches of paper towels and a heavy weight were stacked on top of the filter paper. Blotting of the RNA from the gel to the nylon membrane was allowed to take place overnight in 10x SSC. Evaporation of buffer from the dish and short-circuiting of buffer from the wick to the membrane hence by-passing the gel were eliminated by placing a layer of cling-film between the wick and the membrane. Complete transfer was checked by examining the gel and the membrane under ultraviolet light. The membrane was washed briefly in 2x SSC, dried at room temperature and baked at 80°C for 2 hours. The track containing the RNA markers was cut from the membrane and the markers
were fixed in 5% acetic acid for 10 minutes and then stained in 0.5M sodium acetate, 0.04% methylene blue for 3 minutes. If necessary, the markers were subsequently briefly destained in distilled water. The nylon membrane was subsequently probed with labelled cDNA probes for TIMP-1, TIMP-2, interstitial collagenase and 28S rRNA, as described in 7.2.8.

7.2.6. Preparation of competent DH5α E. coli bacteria

Competent DH5α bacteria were used for the preparation of cDNAs for TIMP-1, TIMP-2, interstitial collagenase, and 28S rRNA as described in 7.2.7. The bacteria were cultured in 300ml of autoclaved Luria-Bertuni (LB) medium (10g/l bacto-tryptone, 5g/l bacto-yeast extract, 10g/l sodium chloride, pH 7.0) until an absorbance of 0.4-0.5 at 550nm was attained. The bacterial culture was then chilled on ice and centrifuged at 2 700g for 10 minutes. The supernate was discarded and the pellet well drained; then the pellet was resuspended in 150ml of 100mM calcium chloride and incubated on ice for 30 minutes with regular agitation. The preparation was centrifuged for a further 10 minutes at 2 700g, the supernatant discarded and the pellet resuspended in 12ml 100mM calcium chloride, 15% glycerol. The preparation was divided into 200μl aliquots, frozen on dry ice for 30 minutes and stored at -70°C until required.

7.2.7. Preparation of TIMP-1, TIMP-2, interstitial collagenase and 28S rRNA cDNA probes for Northern blot hybridization

Each cDNA was prepared from a plasmid vector containing an ampicillin resistance marker gene according to the following protocol.
10ng of each plasmid was added to 50μl of competent DH5α bacteria followed by a 30 minute incubation on ice. The bacteria were then subjected to heat-shock by incubating at 42°C for 90 seconds. 200μl of LB medium was added followed by a 2 minute incubation on ice. The culture was then allowed to recover for 1 hour at 37°C.
The culture was plated out on LB medium containing 15g/l of agar and 50µg/ml ampicillin and incubated overnight at 37°C. A single colony was chosen from the plate and used to inoculate 5ml of autoclaved LB medium containing 50µg/ml ampicillin. After an overnight incubation at 37°C with constant shaking, the 5ml culture was used to inoculate a larger 200ml culture (autoclaved LB with 50µg/ml ampicillin) which was again left to grow overnight. In addition, 800µl of the 5ml culture was reserved and added to 200µl of sterile glycerol in a glass vial. This was mixed, frozen on dry ice for 30 minutes and stored at -70°C.

The plasmids were harvested from the 200ml culture using a commercial kit (Plasmid Maxi kit from Qiagen). This method enables the purification of plasmid DNA from the cells by utilizing alkaline lysis and an anion-exchange silicagel resin. The culture was centrifuged for 15 minutes at 4°C and 6 370g and the supernatant discarded. The pellet was then resuspended in 10ml of buffer P1 (as supplied by the kit) (50mM Tris/hydrochloride, 10mM EDTA, pH 8.0, 100µg/ml RNAase A). 10ml of buffer P2 (200mM NaOH, 1% SDS) was added, the sample was mixed by inversion and incubated at room temperature for 5 minutes. This step results in the lysis of the cells. 10ml of chilled buffer P3 (3.0M potassium acetate, pH 5.5) was added, the sample mixed immediately by inversion and incubated on ice for 20 minutes. The high salt content of this buffer causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate while plasmid DNA renatures and stays in solution. These conditions also result in the digestion of RNA by RNAase A. The preparation was mixed again and centrifuged at 4°C for 30 minutes at 30 000g. The supernatant was removed and filtered through gauze. A Qiagen column which contains the plasmid-binding resin was then equilibrated in 750mM sodium chloride, 50mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100 and the sample was applied by gravity flow. The column was then washed with 60ml of buffer QC (1.0M sodium chloride, 50mM MOPS, 15% ethanol, pH 7.0) (supplied by the kit) followed by elution of the plasmids from the column using 15ml of the elution buffer (1.25M sodium chloride, 50mM Tris/hydrochloride, 15% ethanol, pH 8.5). The eluted plasmid DNA was desalted and
concentrated by isopropanol precipitation. 0.7 volumes of isopropanol was added followed by immediate inversion and centrifugation at 15 000g and 4°C for 30 minutes. Finally, the pellet was washed with cold 70% ethanol, recentrifuged under the same conditions for 10 minutes, briefly air-dried for 5 minutes and then resuspended in 200-400μl of 10mM Tris, 10mM EDTA, pH 8.0. The concentration of plasmid DNA obtained from the preparation was calculated by reading the absorbance at 260nm using a 300-fold dilution.

The cDNA inserts for TIMP-1, TIMP-2, interstitial collagenase, 28S rRNA were cut from each of the plasmid vectors using the following protocol. 20-30μg of each plasmid was digested while the remainder was stored at -20°C for future use. The appropriate restriction enzymes were added in the appropriate buffers depending upon the nature of the sites to be cut using 1 unit of enzyme for each μg of DNA to be digested. The sample was mixed and incubated at 37°C for 90 minutes.

A 1% agarose gel was prepared by dissolving 1g of agarose in 100ml 45mM Tris, 45mM boric acid, 1mM EDTA, pH 8.0 (TBE buffer) and using 33ml of this to pour the gel. An appropriate volume of loading buffer was added to the digested sample to give a ratio of sample to loading buffer of 4:1 followed by mixing. In addition, 2.5μl of loading buffer was added to 10μl of a mixture of DNA standards of known molecular weight followed by mixing. The molecular weight markers and the digested plasmid preparation were loaded onto the gel using a single lane for the markers and 9 lanes fused together for the sample. The gel was subsequently run at 100 volts in TBE buffer for approximately 2 hours. The gel was stained in 1.5μg/ml ethidium bromide in distilled water for 15 minutes and then destained in distilled water for 5 minutes. The DNA was viewed under ultraviolet light; this revealed 2 major bands in the sample track, the upper band corresponding to the plasmid vector and the lower band corresponding to the excised cDNA insert. The lower band was then cut out of the gel using a scalpel and the insert purified using a commercial kit (Prep-A-Gene supplied by Biorad) which relies on a silica-based DNA-binding matrix.
The amount of DNA insert present in the lower band was calculated from the amount of plasmid digested and the relative sizes of the insert and plasmid vector. The excised band was cut into small pieces, and the correct amount of sodium perchlorate buffer (dependent upon the approximate volume of the gel slice and the amount of DNA present) was added followed by gentle agitation to dissolve the gel. The correct amount of matrix required to bind all the DNA was then added. This was followed by gentle agitation for 10 minutes at room temperature. The DNA-binding matrix was pelleted by centrifuging briefly and the supernatant was removed. The pellet was resuspended in sodium perchlorate buffer (25x the volume of added matrix) followed by recentrifuging briefly and discarding of the supernatant. The pellet was then washed twice by resuspension and centrifugation in 25x the matrix volume of wash buffer (supplied by the kit). After the last wash, care was taken to remove all traces of wash buffer. Finally, the bound DNA insert was eluted from the matrix by resuspending the pellet in 1 volume of elution buffer followed by incubation at 37°C for 5 minutes. The sample was briefly centrifuged and the supernatant was removed and respun to remove any residual matrix. In order to maximize the yield of insert, the matrix pellet was resuspended a second time in 1 volume of elution buffer followed by centrifugation and removal of residual matrix as in the first elution. The 2 supernatants from the first and second elution were then combined and mixed. Finally, it was necessary to measure the concentration of DNA in the purified cDNA insert preparation. This was in order to calculate the volume of insert required to make a radiolabelled cDNA probe for Northern blot hybridization. A 1% agarose gel was prepared as described above. The following samples were prepared and loaded onto the gel as previously described: DNA molecular weight markers, 2, 4 and 6μl of the purified cDNA insert (made up to 10μl with distilled water) and 1, 2, 3 and 4μl of EcoRI/HindIII-cut λ (made up to 10μl with distilled water). The gel was run, stained and destained as above. The cDNA insert preparation was quantitated by comparing the intensity of the bands obtained using 2, 4 and 6μl of insert with that of the EcoRI/HindIII-cut λ DNA. The latter produces 13 different bands of various
intensities, each of which corresponds to a known quantity of DNA. The cDNA preparations were stored at -20°C until required for use.

7.2.8. Preparation of labelled cDNA probes and Northern blot hybridization

25ng of each purified cDNA insert was made up to 34μl with DEPC-treated distilled water. The insert was denatured by incubation in a boiling water bath for 5 minutes and then placed immediately on ice to prevent renaturation. The following reagents were then added: 10μl of reaction buffer (containing deoxyadenosine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate and hexameric oligodeoxyribonucleotides of random sequence), 5μl of [α-3²P]-deoxycytidine 5'-triphosphate, tetra-(triethylammonium) salt and 1μl of Klenow fragment using a commercial oligolabelling kit supplied by Pharmacia. The reagents were mixed and the sample incubated at 37°C for 2 hours. The newly synthesized labelled probe was then separated from unincorporated radioactivity by applying to a 0.8ml column of G-25 Sephadex equilibrated in 10mM Tris, 1mM EDTA, pH 7.5. The column was centrifuged for 4 minutes at 290g at room temperature which resulted in the labelled probe passing straight through while unincorporated radioactivity was retained by the Sephadex. 1μl of labelled probe was added to 5ml of Optiphase 'HiSafe' scintillation fluid and counted for 15 seconds on a Wallac 1410 liquid scintillation counter. The probe was then denatured by placing in a boiling water bath for 5 minutes followed by immediate incubation on ice to prevent renaturation.

The baked nylon membrane was prehybridized for 2 to 3 hours at 42°C in prewarmed hybridization buffer (50% formamide, 1% SDS, 1M sodium chloride, 1x Denhardt's solution [0.02% (weight/volume) Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin] and 100μg/ml denatured salmon sperm DNA). The nylon membrane was placed on top of a slightly larger piece of gauze; the two were rolled up together and then unrolled inside a hybridization bottle. The bottle was then placed in an oven and rotated constantly during prehybridization, hybridization and subsequent washings. Labelled probe was added at a sufficient volume to give 10⁷ disintegrations
per minute and hybridization was allowed to take place overnight at 42°C. The hybridization solution was then discarded and the blot was washed twice for 10 minutes at 42°C with 0.2x SSC, 0.5% SDS and then twice for 30 minutes at 56°C with 0.2x SSC, 0.5% SDS. In the case of the 28S rRNA cDNA probe, extra stringency was obtained by hybridizing at 56°C and carrying out the 10 minute washes at 56°C and the 30 minute washes at 65°C. Finally, the blot was rinsed briefly with 0.1x SSC at room temperature, dried on filter paper and wrapped in cling-film. The blot was then exposed to autoradiographic film for various lengths of time at -70°C ranging from 2.5 hours to 1 week until a signal within the linear range of the film's sensitivity was obtained. In many experiments, there were large differences in the intensity of the signal obtained under different test conditions and it was not possible to use a single exposure time in which all the test conditions were within the linear range of the film. In these cases, the most intense signals are above the linear range and the true level of induction of the mRNA is likely to be higher. The size of the mRNA giving rise to the signal was evaluated in each case by aligning the strip of stained markers with the autoradiograph.

The labelled cDNA probe was stripped from the blot by incubating the nylon membrane in boiling 0.5% SDS for 10 minutes 2 to 3 times. The membrane was then exposed to autoradiographic film overnight to ensure that there was no signal from residual probe. The blot could then be reprobed with another radiolabelled cDNA insert.

The strength of the signal obtained with each test condition and with each probe was quantitated by gel scan densitometry using an LKB Ultrascan laser densitometer. A 1-dimensional longitudinal scan was taken across the approximate centre of each band and the area under the curve was calculated. This was then multiplied by the width of the band therefore giving a result which reflected the length, width and intensity of the signal. The cDNA probe for 28S rRNA was used to normalize the signals obtained for TIMP-1, TIMP-2 and interstitial collagenase - i.e. to correct for differences in the quantities of total RNA in each test condition. The intensity of the signals obtained
with probes to TIMP-1, TIMP-2 and collagenase were divided in each case by the intensity obtained for 28S rRNA in the same sample. In cases in which a marked induction of TIMP-1 or collagenase mRNA was seen, these figures probably reflect minimum rather than accurate values due to the most intense signals being above the linear range of the film. Although glyceraldehyde-3-phosphate dehydrogenase and actin are commonly used as housekeeping genes in this type of experiment, it was decided that both of these probes were unsuitable since bFGF and EGF are known to induce actin mRNA and to stimulate glycolysis respectively (Fox et al., 1982, Rybak et al., 1988).

The nylon membranes were hybridized with cDNAs for TIMP-1, TIMP-2 and collagenase within a few days or weeks of blotting, but the 28S rRNA probe was not used until a few months later. In a small minority of samples, it was found that significant RNA degradation had occurred between using the TIMP-1, TIMP-2 and collagenase probes and the later use of the ribosomal probe. In these cases, TIMP-1, TIMP-2 and collagenase were not quantitated with respect to 28S rRNA as such corrections were not accurate. These samples are denoted as 'not quantitated' in the Results section and the ethidium bromide stain of total RNA levels for the experiment in question is shown in addition to results using the 28S rRNA probe.

7.2.9. Measurement of total cellular protein synthesis in response to cycloheximide by $^3$H-leucine incorporation

Human skin fibroblasts were seeded into 24-well plates and prepared for stimulation as described in chapter 2. The cells were then treated with various concentrations of cycloheximide for 24 hours in the additional presence of 50μCi/ml of L-[4,5-$^3$H]-leucine. The medium was removed and the cell monolayers washed 3x in HBSS. The 24-well plates were then placed on ice and 1ml of ice-cold 10% trichloroacetic acid was added to each well followed by a 10 minute incubation in order to remove unincorporated $^3$H-leucine. This step was repeated twice with 5 minute incubations each time. 0.5ml of 0.3M sodium hydroxide, 1% SDS was added to each well
followed by a 30 minute incubation at room temperature. The contents of each well were mixed well and transferred to scintillation vials. 5ml of Optiphase 'HiSafe' scintillation fluid was added to each vial and followed by counting for 1 minute on a Wallac 1410 liquid scintillation counter.

7.2.10. Materials

Petri dishes and cell scrapers were from COSTAR U.K. Ltd., High Wycombe, Bucks., U.K.; RNeasy™ and plasmid maxi kits were from Qiagen, Dorking, Surrey, U.K.; DEPC, ethidium bromide, bromophenol blue, methylene blue, G-25 Sephadex, Denhardt's solution, EcoRI/HindIII-cut λ, ampicillin, cycloheximide and all molecular biology grade chemicals were from Sigma Chemical Company Ltd., Poole, Dorset, U.K.; agarose, formamide, DNA standards, RNA standards and glycerol were from GIBCO BRL Life Technologies Ltd., Paisley, Scotland, U.K.; filter paper was from Whatman International Ltd., Maidstone, Kent, U.K.; nylon membrane (GeneScreen Plus®), was from Biotechnology Systems, NEN® Research Products, 549 Albany Street, Boston, U.S.A.; Saran wrap cling-film was from the Dow Chemical Company, bacto-tryptone, bacto-yeast extract and bacto-agar were from Difco Laboratories, Detroit, Michigan, U.S.A.; isopropanol and ethanol were from BDH, Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics., U.K.; restriction enzymes and restriction enzyme buffers were from Boehringer Mannheim U.K., Lewes, East Sussex, U.K.; gel electrophoresis equipment, hybridization gauze and hybridization bottles were from Hybaid Limited, Teddington, Middlesex, U.K.; Prep-A-Gene kits were from Biorad Ltd., Hemel Hempstead, Herts., U.K., oligolabelling kits were from Pharmacia Biotech Ltd., St. Albans, Herts. U.K.; scintillation fluid was from Wallac, Milton Keynes, U.K., polypropylene tubes were from Corning Ltd., Stone, Staffordshire, U.K., Eppendorf tubes were from Greiner Labortechnik Ltd., Gloucestershire, U.K., L-[4,5-3H]-leucine was from Amersham Bucks., U.K. and autoradiographic film was from Fuji Photo Film (U.K.) Ltd., Swindon, Wiltshire, U.K. The plasmid vectors containing the cDNA inserts used
were obtained as follows: TIMP-1 and TIMP-2 were a generous gift from Dr. A. Galloway, R. & D. Systems Europe, Abingdon, Oxon, U.K.; 28S rRNA and interstitial collagenase were kindly donated by Prof. C. Brinckerhoff, Dartmouth Medical School, Hanover, New Hampshire, U.S.A. DH5α bacteria were kindly donated by Dr. A. Ryan, Department of Zoology, Cambridge University, Cambridge, U.K.
7.3. RESULTS

7.3.1. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and TGF-β alone and in combination on steady-state levels of TIMP-1 mRNA

Using the cell assay procedure described above, the effect of the following test reagents on TIMP-1 mRNA levels was examined in 3 different human skin fibroblast cells lines. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF, recombinant PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml
3. all-trans-retinoic acid at 10⁻⁸M
4. all-trans-retinoic acid at 10⁻⁸M + recombinant bFGF, recombinant PDGF-BB or purified TGF-β at 100ng/ml or recombinant EGF at 10ng/ml.

After 72 hours, the cell monolayers were harvested and used to prepare total RNA as described above. Purified total RNA was then subjected to electrophoresis, blotting onto nylon and hybridization with a TIMP-1 cDNA probe as described above. Figures 7.1. to 7.4. show photographs of the autoradiograms obtained from these experiments. The expression of TIMP-1 mRNA induced by the test conditions is compared to the levels of 28S rRNA in the same sample detected using the ribosomal cDNA probe. Detection of ribosomal RNA using the ethidium bromide stain of the gel is also shown for some experiments in cases in which this was thought to be more reliable. Tables 7.1. to 7.4. show the results of quantifying the signal intensities obtained by gel scan densitometry and correcting with respect to the level of 28S rRNA in the same sample.

In all experiments, TIMP-1 mRNA was detectable in unstimulated cells as a single species which usually migrated below the 1.4 kilobase (kb) molecular weight marker.
This agrees with previously published work in which a single mRNA band with a size of 0.9kb has been detected in response to hybridization with a TIMP-1 probe (e.g. Clark et al., 1987, Edwards et al., 1987, Overall et al., 1989). Retinoic acid alone caused a significant stimulation of TIMP-1 mRNA levels in all experiments. The level of induction varied in different experiments between approximately 4 fold to 11 fold with the exception of 1 experiment in which a very modest stimulation of only 1.6 fold was seen. The addition of growth factor alone to the cells also caused approximately 2 to 4 fold inductions of TIMP-1 mRNA levels. This occurred in all 3 cell lines in the case of bFGF, in 2 out of 3 cell lines with EGF or TGF-β and in 1 cell line out of 3 in the case of PDGF-BB.

When retinoic acid was applied in combination with bFGF, this resulted in a marked induction of TIMP-1 mRNA levels compared to unstimulated cells. The magnitude of these inductions were approximately 14 fold, 24 fold and 40 fold for the cell lines hsf 10, hsf 15 and hsf 13 respectively. In all cases, the induction of TIMP-1 mRNA by retinoic acid and bFGF was greater than the additive effect of the factors and was therefore synergistic. Retinoic acid applied in combination with EGF caused a more modest stimulation of TIMP-1 mRNA levels (9 fold, 14 fold and 17 fold compared to control cells for hsf 9, hsf 13 and hsf 10 respectively). In all cases, these inductions were synergistic rather than merely additive. Retinoic acid in combination with PDGF-BB also caused a synergistic stimulation of TIMP-1 mRNA in all 3 cell lines (5 fold, 11 fold and 50 fold above control levels for hsf 13, hsf 10 and hsf 15 respectively). A similar effect was seen in the case of retinoic acid and TGF-β applied together in which TIMP-1 mRNA levels were stimulated 22 fold, 26 fold and 29 fold above control levels in hsf 13, hsf 9 and hsf 10 cells respectively. These inductions of TIMP-1 mRNA were again synergistic rather than merely additive. These experiments therefore show that retinoic acid in combination with each of the 4 growth factors causes a synergistic induction of TIMP-1 mRNA.
7.3.2. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and TGF-β alone and in combination on steady-state levels of TIMP-2 mRNA

The RNA samples from the above experiments were also hybridized with a cDNA for TIMP-2 as described in Materials and Methods. The autoradiographs obtained are shown in figures 7.1. to 7.4. and the results of quantifying the signals and correcting with respect to levels of 28S rRNA are shown in tables 7.1. to 7.4.

2 species of TIMP-2 mRNA were detected in all samples; the major band migrated level with or below the 4.4kb marker while a much fainter, minor band migrated either level with or below the 1.4kb marker. This result agrees with previously published work in which 2 distinct transcripts have been detected with sizes of 3.5kb and 1.0kb of which the 3.5kb form predominates in fibroblasts (Stetler-Stevenson et al., 1990).

The addition of retinoic acid alone at 10⁻⁴M to the cells did not have any significant effect on TIMP-2 mRNA levels. None of the growth factors applied alone had any significant effect on TIMP-2 mRNA levels either. Furthermore, TIMP-2 mRNA was not altered in a consistent manner by 10⁻⁴M retinoic acid in combination with 100ng/ml of bFGF or PDGF-BB or 10ng/ml of EGF. However, retinoic acid at 10⁻⁴M and TGF-β at 100ng/ml in combination appeared to stimulate the levels of both the 3.5kb mRNA species and the 1.0kb mRNA band in all 3 cell lines. The 3.5kb mRNA species was stimulated approximately 1.7 fold, 2 fold and 4 fold above control levels in the cell lines hsf 13, hsf 9 and hsf 10 respectively. The 1.0kb species was stimulated approximately 3 fold and 4 fold in the cell lines hsf 13 and hsf 9 respectively; in the cell line hsf 10, the level of induction could not be calculated as the control level was too faint to be accurately quantitated. Hence it appears that both TIMP-1 and TIMP-2 mRNA levels are stimulated in human skin fibroblasts by retinoic acid in combination with TGF-β; however the effect on TIMP-2 is very modest compared to the effect on TIMP-1.
7.3.3. The effect of all-trans-retinoic acid, bFGF, EGF, PDGF-BB and TGF-β alone and in combination on steady-state levels of collagenase mRNA

The RNA samples from the experiments described in sections 7.3.1. and 7.3.2. were also hybridized with the cDNA probe for interstitial collagenase. The autoradiographs obtained are shown in figures 7.1. to 7.4. and the results of quantifying the signals and correcting with respect to levels of 28S rRNA are shown in tables 7.1. to 7.4.

The collagenase cDNA probe detected a single mRNA species which migrated level with the 2.4kb marker. This agrees with previous work investigating the regulation of MMP-1 mRNA levels in fibroblasts, in which a single mRNA species with a size of 2.5kb has been detected (e.g. Clark et al., 1987, Edwards et al., 1987).

In 6 experiments, collagenase mRNA was detectable in unstimulated cells while in a further 6 experiments no collagenase mRNA was detectable under these conditions. In all experiments in which control levels of collagenase mRNA were detectable, 10⁻⁶M retinoic acid caused repression of this signal either to a lower level or to undetectable levels. The addition of 100ng/ml of bFGF or PDGF-BB or 10ng/ml of EGF to the cells caused a potent induction of collagenase mRNA levels in all experiments. In experiments in which control levels of collagenase were detectable, these inductions were calculated to be 19 fold above control levels in the case of EGF and 24-55 fold above control levels in the case of PDGF-BB. In the additional presence of 10⁻⁶M retinoic acid, bFGF and PDGF-BB-stimulated collagenase mRNA levels were markedly reduced although not to control levels. bFGF-stimulated collagenase mRNA was reduced 5.5 fold, 6 fold and 11 fold in the cell lines hsf 13, hsf 15 and hsf 10 respectively and PDGF-BB-stimulated collagenase was reduced 3.4 fold, 6 fold and 10 fold in the cell lines hsf 10, hsf 15 and hsf 13 respectively. EGF-stimulated collagenase levels were reduced to control levels by the additional presence of 10⁻⁶M retinoic acid in the cell lines hsf 13 and hsf 9. In the cell line hsf 10, EGF-stimulated collagenase mRNA was also markedly reduced by retinoic acid, possibly to control levels although this was uncertain due to control levels of collagenase being undetectable.
Surprisingly, the addition of 100ng/ml of TGF-β to the cells did not have any significant effect on collagenase mRNA levels in 2 cell lines out of 3; all collagenase mRNA levels were below detection in the third cell line. The effect of 10^5 M retinoic acid in combination with 100ng/ml TGF-β on collagenase mRNA levels was difficult to assess due to the relevant signal intensities being undetectable or at the limits of detection.
Figure 7.1.
Induction of TIMP-1, TIMP-2 and collagenase (CL) mRNA by retinoic acid and bFGF either alone or in combination after 72 hours of stimulation. The following test conditions are shown: lane 1: No additions, lane 2: Retinoic acid 10^{-5} M, lane 3: bFGF 100ng/ml, lane 4: Retinoic acid 10^{-5} M + bFGF 100ng/ml. The figure shows results from the following cell lines: a) hsf 13 (passage 7), b) hsf 15 (passage 4), c) hsf 10 (passage 4). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.2.
Induction of TIMP-1, TIMP-2 and collagenase (CL) mRNA by retinoic acid and EGF either alone or in combination after 72 hours of stimulation. The following test conditions are shown: lane 1: No additions, lane 2: Retinoic acid $10^{-5}$M, lane 3: EGF 10ng/ml, lane 4: Retinoic acid $10^{-5}$M + EGF 10ng/ml. The figure shows results from the following cell lines: a) hsf 13 (passage 7), b) hsf 9 (passage 6), c) hsf 10 (passage 11). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.3.
Induction of TIMP-1, TIMP-2 and collagenase (CL) mRNA by retinoic acid and PDGF-BB either alone or in combination after 72 hours of stimulation. The following test conditions are shown: lane 1: No additions, lane 2: Retinoic acid 10⁻⁶M, lane 3: PDGF-BB 100ng/ml, lane 4: Retinoic acid 10⁻⁶M + PDGF-BB 100ng/ml. The figure shows results from the following cell lines: a) hsf 13 (passage 8), b) hsf 15 (passage 10), c) hsf 10 (passage 7). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.4.
Induction of TIMP-1, TIMP-2 and collagenase (CL) mRNA by retinoic acid and TGF-β either alone or in combination after 72 hours of stimulation. The following test conditions are shown: lane 1: No additions, lane 2: Retinoic acid 10⁻⁵M, lane 3: TGF-β 100ng/ml, lane 4: Retinoic acid 10⁻⁵M + TGF-β 100ng/ml. The figure shows results from the following cell lines: a) hsf 13 (passage 4), b) hsf 9 (passage 8), c) hsf 10 (passage 12). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Table 7.1.

Steady-state levels of TIMP-1, TIMP-2 and collagenase mRNA in human skin fibroblasts following treatment for 72 hours with bFGF either alone or in the additional presence of retinoic acid. The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample. In the case of TIMP-2, the intensity of the 3.5kb mRNA band is shown; the intensity of the 1.0kb band is additionally shown in brackets in samples where quantitation of this band was possible.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>mRNA</th>
<th>No additions</th>
<th>Retinoic acid 10^M</th>
<th>EGF 10ng/ml</th>
<th>Retinoic acid 10^M + EGF 10ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13</td>
<td>TIMP-1</td>
<td>0.163</td>
<td>0.827</td>
<td>0.512</td>
<td>2.301</td>
</tr>
<tr>
<td>(passage 7)</td>
<td>TIMP-2</td>
<td>1.006</td>
<td>1.162</td>
<td>0.851</td>
<td>1.026</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>0.752</td>
<td>0.058</td>
<td>14.180</td>
<td>1.024</td>
</tr>
<tr>
<td>hsf 9</td>
<td>TIMP-1</td>
<td>0.069</td>
<td>0.491</td>
<td>0.059</td>
<td>0.636</td>
</tr>
<tr>
<td>(passage 6)</td>
<td>TIMP-2</td>
<td>1.873</td>
<td>2.427</td>
<td>1.026</td>
<td>1.556</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>0.098</td>
<td>not detectable</td>
<td>1.883</td>
<td>0.063</td>
</tr>
<tr>
<td>hsf 10</td>
<td>TIMP-1</td>
<td>0.276</td>
<td>2.231</td>
<td>0.875</td>
<td>4.802</td>
</tr>
<tr>
<td>(passage 11)</td>
<td>TIMP-2</td>
<td>0.637</td>
<td>0.935 (0.057)</td>
<td>0.669 (0.100)</td>
<td>1.360 (0.192)</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>not detectable</td>
<td>not detectable</td>
<td>0.286</td>
<td>not detectable</td>
</tr>
</tbody>
</table>

Table 7.2.

Steady-state levels of TIMP-1, TIMP-2 and collagenase mRNA in human skin fibroblasts following treatment for 72 hours with EGF either alone or in the additional presence of retinoic acid. The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample. In the case of TIMP-2, the intensity of the 3.5kb mRNA band is shown; the intensity of the 1.0kb band is additionally shown in brackets in samples where quantitation of this band was possible.
Table 7.3.

Steady-state levels of TIMP-1, TIMP-2 and collagenase mRNA in human skin fibroblasts following treatment for 72 hours with PDGF-BB either alone or in the additional presence of retinoic acid. The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample. In the case of TIMP-2, the intensity of the 3.5kb mRNA band is shown; the intensity of the 1.0kb band is additionally shown in brackets in samples where quantitation of this band was possible.
<table>
<thead>
<tr>
<th>Cell Line (passage)</th>
<th>mRNA</th>
<th>No additions</th>
<th>Retinoic acid 10^{-5}M</th>
<th>TGF-β 100ng/ml</th>
<th>Retinoic acid 10^{-5}M + TGF-β 100ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13 (4)</td>
<td>TIMP-1</td>
<td>0.073</td>
<td>0.265</td>
<td>0.141</td>
<td>1.584</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td>0.974 (0.088)</td>
<td>0.822 (0.073)</td>
<td>1.062 (0.109)</td>
<td>1.637 (0.271)</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>0.045</td>
<td>0.0092</td>
<td>0.068</td>
<td>not detectable</td>
</tr>
<tr>
<td>hsf 9 (8)</td>
<td>TIMP-1</td>
<td>0.094</td>
<td>0.520</td>
<td>0.084</td>
<td>2.398</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td>1.548 (0.152)</td>
<td>2.029 (0.099)</td>
<td>1.290 (0.231)</td>
<td>3.364 (0.651)</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>0.651</td>
<td>not detectable</td>
<td>0.802</td>
<td>not detectable</td>
</tr>
<tr>
<td>hsf 10 (12)</td>
<td>TIMP-1</td>
<td>0.118</td>
<td>1.354</td>
<td>0.467</td>
<td>3.471</td>
</tr>
<tr>
<td></td>
<td>TIMP-2</td>
<td>0.585</td>
<td>0.714</td>
<td>0.381</td>
<td>2.276 (0.137)</td>
</tr>
<tr>
<td></td>
<td>collagenase</td>
<td>not detectable</td>
<td>not detectable</td>
<td>not detectable</td>
<td>not detectable</td>
</tr>
</tbody>
</table>

Table 7.4.

Steady-state levels of TIMP-1, TIMP-2 and collagenase mRNA in human skin fibroblasts following treatment for 72 hours with TGF-β either alone or in the additional presence of retinoic acid. The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample. In the case of TIMP-2, the intensity of the 3.5kb mRNA band is shown; the intensity of the 1.0kb band is additionally shown in brackets in samples where quantitation of this band was possible.
7.3.4. Time course of the synergistic induction of TIMP-1 mRNA by all-trans-retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β

The data shown in section 7.3.1. indicates that retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β causes a synergistic induction of TIMP-1 mRNA at 72 hours after stimulation. In this section, the kinetics of this synergistic induction of TIMP-1 mRNA is investigated in order to establish the length of time required to give the maximal effect.

Using the cell assay procedure described above, the effect of the following test reagents on TIMP-1 mRNA levels was examined in 3 different human skin fibroblast cells lines. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells simultaneously.

1. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml for 0, 12, 24, 48 and 72 hours
2. all-trans-retinoic acid at 10⁻⁴M + recombinant EGF at 10ng/ml for 0, 12, 24, 48 and 72 hours
3. all-trans-retinoic acid at 10⁻⁴M + recombinant PDGF-BB at 100ng/ml for 0, 12, 24, 48 and 72 hours
4. all-trans-retinoic acid at 10⁻⁴M + purified TGF-β at 100ng/ml for 0, 12, 24, 48 and 72 hours.

After the indicated time periods, the cell monolayers were harvested and used to prepare total RNA as described above. Purified total RNA was then subjected to electrophoresis, blotting onto nylon and hybridization with a TIMP-1 cDNA probe as described above. Figures 7.5. to 7.8. show photographs of the autoradiograms obtained from these experiments. The expression of TIMP-1 mRNA induced by the test conditions is compared to the levels of 28S rRNA in the same sample detected using the ribosomal cDNA probe. Detection of ribosomal RNA using the ethidium bromide stain of the gel is also shown for some experiments in cases in which this was
thought to be more reliable. Tables 7.5. to 7.8. show the results of quantifying the
signal intensities obtained by gel scan densitometry and correcting with respect to the
level of 28S rRNA in the same sample.

In all experiments except 2, TIMP-1 mRNA was detectable at 0 hours on the
autoradiograph which gave optimum intensities for the other time points. TIMP-1
mRNA was detectable in the remaining 2 experiments at 0 hours when the
autoradiographic exposure time was increased to a level which gave greater than
optimal intensities for the other time points.

After 12 hours of exposure to retinoic acid and bFGF in combination, a significant
stimulation of TIMP-1 mRNA was seen. This induction of TIMP-1 mRNA continued
to increase across the remaining time points becoming maximal after 72 hours of
stimulation. The level of induction seen after 72 hours was approximately 11.5 fold,
24 fold and 27 fold above the 0 hour time point for hsf 15, hsf 12 and hsf 13
respectively. Stimulation of the cells with retinoic acid and EGF in combination for
12 hours also stimulated TIMP-1 mRNA levels such that they were approximately 2
d fold (hsf 13), 6 fold (hsf 15) and 7.5 fold (hsf 9) above that seen prior to stimulation.
In the case of hsf 15 and hsf 9, the induction of TIMP-1 mRNA at 12 hours was
maximal and gradually declined thereafter. In the case of hsf 13, however, TIMP-1
mRNA continued to accumulate to a maximum at 72 hours after stimulation.

Retinoic acid and PDGF-BB in combination also caused a marked stimulation of
TIMP-1 mRNA after 12 hours; the maximum effect was seen either 12 hours after
treatment (hsf 15 and hsf 10) or 24 hours after treatment (hsf 13) and slowly declined
thereafter. TIMP-1 mRNA was also stimulated by 12 hours of treatment with retinoic
acid and TGF-β. In this case, TIMP-1 mRNA continued to accumulate until 48-72
hours after stimulation and the maximum level of induction was approximately 6.5
fold.

The kinetics of the induction of TIMP-1 mRNA in response to retinoic acid and
growth factors therefore appear to vary depending upon the growth factor.
Stimulation by retinoic acid and EGF or PDGF-BB in general is maximal after 12-24
hours. A slower response is seen with retinoic acid and bFGF or TGF-β which reaches an optimum after 48-72 hours of stimulation. bFGF appears to give a more marked induction of TIMP-1 mRNA in these experiments than EGF or TGF-β; induction by PDGF-BB could only be quantitated in 1 experiment out of 3 and is therefore inconclusive.
Figure 7.5.
Induction of TIMP-1 mRNA by retinoic acid (10^{-6}M) and bFGF (100ng/ml) in combination at 0, 12, 24, 48 and 72 hours after stimulation in the following cell lines: a) hsf 13 (passage 4), b) hsf 15 (passage 6) and c) hsf 12 (passage 12). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.6.
Induction of TIMP-1 mRNA by retinoic acid (10^{-8}M) and EGF (10ng/ml) in combination at 0, 12, 24, 48 and 72 hours after stimulation in the following cell lines: a) hsf 13 (passage 3), b) hsf 15 (passage 5) and c) hsf 9 (passage 4). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.7.
Induction of TIMP-1 mRNA by retinoic acid (10^{-5}M) and PDGF-BB (100ng/ml) in combination at 0, 12, 24, 48 and 72 hours after stimulation in the following cell lines: a) hsf 13 (passage 6), b) hsf 15 (passage 8) and c) hsf 10 (passage 5). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
Figure 7.8.
Induction of TIMP-1 mRNA by retinoic acid (10^{-5}M) and TGF-β (100ng/ml) in combination at 0, 12, 24, 48 and 72 hours after stimulation in the following cell lines: a) hsf 13 (passage 10), b) hsf 10 (passage 9) and c) hsf 9 (passage 7). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13</td>
<td>0.095</td>
<td>1.631</td>
<td>1.498</td>
<td>not quantitated</td>
<td>2.557</td>
</tr>
<tr>
<td>(passage 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsf 15</td>
<td>0.058</td>
<td>0.288</td>
<td>0.482</td>
<td>0.643</td>
<td>0.667</td>
</tr>
<tr>
<td>(passage 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsf 12</td>
<td>0.165</td>
<td>1.322</td>
<td>2.321</td>
<td>3.337</td>
<td>3.903</td>
</tr>
<tr>
<td>(passage 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.5.

Steady-state levels of TIMP-1 mRNA in human skin fibroblasts at increasing time after stimulation with retinoic acid (10^{-5}M) and bFGF (100ng/ml). The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13</td>
<td>0.128</td>
<td>0.252</td>
<td>0.298</td>
<td>0.389</td>
<td>0.437</td>
</tr>
<tr>
<td>(passage 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsf 15</td>
<td>0.110</td>
<td>0.663</td>
<td>0.588</td>
<td>0.469</td>
<td>0.374</td>
</tr>
<tr>
<td>(passage 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsf 9</td>
<td>0.211</td>
<td>1.582</td>
<td>1.205</td>
<td>0.863</td>
<td>0.595</td>
</tr>
<tr>
<td>(passage 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.6.

Steady-state levels of TIMP-1 mRNA in human skin fibroblasts at increasing time after stimulation with retinoic acid (10^{-5}M) and EGF (10ng/ml). The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13 (passage 6)</td>
<td>0.097</td>
<td>3.225</td>
<td>5.998</td>
<td>2.089</td>
<td>1.789</td>
</tr>
<tr>
<td>hsf 15 (passage 8)</td>
<td>not detectable (&lt;0.759)</td>
<td>3.283</td>
<td>1.703</td>
<td>1.686</td>
<td>1.017</td>
</tr>
<tr>
<td>hsf 10 (passage 5)</td>
<td>not detectable (&lt;0.293)</td>
<td>0.437</td>
<td>0.403</td>
<td>0.276</td>
<td>0.223</td>
</tr>
</tbody>
</table>

**Table 7.7.**

Steady-state levels of TIMP-1 mRNA in human skin fibroblasts at increasing time after stimulation with retinoic acid (10^{-5} M) and PDGF-BB (100 ng/ml). The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsf 13 (passage 10)</td>
<td>0.422</td>
<td>0.801</td>
<td>1.112</td>
<td>1.660</td>
<td>2.698</td>
</tr>
<tr>
<td>hsf 10 (passage 9)</td>
<td>0.481</td>
<td>1.819</td>
<td>2.178</td>
<td>not quantitated</td>
<td>3.272</td>
</tr>
<tr>
<td>hsf 9 (passage 7)</td>
<td>not quantitated</td>
<td>not done</td>
<td>2.265</td>
<td>2.532</td>
<td>1.237</td>
</tr>
</tbody>
</table>

**Table 7.8.**

Steady-state levels of TIMP-1 mRNA in human skin fibroblasts at increasing time after stimulation with retinoic acid (10^{-5} M) and TGF-β (100 ng/ml). The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample.
7.3.5. The effect of the protein synthesis inhibitor cycloheximide on the synergistic induction of TIMP-1 mRNA by all-trans-retinoic acid and bFGF

Cycloheximide interferes with peptide bond formation in cells (Shaw and Kamen, 1986) resulting in 85%-90% inhibition of protein synthesis (Schöntal et al., 1988). Initially, the effect of cycloheximide on total cell protein synthesis was measured in order to check that protein synthesis was indeed being blocked under the experimental conditions used. This was carried out using the human skin cell line hsf 13 (passage 3) using the protocol described above. The following test conditions were examined in triplicate:

1. Control - DMEM + 1%ATFCS only.
2. Cycloheximide at 10, 25 and 50µg/ml.

A 24 hour period of treatment with cycloheximide was chosen rather than a longer period as it was thought likely that longer incubations would lead to significant cytotoxicity. The data shown in section 7.3.4. demonstrates that retinoic acid and bFGF in combination give a good induction of TIMP-1 mRNA after 24 hours of treatment although the maximum effect is not seen until 72 hours. It is therefore feasible to investigate the effect of cycloheximide on TIMP-1 induction by retinoic acid and bFGF over a 24 hour time period.

The effect of cycloheximide on protein synthesis is shown in figure 7.9. Cycloheximide was found to block 3H-leucine incorporation and therefore protein synthesis by approximately 90% at each concentration. 25µg/ml gave a marginally greater effect than 10µg/ml but no further increase was seen with 50µg/ml. 25µg/ml was therefore chosen as the concentration to be used for further experiments.

The cell supernates from this experiment were assayed for LDH activity as previously described in order to assess the cytotoxicity of cycloheximide. Figure 7.10. shows that LDH release from cells treated with cycloheximide at all concentrations is similar.
Figure 7.9. (top)
The effect of cycloheximide on ³H-leucine incorporation in the human skin cell line hsf 13 (passage 3).

Figure 7.10. (bottom)
Production of LDH activity by the human skin cell line hsf 13 (passage 3) following treatment with cycloheximide.
to that released by untreated cells. In addition, the appearance of the cells was unchanged by the presence of cycloheximide (not shown). Cycloheximide is therefore not cytotoxic under these experimental conditions.

The effect of 25μg/ml cycloheximide on the synergistic induction of TIMP-1 mRNA by 10⁻⁵ M retinoic acid and 100ng/ml bFGF was investigated in 3 human skin cell lines (hsf 13, hsf 10 and hsf 9). The experiments were performed as described in Materials and Methods and the cells were stimulated with the following test reagents for 24 hours:

1. control - DMEM + 1% ATFCS only
2. all-trans-retinoic acid at 10⁻⁴ M
3. recombinant bFGF at 100ng/ml
4. all-trans-retinoic acid at 10⁻⁴ M + recombinant bFGF at 100ng/ml
5. cycloheximide at 25μg/ml
6. all-trans-retinoic acid at 10⁻⁴ M + cycloheximide at 25μg/ml
7. recombinant bFGF at 100ng/ml + cycloheximide at 25μg/ml
8. all-trans-retinoic acid at 10⁻⁴ M + recombinant bFGF at 100ng/ml + cycloheximide at 25μg/ml.

Total RNA was prepared from the cell monolayers, electrophoresed, blotted and hybridized with the TIMP-1 and ribosomal cDNA probes as described in Materials and Methods. The results of these experiments are shown in figure 7.11. and table 7.9. Figure 7.11. shows autoradiographs of blots hybridized with the TIMP-1 cDNA probe and with the housekeeping ribosomal probe. Table 7.9. shows the quantitation of these results by gel scan densitometry including corrections for differences in levels of the ribosomal mRNA.

The data shows that TIMP-1 mRNA is induced by retinoic acid alone and by bFGF alone (2 cell lines out of 3), and that a synergistic induction of TIMP-1 mRNA occurs
in the presence of retinoic acid and bFGF. These results are similar to those shown above at 72 hours after stimulation (section 7.3.1.).

In the presence of cycloheximide, a stimulation of TIMP-1 mRNA occurred in response to retinoic acid treatment in only 1 experiment out of 3, and this effect was less than that seen without cycloheximide. This suggests that retinoic acid stimulation of TIMP-1 mRNA occurs partially or totally through de novo protein synthesis. It was also found that cycloheximide completely blocked the induction of TIMP-1 mRNA by bFGF in the 2 experiments in which bFGF had a stimulatory effect. This indicates that stimulation of TIMP-1 mRNA by bFGF is dependent on new protein synthesis. The synergistic induction of TIMP-1 mRNA by retinoic acid and bFGF together was greatly diminished by cycloheximide. A slight residual effect in the presence of cycloheximide can probably be explained by the fact that 10-15% of normal protein synthesis still occurs under these conditions. It is therefore concluded that the synergistic induction of TIMP-1 mRNA by retinoic acid and bFGF requires de novo protein synthesis by the cells.
Figure 7.11. Induction of TIMP-1 mRNA by retinoic acid and bFGF either alone or in combination and in the additional presence of cycloheximide after 24 hours of stimulation. The following test conditions are shown: lane 1: No additions, lane 2: Retinoic acid 10⁻⁵M, lane 3: bFGF 100ng/ml, lane 4: Retinoic acid 10⁻⁵M + bFGF 100ng/ml, lane 5: Cycloheximide 25μg/ml, lane 6: Retinoic acid 10⁻⁵M + cycloheximide 25μg/ml, lane 7: bFGF 100ng/ml + cycloheximide 25μg/ml, lane 8: Retinoic acid 10⁻⁵M + bFGF 100ng/ml + cycloheximide 25μg/ml. The figure shows results from the following cell lines: a) hsf 13 (passage 4), b) hsf 9 (passage 8), c) hsf 10 (passage 11). The positions and sizes in kb of molecular weight markers are shown. The levels of each mRNA are compared to the detection of 28S rRNA in each sample.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 13 passage 4</td>
<td>hsf 9 passage 8</td>
<td>hsf 10 passage 11</td>
</tr>
<tr>
<td>No additions</td>
<td>0.157</td>
<td>0.513</td>
<td>0.053</td>
</tr>
<tr>
<td>Retinoic acid $10^6$M</td>
<td>0.496</td>
<td>1.605</td>
<td>0.350</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>0.252</td>
<td>0.371</td>
<td>0.320</td>
</tr>
<tr>
<td>Retinoic acid $10^6$M + bFGF 100ng/ml</td>
<td>2.217</td>
<td>2.634</td>
<td>1.218</td>
</tr>
<tr>
<td>Cycloheximide 25μg/ml</td>
<td>0.182</td>
<td>0.295</td>
<td>0.111</td>
</tr>
<tr>
<td>Retinoic Acid $10^6$M + cycloheximide 25μg/ml</td>
<td>0.135</td>
<td>0.684</td>
<td>0.121</td>
</tr>
<tr>
<td>bFGF 100ng/ml + cycloheximide 25μg/ml</td>
<td>0.099</td>
<td>0.424</td>
<td>0.101</td>
</tr>
<tr>
<td>Retinoic Acid $10^6$M + bFGF 100ng/ml + cycloheximide 25μg/ml</td>
<td>0.132</td>
<td>0.915</td>
<td>0.242</td>
</tr>
</tbody>
</table>

Table 7.9.

Steady-state levels of TIMP-1 mRNA in human skin fibroblasts following treatment for 24 hours with bFGF and retinoic acid in the absence and additional presence of cycloheximide. The intensity of each autoradiographic band (arbitrary units) is corrected with respect to the level of 28S rRNA measured in the same sample.
7.4. DISCUSSION

The data in this chapter shows that retinoic acid in combination with each of the 4 growth factors causes a synergistic induction of TIMP-1 mRNA after 72 hours of stimulation. These effects on TIMP-1 mRNA are similar to the effects of the same agents on TIMP-1 protein secretion as shown in chapters 3 and 4. Changes in TIMP-1 protein production are therefore paralleled by changes in TIMP-1 mRNA in the cells indicating that regulation is occurring at a pretranslational level. This regulation is most likely to be via modulation of TIMP-1 gene transcription and/or TIMP-1 mRNA stability.

Investigation into the kinetics of this synergistic induction of TIMP-1 mRNA show that a maximal effect is obtained in response to retinoic acid and EGF or PDGF-BB after 12-24 hours of stimulation. In chapter 6, it was demonstrated that the optimum induction of TIMP-1 protein occurred at least 72 hours after stimulation with these agents. This peak induction of TIMP-1 protein is therefore preceded by a peak induction of TIMP-1 mRNA as would be expected. In experiments in which the maximum effect on mRNA was seen after 12 hours, it is possible that the peak induction in fact occurred at an earlier time point since 12 hours was the earliest time point investigated. However, since maximum protein induction does not occur until at least 72 hours after stimulation, this seems unlikely. In the case of retinoic acid and EGF, the rather modest induction of TIMP-1 mRNA by these factors combined at 72 hours after stimulation is probably because the response has declined by this time point.

In the case of retinoic acid and bFGF or retinoic acid and TGF-β, the peak effect on TIMP-1 mRNA is seen after 48-72 hours of stimulation. Hence the maximum induction of TIMP-1 mRNA by these agents appears to occur at the same time as, rather than to precede the peak induction of TIMP-1 protein. Perhaps this is because not all of the TIMP-1 mRNA induced at 72 hours is translated. It is also possible that TIMP-1 protein levels continue to increase beyond 72 hours in response to a peak induction of TIMP-1 mRNA at or later than 72 hours. However, in chapter 6, it was
suggested that for retinoic acid and bFGF, TIMP-1 protein induction declines after 72 hours since the response is reduced if incubation with the agents is continued to 96 hours. However there is no evidence to suggest that TIMP-1 protein induction by retinoic acid and TGF-β, does not continue to increase after 72 hours. The slow kinetics of TIMP-1 mRNA induction in response to retinoic acid and TGF-β correlate with the data in chapter 6 showing that a prolonged incubation with TGF-β (72 hours) followed by retinoic acid treatment produces the same effect as both agents added together. Taken together, these findings suggest that TIMP-1 induction by TGF-β and retinoic acid is delayed because it depends upon the slow induction of an intracellular event by TGF-β.

The kinetics of TIMP-1 mRNA induction in response to other stimulatory agents are similar to the data reported here. In human lung fibroblast cultures, the maximum induction of TIMP-1 mRNA in response to oncostatin M occurs 18-24 hours after stimulation (Richards et al., 1993). In rat osteoblasts, the effects of retinoic acid or TGF-β on decreasing or increasing TIMP-1 mRNA levels respectively were found to be greater at 72 hours than at 24 hours (Overall, 1995). Similarly, concanavalin A does not have any appreciable effect on TIMP-1 mRNA in human gingival fibroblasts after 24 hours but a reduction is seen after 72 hours (Overall and Sodek, 1990). Hence, the kinetics of TIMP-1 mRNA elevation and repression in response to modulatory factors suggests that these are secondary rather than a primary responses of the cells to the agents.

Previous investigations into the regulation of TIMP-1 mRNA levels in cells have attempted to distinguish between transcriptional and posttranscriptional effects. In human foetal lung fibroblasts, the upregulation of TIMP-1 gene expression by bFGF and TGF-β in combination is caused mainly by an increase in TIMP-1 gene transcription (Edwards et al., 1987). Other reports also indicate that the induction of TIMP-1 mRNA by TGF-β is a transcriptional effect (Overall et al., 1991, Overall, 1994, 1995). In rat osteoblasts where retinoic acid has a suppressive effect on TIMP-1 mRNA levels, this negative regulation again appears to be transcriptional (Overall,
1994, 1995); transcriptional changes are also involved in the repression of TIMP-1 gene expression by concanavalin A (Overall and Sodek, 1990). Taken together, these reports suggest that regulation of TIMP-1 gene expression is usually transcriptional with stability of TIMP-1 mRNA having little or no role. Notably, the 3' end of TIMP-1 mRNA does not contain an AUUUA element in contrast to metalloproteinase enzymes such as collagenase and 72 000-Mr gelatinase (Vincenti et al., 1994, Overall et al., 1991). On this basis, it can be postulated that the synergistic inductions of TIMP-1 mRNA by retinoic acid and each growth factor are also transcriptional rather than mRNA stability effects. However, 1 report has indicated that the induction of TIMP-1 mRNA by TPA in human promonocyte-like cells is independent of transcriptional effects and results from an increase in TIMP-1 mRNA stability (Shapiro et al., 1993). In the case of retinoic acid and bFGF, it is noted from the time course experiments that an initial large increase in TIMP-1 mRNA takes place during the first 12 hours followed by a more modest increase from 12 to 72 hours. These data may result from an initial burst of transcriptional activity giving the effect seen at 12 hours followed by a subsequent slower accumulation of mRNA up to 72 hours due to an additional effect on mRNA stability. Further experiments are needed to investigate this.

The experiments using the protein synthesis inhibitor cycloheximide indicate that new protein synthesis is required for the synergistic induction of TIMP-1 mRNA by retinoic acid and bFGF. In chapter 6, it was demonstrated that bFGF causes a transient intracellular event to take place which is responsible for the effect on TIMP-1 protein. It was suggested that this event was most likely to be the induction of a new protein by the growth factor. The effect of cycloheximide therefore correlates well with this hypothesis. However, the possibility that retinoic acid is inducing the synthesis of a new protein and that this accounts for the results shown cannot be ruled out. Such a theory, nevertheless is not supported by the data in chapter 6 showing that retinoic acid treatment of cells followed by bFGF cannot synergistically induce TIMP-1.
This chapter also shows that the induction of TIMP-1 mRNA by retinoic acid alone is partially or totally dependent on new protein synthesis. Previous work has demonstrated that immortalized rabbit synovial fibroblasts constitutively express retinoic acid receptors (Pan and Brinckerhoff, 1994, Pan et al., 1995) and that the expression of RAR-α, RAR-β and RAR-γ mRNAs is induced by treatment with all-trans- or 9-cis-retinoic acid (Pan and Brinckerhoff, 1994, Pan et al., 1995). This induction was observed within 3 hours and peaked between 6 and 12 hours of treatment. In contrast, RXR-α mRNA levels are not modulated by either all-trans- or 9-cis-retinoic acid. It can be postulated that stimulation of TIMP-1 mRNA by retinoic acid first requires the upregulation of retinoic acid receptor expression as a primary event. Sometimes, however the presence of pre-existing constitutively expressed receptors is sufficient to drive a partial response which is resistant to cycloheximide.

The sensitivity of TIMP-1 gene expression to cycloheximide has been previously investigated by others. It appears that new protein synthesis is sometimes required for changes in TIMP-1 mRNA levels to occur and that in other cases it is not required. The induction of TIMP-1 mRNA in response to PDGF in skin fibroblasts appears to be independent of new protein synthesis (Circolo et al., 1991). The negative effect of retinoic acid on TIMP-1 mRNA levels in rat osteoblasts is also not affected by cycloheximide (Overall, 1995). However, the induction of TIMP-1 mRNA by TGF-β in these cells and in human gingival fibroblasts does appear to require new protein synthesis (Overall et al., 1991, Overall, 1995). The repressive effect of concanavalin A on TIMP-1 gene expression in human gingival fibroblasts also requires new protein synthesis (Overall and Sodek, 1990).

In sharp contrast to the effects on TIMP-1, TIMP-2 mRNA levels were not modulated by retinoic acid either alone or in combination with bFGF, EGF or PDGF-BB. None of the growth factors applied alone had any effect on TIMP-2 mRNA levels either. However, retinoic acid in combination with TGF-β did consistently elevate both species of TIMP-2 mRNA although this was a modest induction compared to the
effect on TIMP-1. The lack of effect of TGF-β alone on TIMP-2 mRNA expression correlates with previous work in which no significant effect was seen in foetal lung fibroblasts (Stetler-Stevenson et al., 1990). In addition, the modest effect of retinoic acid and TGF-β on TIMP-2 compared to their effect on TIMP-1 is similar to the effect of TPA in human foetal lung fibroblasts. In these cells, TPA caused an 8 fold induction of TIMP-1 mRNA but only a 2 fold induction of TIMP-2 mRNA (Stetler-Stevenson et al., 1990). TIMP-1 and TIMP-2 mRNA levels and protein production are also co-ordinately upregulated in response to follicle-stimulating hormone in rat Sertoli cells; again the effect on TIMP-1 is greater than the effect on TIMP-2 (Ulisse et al., 1994). Other agents which have been shown to upregulate TIMP-2 mRNA in cells include dexamethasone alone and in combination with various cytokines (Roeb et al., 1995). Finally, the fact that most of the agents examined in this chapter had no effect on TIMP-2 mRNA levels fits in with the general trend that TIMP-2 gene expression is mainly constitutive and non-inducible.

In agreement with previous reports (Circolo et al., 1991, Edwards et al., 1987) it was found that bFGF, EGF or PDGF-BB potently increased collagenase mRNA levels in human skin fibroblasts. The additional presence of retinoic acid caused a marked inhibition of growth factor-stimulated collagenase mRNA in all experiments. Changes in collagenase protein levels secreted by the cells as demonstrated in chapters 3 and 4 are therefore paralleled by changes in steady-state levels of mRNA, demonstrating that these effects are pretranslational.

EGF stimulates collagenase primarily by increasing the stability of collagenase mRNA (Delany and Brinckerhoff, 1992) and it has been suggested that this takes place via the production of an RNA-stabilizing factor which prohibits the action of a constitutively expressed specific metalloproteinase mRNA ribonuclease. Retinoic acid may therefore block EGF-stimulated collagenase either by stimulating the production of the ribonuclease or inhibiting the production of the RNA-stabilizing factor. However, other work has shown that the half-life of collagenase mRNA is not modulated by retinoic acid when applied alone (Brinckerhoff et al., 1986) which
presumably precludes the possibility of retinoic acid interfering with a constitutively expressed ribonuclease. The inhibition of RNA-stabilizing factor production is therefore more likely, since it only necessitates that retinoic acid alters the stability of collagenase mRNA in the additional presence of EGF.

bFGF appears to stimulate collagenase primarily via increased transcription of the collagenase gene (Edwards et al., 1987), and it is possible that this occurs via c-fos and the AP-1 complex since it is known that FGF treatment of fibroblasts results in the induction of c-fos (Müller et al., 1984). If this is the case, then the observed inhibition of bFGF-stimulated collagenase by retinoic acid could result from a downregulation of c-fos by retinoic acid as has been previously suggested in the case of IL-1-stimulated collagenase production (Lafyatis et al., 1990). Other reports suggest alternative mechanisms of action for retinoic acid repression of collagenase involving protein-protein interactions (Schüle et al., 1991, Pan et al., 1992, Pan and Brinckerhoff, 1994, Pan et al., 1995). Retinoid receptors are believed to bind to the AP-1-binding proteins c-fos and c-jun thereby preventing them from interacting with gene regulatory sequences such as the TRE. bFGF-stimulated collagenase may also be modulated by retinoic acid in this manner.

The mechanism by which PDGF stimulates human fibroblast collagenase gene expression has not received very much attention although the induction of collagenase mRNA has been shown to be insensitive to cycloheximide in these cells (Circolo et al., 1991). This suggests that the induction of AP-1-binding proteins such as c-fos by PDGF (Kruijer et al., 1984) is probably not involved in the upregulation of collagenase mRNA. However, in bone cell cultures, stimulation of collagenase (MMP-13) mRNA by PDGF-BB does require new protein synthesis and results from both transcriptional and posttranscriptional effects (Varghese et al., 1996). The effect of PDGF-BB is also extremely transient such that MMP-13 mRNA levels return to control levels after 16 hours. The data in this chapter by contrast shows a marked elevation of collagenase (MMP-1) mRNA in skin fibroblasts 72 hours after
stimulation. It therefore seems likely that MMP-1 and MMP-13 gene expression in response to PDGF-BB are modulated by different mechanisms.

Surprisingly, TGF-β was found to have no effect on collagenase mRNA levels 72 hours after stimulation, in contrast to its inhibitory effect on collagenase protein secretion at the same time point as shown in chapter 4. It is possible that collagenase steady-state mRNA levels are in fact altered by TGF-β but at an earlier time point followed by a return to basal levels by 72 hours after stimulation. However, the data contrast with the findings of Overall et al. (1991) in which a reduction in collagenase mRNA levels was observed 24, 48 and 72 hours after stimulation of human gingival fibroblasts with TGF-β. In this report, the effect after 72 hours was greater than the effect after 24 hours. Another possibility is that TGF-β regulates collagenase protein levels via effects on translation. However this does not seem likely since in human foetal lung and gingival fibroblasts the effect of TGF-β on collagenase has been shown to be transcriptional (Edwards et al., 1987, Overall et al., 1991).

In summary, therefore, this chapter has demonstrated that the synergistic induction of TIMP-1 protein by retinoic acid combined with bFGF, EGF, PDGF-BB or TGF-β is paralleled by similar changes in steady-state levels of TIMP-1 mRNA. This demonstrates that control of TIMP-1 gene expression by these agents occurs at a pretranslational level, possibly involving the modulation of transcription and/or mRNA stability. The effects of the same factors on collagenase protein production are also paralleled by changes in steady-state levels of collagenase mRNA, again demonstrating pretranslational regulation. In contrast to TIMP-1, TIMP-2 gene expression appears to be mainly constitutive and was not inducible by most of the agents investigated. A modest induction of TIMP-2 mRNA occurred in response to retinoic acid and TGF-β although this effect was small compared to the effect on TIMP-1. Finally, the synergistic induction of TIMP-1 mRNA by retinoic acid and bFGF requires new protein synthesis since it is sensitive to cycloheximide.
CHAPTER EIGHT

THE EFFECT OF PROTEIN KINASE INHIBITORS ON THE SYNERGISTIC INDUCTION OF TIMP-1 PROTEIN BY ALL-TRANS-RETINOIC ACID AND bFGF

8.1. INTRODUCTION

The aim of this chapter is to understand further the mechanism by which retinoic acid and bFGF synergistically stimulate the production of TIMP-1 protein by investigating the bFGF signalling pathway. bFGF, in common with other mitogenic growth factors is believed to elicit its effects on cells via high affinity receptors with intrinsic tyrosine kinase activity as discussed in detail in chapter 1. These receptors dimerize in response to binding of growth factor (Ullrich and Schlessinger, 1990) and the tyrosine kinase is activated. This results in autophosphorylation of the cytoplasmic domain (Bellot et al., 1991) followed by the phosphorylation and activation of intracellular substrates and pathways including phospholipase Cγ, phosphatidylinositol 3'-kinase and the Ras pathway (Jaye et al., 1992, Mason, 1994, Friesel and Maciag, 1995) (see figure 1.3.).

All of these events depend upon the initial activation of receptor tyrosine kinase activity and the resultant autophosphorylation of the receptor cytoplasmic domain. In theory, therefore, the inhibition of tyrosine kinase activity in cells should abolish the bFGF signal transduction pathway and therefore prevent the synergistic induction of TIMP-1 by retinoic acid and bFGF. In order to find out whether this is indeed the case, the effect of 2 tyrosine kinase inhibitors, herbimycin A (HERB A) and genistein on the synergistic induction of TIMP-1 protein in response to retinoic acid and bFGF was examined.

Receptor autophosphorylation is followed by the phosphorylation and activation of various intracellular substrates, and at this point the transduction of the signal to induce TIMP-1 production could take 1 or more of a number of different pathways.
The activation of phospholipase C\textsubscript{Y} which hydrolyses phosphatidylinositol 4,5-bisphosphate to produce inositol trisphosphate and diacylglycerol is probably the best understood of these pathways (Rhee et al., 1989, Burgess et al., 1990, Wahl and Carpenter, 1991). Inositol trisphosphate acts as a second messenger molecule to liberate stored calcium from the endoplasmic reticulum and thereby activates calcium-requiring enzymes or processes. Diacylglycerol is an activator of protein kinase C, an important intracellular signalling molecule which leads to the activation of c\textit{jun} (reviewed by Angel and Karin, 1991). The involvement of this pathway in the synergistic induction of TIMP-1 protein by retinoic acid and bFGF was investigated using the commercially available inhibitor bisindolylmaleimide (BIS) which is a protein kinase C inhibitor (Toullec et al., 1991).

Finally, the effect of a highly specific MAP kinase inhibitor was investigated. This inhibitor is a pyridinyl-imidazole compound (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole) and is referred to as 203580. It is not commercially available and was supplied by SmithKline Beecham Pharmaceuticals. These compounds were discovered when it was found that a bicyclic imidazole had an inhibitory effect on lipopolysaccharide-stimulated human monocyte IL-1 and TNF-\(\alpha\) production (Lee et al., 1990, Lee et al., 1993). Lipopolysaccharide and cytokines such as IL-1 have been shown to stimulate the activity of the stress-response p38 MAP kinase (Han et al., 1994, Freshney et al., 1994). Activation of stress-response MAP kinases leads to increased transcription of certain genes (Han et al., 1994). MAP kinases belonging to different phosphorylation cascades, controlling distinct physiological processes have been identified in yeast cells (Ammerer, 1994). The molecular target of the pyridinyl-imidazole compounds has been identified as a protein which is closely related to the murine p38 MAP kinase (Lee et al., 1994). These compounds do not inhibit the activity of the mitogen-driven MAP kinase Erk-1 (extracellular signal-regulated kinase-1) or the stress-response MAP kinase JNK1, suggesting very high selectivity. This high selectivity contrasts with many commercially available inhibitors which have a far broader substrate specificity.
8.2. METHODS AND RESULTS

8.2.1. The effect of the tyrosine kinase inhibitor HERB A on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

The tyrosine kinase inhibitor HERB A which inhibits tyrosine kinase activity by reacting with free SH groups (IC_{50} = 900nM) (Uehara and Fukazawa, 1991) was used to investigate the role of tyrosine phosphorylation in the synergistic induction of TIMP-1 by retinoic acid and bFGF. HERB A selectively inhibits tyrosine kinases and has no effect on protein kinase A and protein kinase C.

Initially, the cytotoxicity of HERB A was evaluated in order to find the maximum concentration which could be used without causing significant toxicity to the cells. The effect of increasing concentrations of HERB A (5 x 10^{-8} - 10^{-4}M) on LDH release from the human skin cell line hsf15 was assessed using the basic cell assay system described in chapter 2. LDH was quantitated using a commercial kit as described in chapter 3. Since retinoic acid is cytotoxic at concentrations above 10^{-3}M, it was postulated that HERB A and retinoic acid at 10^{-3}M in combination might be more toxic than HERB A alone. Hence, the effect of increasing concentrations of HERB A in the additional presence of 10^{-3}M retinoic acid on LDH release was also measured in a similar manner.

The results of this experiment shown in figure 8.1 indicate that the maximum concentration of HERB A which can be used without causing cytotoxicity is 10^{-4}M. At 5 x 10^{-4}M HERB A, a small but significant increase in LDH release compared to control cells was seen. Further increases in the concentration of HERB A caused a dose-dependent increase in the level of cytotoxicity observed. In the additional presence of retinoic acid, 5 x 10^{-4}M HERB A caused a more potent cytotoxic effect and further increases in the concentration of HERB A again caused a dose-dependent increase in cytotoxicity.

The effect of 10^{-4}M HERB A on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF was then investigated using the cell assay system described in
chapter 2. Three different experiments were performed using 3 different skin cell 
lines (hsf 13, hsf 15 and hsf 10). The effect of the following test conditions was 
examined in triplicate in each experiment. In cases in which more than 1 test reagent 
was added to the wells, the test reagents were mixed and added to the cells 
simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF at 100ng/ml
3. all-trans-retinoic acid at 10⁻⁴M
4. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml
5. HERB A at 10⁻⁴M
6. recombinant bFGF at 100ng/ml + HERB A at 10⁻⁴M
7. all-trans-retinoic acid at 10⁻⁴M + HERB A at 10⁻⁴M
8. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml + HERB A at 
10⁻⁴M

After 72 hours, the production of TIMP-1 protein by the cells under these various 
conditions was assessed by ELISA and corrected with respect to total cell protein as 
described in chapter 3. The release of LDH from the cells was also measured as 
previously described. Statistical analysis was by one way analysis of variance or 
multiple regression as described in chapter 2.

In each experiment, 10⁻⁴M retinoic acid and 100ng/ml bFGF each caused a small 
increase in TIMP-1 protein production when applied alone and a larger synergistic 
increase when applied together. When 10⁻⁴M retinoic acid and 100ng/ml bFGF were 
applied to the cells in the additional presence of 10⁻⁴M HERB A there was either no 
inhibition or a small inhibition (16-25%) of TIMP-1 protein induction. Figure 8.2. 
shows the results of 1 experiment in which no inhibition is seen and table 8.1. shows 
the results of all experiments. No cytotoxicity was seen in any of these experiments 
as shown by LDH release (data not shown).
Unconditioned culture medium
■ No Additions
 Retinoic Acid $10^{-3}$M
 HERB A at the indicated concentrations
 Retinoic acid $10^{-3}$M + HERB A
 at the indicated concentrations
 positive control

*** p<0.001 compared to No Additions

Figure 8.1. (top)
Production of LDH activity by the human skin cell line hsf 15 (passage 10) following treatment with HERB A alone and in the additional presence of retinoic acid.

Figure 8.2. (bottom)
The effect of HERB A on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF in the human skin cell line hsf 10 (passage 3).
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>Retinoic Acid 10⁻⁵ M</th>
<th>bFGF 100ng/ml</th>
<th>Retinoic Acid 10⁻⁵ M + bFGF 100ng/ml</th>
<th>HERB A 10⁻⁸ M</th>
<th>Retinoic Acid 10⁻⁵ M + HERB A 10⁻⁸ M</th>
<th>bFGF 100ng/ml + HERB A 10⁻⁸ M</th>
<th>Retinoic Acid 10⁻⁵ M + bFGF 100ng/ml + HERB A 10⁻⁸ M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsf 13</td>
<td>4.37 +/- 0.34</td>
<td>10.79 +/- 0.38 ***</td>
<td>6.93 +/- 0.43 ***</td>
<td>36.22 +/- 0.37 ♦♦♦</td>
<td>3.55 +/- 0.07</td>
<td>8.87 +/- 0.27</td>
<td>7.08 +/- 0.11</td>
<td>29.61 +/- 1.39 ***</td>
</tr>
<tr>
<td>passage 9</td>
<td>3.27 +/- 0.07</td>
<td>8.79 +/- 0.35 ***</td>
<td>5.38 +/- 0.33 ***</td>
<td>31.74 +/- 0.63 ***</td>
<td>2.71 +/- 0.043</td>
<td>7.22 +/- 0.31</td>
<td>6.00 +/- 0.18</td>
<td>26.04 +/- 1.39 ***</td>
</tr>
<tr>
<td></td>
<td>6.98 +/- 0.22</td>
<td>16.96 +/- 0.54 ***</td>
<td>9.95 +/- 0.56 ***</td>
<td>47.91 +/- 1.40 ***</td>
<td>6.97 +/- 0.08</td>
<td>15.15 +/- 0.38</td>
<td>10.14 +/- 0.09</td>
<td>51.10 +/- 3.61 **</td>
</tr>
<tr>
<td>hsf 15</td>
<td>3.27 +/- 0.07</td>
<td>8.79 +/- 0.35 ***</td>
<td>5.38 +/- 0.33 ***</td>
<td>31.74 +/- 0.63 ***</td>
<td>2.71 +/- 0.043</td>
<td>7.22 +/- 0.31</td>
<td>6.00 +/- 0.18</td>
<td>26.04 +/- 1.39 ***</td>
</tr>
<tr>
<td>passage 11</td>
<td>6.98 +/- 0.22</td>
<td>16.96 +/- 0.54 ***</td>
<td>9.95 +/- 0.56 ***</td>
<td>47.91 +/- 1.40 ***</td>
<td>6.97 +/- 0.08</td>
<td>15.15 +/- 0.38</td>
<td>10.14 +/- 0.09</td>
<td>51.10 +/- 3.61 **</td>
</tr>
<tr>
<td>hsf 10</td>
<td>6.98 +/- 0.22</td>
<td>16.96 +/- 0.54 ***</td>
<td>9.95 +/- 0.56 ***</td>
<td>47.91 +/- 1.40 ***</td>
<td>6.97 +/- 0.08</td>
<td>15.15 +/- 0.38</td>
<td>10.14 +/- 0.09</td>
<td>51.10 +/- 3.61 **</td>
</tr>
<tr>
<td>passage 3</td>
<td>6.98 +/- 0.22</td>
<td>16.96 +/- 0.54 ***</td>
<td>9.95 +/- 0.56 ***</td>
<td>47.91 +/- 1.40 ***</td>
<td>6.97 +/- 0.08</td>
<td>15.15 +/- 0.38</td>
<td>10.14 +/- 0.09</td>
<td>51.10 +/- 3.61 **</td>
</tr>
</tbody>
</table>

Table 8.1. The production of TIMP-1 protein by human skin fibroblasts following treatment with bFGF and retinoic acid in the absence and additional presence of HERB A. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001. Statistical comparisons with the effect of retinoic acid and bFGF without HERB A are as follows: * p<0.05, ** p<0.01, *** p<0.001.
This result is surprising given the important role of tyrosine kinases in mediating signal transduction by bFGF. The effect of 10^8 M HERB A on bFGF-stimulated collagenase was therefore also investigated to see whether HERB A was any more effective in inhibiting this response. The levels of collagenase protein in the cell supernates harvested from the above experiments were measured by ELISA as previously described and corrected with respect to total cell protein. The results of these experiments are shown in table 8.2. and figure 8.3. bFGF at 100ng/ml was found to stimulate collagenase protein production in all experiments. Furthermore, in the additional presence of HERB A at 10^8 M, bFGF-stimulated collagenase was partially inhibited (27-70%) in all experiments.

These experiments seem to show that HERB A at 10^8 M is ineffective or only slightly effective in inhibiting the synergistic induction of TIMP-1 protein by retinoic acid and bFGF. It is however more effective in inhibiting bFGF-stimulated collagenase production by the cells although a total inhibition is still not seen. The results may indicate that HERB A does not cause sufficient inhibition of cellular tyrosine kinases at the concentration used. Higher concentrations of HERB A cannot be used as these are cytotoxic as indicated above. In order to investigate further the role of tyrosine kinases in the synergistic induction of TIMP-1 by retinoic acid and bFGF, it was decided to examine the effect of genistein which is another tyrosine kinase inhibitor on this response. Genistein inhibits cellular tyrosine kinases by a different mechanism to HERB A which raises the possibility that it may give effective inhibition without also causing cytotoxicity.

8.2.2. The effect of the tyrosine kinase inhibitor genistein on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

Genistein (4',5,7-trihydroxyisoflavone) inhibits protein tyrosine kinases and other protein kinases by acting as a competitive inhibitor of ATP binding (Kᵢ = 13.7μM) (Dean et al., 1989, Akiyama and Ogawara, 1991). The cytotoxicity of genistein was first evaluated in order to find the maximum concentration which could be used...
Figure 8.3.
The effect of HERB A on collagenase induction by bFGF in the human skin cell line hsf 15 (passage 11).
Table 8.2. The production of collagenase protein by human skin fibroblasts following treatment with bFGF in the absence and additional presence of HERB A. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of bFGF alone are as follows: * p<0.05, ** p<0.01, *** p<0.001.
without causing significant toxicity to the cells. The effect of increasing concentrations of genistein (5 x 10⁻¹M - 4 x 10⁻¹M) on LDH release from the human skin cell line hsf 15 (passage 11) was measured as described above in section 8.2.1. The results of this experiment shown in figure 8.4, indicate that none of the concentrations of genistein investigated caused significant cytotoxicity to the cells. However, marked changes in the appearance of the cell monolayer were seen using 2 x 10⁻⁴ and 4 x 10⁻⁴M genistein. The cells appeared to be surrounded by extracellular 'debris' although no dead or dying cells were apparent.

The effect of genistein on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF was then investigated using the cell assay system described in chapter 2. Five different experiments were performed using 4 different skin cell lines. The skin cell lines hsf 9, hsf 13 and hsf 15 were each investigated once and the cell line hsf 10 was investigated twice. The effect of the following test conditions was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF at 100ng/ml
3. all-trans-retinoic acid at 10⁻⁴M
4. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml
5. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml + genistein at 10⁻⁴M
6. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml + genistein at 2 x 10⁻⁴M
7. all-trans-retinoic acid at 10⁻⁴M + recombinant bFGF at 100ng/ml + genistein at 4 x 10⁻⁴M

After 72 hours, the production of TIMP-1 protein by the cells under these various conditions was assessed by ELISA as described in chapter 3. The release of LDH
Figure 8.4. (top).
Production of LDH activity by the human skin cell line hsf 15 (passage 11) following treatment with genistein.

Figure 8.5. (bottom).
The effect of genistein on $^3$H-thymidine incorporation in the human skin cell line hsf 10 (passage 8).
from the cells was also measured as previously described. Statistical analysis was by one way analysis of variance or multiple regression as described in chapter 2.

When the Lowry assay was used to measure the protein content of the cell monolayers, it was found that abnormally high levels of protein were observed in the presence of $2 \times 10^{-4}$M and $4 \times 10^{-4}$M genistein. It was thought that this phenomenon was related to the marked changes in the appearance of the cell monolayer which occur in the presence of these concentrations of genistein. It is possible that genistein interferes with the normal turnover of an extracellular protein or proteins by the cells which results in the appearance of 'debris' surrounding the cells and abnormally high levels of protein measured in the assay. The synthesis or release of such a protein or proteins could be stimulated by genistein, alternatively normal pathways of protein degradation may be blocked. For this reason, it was not possible to use measurement of the protein content of the cell monolayers to correct for stimulation or inhibition of cell division caused by the test reagents. In order to find out whether genistein had any significant effect on cell division, the effect of genistein on $^3$H-thymidine incorporation was measured using the following protocol:

Human skin fibroblasts (hsf 10, passage 8) were plated out as for the cell assay system described in chapter 2 but with half the number of cells per well ($1.25 \times 10^4$ cells per cm$^2$). This was to ensure that the cells were subconfluent as this is necessary for reasonable levels of $^3$H-thymidine incorporation to occur. The cells were then prepared for stimulation according to the basic cell assay system. The following test reagents were added in triplicate; in cases in which more than 1 test reagent was added, the test reagents were mixed and added simultaneously. The effect of genistein was compared to the effect of retinoic acid or retinoic acid in combination with bFGF. This is because the purpose of the experiments in this section is to examine the effect of genistein on TIMP-1 production by retinoic acid and bFGF to see whether it can be reduced to the level seen with retinoic acid alone.

1. Retinoic acid at $10^{-4}$M
2. Retinoic acid at 10^{-4} M + bFGF 100 ng/ml
3. genistein at 10^{-4} M
4. genistein at 2 \times 10^{-4} M
5. genistein at 4 \times 10^{-4} M.

After 24 hours, 0.5 \mu Ci of 6-^3H-thymidine (supplied by Amersham, Bucks., U.K.) was added to each well and the cells were incubated for a further 6 hours. The medium was then removed, the wells were washed 3 times with HBSS and 1 ml of 3 \mu M unlabelled thymidine (Sigma Chemical Company Ltd., Poole, Dorset, U.K.) in DMEM containing 1\% ATFCS was added to each well. The cells were incubated overnight and then washed 3 times with HBSS. 300 \mu l of 0.25 M ammonia was added to each well followed by agitation for 2 hours at room temperature. The contents of each well were mixed thoroughly and 200 \mu l was removed from each and added to 3 ml of Optiphase 'HiSafe' scintillation fluid. The samples were then counted for 1 minute on a Wallac 1410 liquid scintillation counter.

It was found that genistein at all 3 concentrations almost completely abolished 3H-thymidine incorporation by the cells. The results of this experiment are shown in figure 8.5. Such an effect is not surprising since stimulation of cell growth under these experimental conditions is likely to result from growth factors in the serum which operate through receptor tyrosine kinases. The abolition of 3H-thymidine incorporation by genistein therefore suggests that it is inhibiting cellular tyrosine kinases effectively. However, since genistein has such a marked effect on cell division, it is likely that there will be fewer cells in wells which have been treated with genistein than those treated with retinoic acid or retinoic acid in combination with bFGF after 72 hours of stimulation. The data in this section is plotted in ng/ml of TIMP instead of ng/\mu g of cell protein due to the difficulty with the Lowry assay and the effect of genistein on cell division must be borne in mind when interpreting the results. Nevertheless, the differences in cell number in the presence and absence of genistein are not likely to be vast for 2 reasons: firstly the design of the cell assay
system is such that the cells are confluent at the time of stimulation, hence further
growth is suppressed by contact inhibition, secondly the cells are stimulated in the
presence of low serum which does not support rapid growth.

In each experiment, 10⁻⁴M retinoic acid and 100ng/ml bFGF each caused a small
increase in TIMP-1 protein production when applied alone and a much bigger
synergistic increase when applied together. When 10⁻⁴M retinoic acid and 100ng/ml
bFGF were applied to the cells in the additional presence of increasing concentrations
of genistein there was a dose-dependent inhibition of TIMP-1 protein induction.
TIMP-1 protein production was reduced to the level seen with retinoic acid alone or to
below the level seen with retinoic acid alone in all experiments. The fact that TIMP-1
in ng/ml is reduced to less than the effect seen with retinoic acid alone in some cases
may well reflect the inhibitory effect of genistein on cell division as discussed above.

Figure 8.6. shows the results of a representative experiment and table 8.3. shows the
results of all experiments. No cytotoxicity was seen in any of the experiments as
shown by LDH release (data not shown) with the exception of hsf 10 (passage 8) in
which the 2 higher concentrations of genistein were toxic. In this experiment in
contrast to the others, an almost complete inhibition of the synergistic induction of
TIMP-1 protein was seen with the lowest concentration of genistein. It is thought that
the explanation for this variability is that a different and perhaps more potent stock of
genistein was used for this particular experiment.

The data shown in this section indicate that the tyrosine kinase inhibitor genistein is
able to block the synergistic induction of TIMP-1 protein by retinoic acid and bFGF at
concentrations which do not also cause cytotoxicity. Such results provide evidence
that protein phosphorylation is required for the synergistic induction of TIMP-1 by
these agents. In particular, genistein may be abolishing the activity of the bFGF
receptor tyrosine kinases which are thought to be the first step in bFGF signal
transduction.
Figure 8.6.
The effect of genistein on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF in the human skin cell line hsf 10 (passage 4).
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 9 passage 4</td>
<td>hsf 10 passage 4</td>
<td>hsf 13 passage 5</td>
<td>hsf 15 passage 12</td>
<td>hsf 10 passage 8</td>
</tr>
<tr>
<td>Control</td>
<td>21.25 +/- 0.66</td>
<td>153.6 +/- 5.49</td>
<td>114.18 +/- 2.58</td>
<td>85.37 +/- 4.55</td>
<td>149.22 +/- 6.31</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>34.66 +/- 1.62***</td>
<td>239.2 +/- 8.50***</td>
<td>244.05 +/- 5.84***</td>
<td>181.6 +/- 3.95***</td>
<td>407.13 +/- 8.48***</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>38.76 +/- 0.85***</td>
<td>238.87 +/- 16.37***</td>
<td>282.77 +/- 7.08***</td>
<td>189.62 +/- 4.92***</td>
<td>389.00 +/- 12.19***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 100ng/ml</td>
<td>310.08 +/- 18.35***</td>
<td>954.58 +/- 32.08***</td>
<td>1135.92 +/- 59.84***</td>
<td>549.75 +/- 16.09***</td>
<td>1640 +/- 43.56***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 100ng/ml + genistein 10^-4 M</td>
<td>299.3 +/- 6.32</td>
<td>450.7 +/- 10.07***</td>
<td>338 +/- 13.07***</td>
<td>272.43 +/- 7.06***</td>
<td>445.98 +/- 7.08***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-4 M + bFGF 100ng/ml + genistein 2 x 10^-4 M</td>
<td>109.06 +/- 7.36***</td>
<td>225.97 +/- 14.11***</td>
<td>202.22 +/- 10.00***</td>
<td>146.33 +/- 9.10***</td>
<td>toxic</td>
</tr>
<tr>
<td>Retinoic Acid 10^-4 M + bFGF 100ng/ml + genistein 4 x 10^-4 M</td>
<td>29.36 +/- 0.99***</td>
<td>143.8 +/- 6.40***</td>
<td>138.88 +/- 4.98***</td>
<td>101.15 +/- 2.41***</td>
<td>toxic</td>
</tr>
</tbody>
</table>

Table 8.3. The production of TIMP-1 protein by human skin fibroblasts following treatment with bFGF and retinoic acid in the absence and additional presence of genistein. The results are expressed as TIMP-1 in ng per ml. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: • p<0.05, •• p<0.01, ••• p<0.001. Statistical comparisons with the effect of retinoic acid and bFGF without genistein are as follows: • p<0.05, • p<0.01, •• p<0.001.
8.2.3. The effect of the protein kinase C inhibitor BIS on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

BIS is a highly selective cell-permeable protein kinase C inhibitor which acts as a competitive inhibitor of the ATP-binding site on protein kinase C (see e.g. Toullec et al., 1991). It is structurally similar to staurosporine, another protein kinase C inhibitor but displays greater selectivity; in particular it has no significant effect on any tyrosine kinases studied. It is also less toxic than staurosporine. IC$_{50}$ values obtained in cellular assays for BIS vary between 0.2μM and 2μM depending on the nature of the cell system and the stimuli used to activate protein kinase C. BIS does not differentiate between different protein kinase C isoenzymes.

Initially, the cytotoxicity of BIS was evaluated in order to find the maximum concentration which could be used without causing significant toxicity to the cells. The effect of increasing concentrations of BIS (5 x 10$^{-8}$M - 5 x 10$^{-6}$M) on LDH release from the human skin cell line hsf 15 (passage 10) was assessed as described above for HERB A. The cytotoxicity of BIS was also monitored in the additional presence of 10$^{-6}$M retinoic acid in a similar manner, in case the 2 combined were more toxic than BIS alone.

The results of this experiment shown in figure 8.7. indicate that the maximum concentration of BIS which can be used without causing cytotoxicity is 10$^{-6}$M. At 5 x 10$^{-6}$M BIS, a small but significant increase in LDH release compared to control cells is seen. In the additional presence of retinoic acid, 5 x 10$^{-6}$M BIS caused a more potent cytotoxic effect.

The effect of 10$^{-6}$M BIS on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF was then investigated using the same protocol as for HERB A described above. Five different experiments were performed using 3 different cell lines. The human skin cell lines hsf 13 and hsf 15 were each investigated twice and the cell line hsf 10 was investigated once. The effect of the following test conditions was examined in triplicate in each experiment. In cases in which more than 1 test reagent was added to the wells, the test reagents were mixed and added to the cells
Figure 8.7. (top)
Production of LDH activity by the human skin cell line hsf 15 (passage 10) following treatment with BIS alone and in the additional presence of retinoic acid.

Figure 8.8. (bottom)
The effect of BIS on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF in the human skin cell line hsf 15 (passage 7).
simultaneously.

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF at 100ng/ml
3. all-trans-retinoic acid at 10^-M
4. all-trans-retinoic acid at 10^-M + recombinant bFGF at 100ng/ml
5. BIS at 10^-M
6. recombinant bFGF at 100ng/ml + BIS at 10^-M
7. all-trans-retinoic acid at 10^-M + BIS at 10^-M
8. all-trans-retinoic acid at 10^-M + recombinant bFGF at 100ng/ml + BIS at 10^-M

After 72 hours, the production of TIMP-1 protein by the cells under these various conditions was assessed by ELISA and corrected with respect to total cell protein as previously described. The release of LDH from the cells was also measured. Statistical analysis was by one way analysis of variance or multiple regression.

In all experiments, 10^-M retinoic acid and 100ng/ml bFGF each caused a small increase in TIMP-1 protein production by the cells and a much greater synergistic increase in TIMP-1 when applied together. When 10^-M retinoic acid and 100ng/ml bFGF were applied to the cells in the additional presence of 10^-M BIS, no inhibition of TIMP-1 protein production was seen in any of the experiments. In 2 of the 5 experiments, a slight enhancement of TIMP-1 protein production occurred in the additional presence of BIS but this probably reflects experimental error. The results of a typical experiment are shown in figure 8.8, and the results of all experiments are shown in table 8.4. 10^-M BIS did not cause cytotoxicity in any of the experiments performed (data not shown). In order to ensure that the maximum possible concentration of BIS was being used, 2 additional experiments were performed using BIS at the slightly higher concentration of 2 x 10^-M (data not shown). In these experiments, a significant inhibition of TIMP-1 protein production by 10^-M retinoic acid and 100ng/ml bFGF was seen in the additional presence of BIS. However, this
### Table 8.4

The production of TIMP-1 protein by human skin fibroblasts following treatment with bFGF and retinoic acid in the absence and additional presence of BIS. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001. Statistical analysis showed that the induction of TIMP-1 by bFGF and retinoic acid is not significantly inhibited by BIS.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.75 +/- 0.39</td>
<td>4.37 +/- 0.34</td>
<td>3.86 +/- 0.087</td>
<td>3.27 +/- 0.07</td>
<td>6.98 +/- 0.22</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁶M</td>
<td>11.43 +/- 0.80 ***</td>
<td>10.79 +/- 0.38 ***</td>
<td>11.08 +/- 0.39 ***</td>
<td>8.79 +/- 0.35 ***</td>
<td>16.96 +/- 0.54 ***</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>10.24 +/- 0.17 ***</td>
<td>6.93 +/- 0.43 ***</td>
<td>5.96 +/- 0.082 ***</td>
<td>5.38 +/- 0.33 ***</td>
<td>9.95 +/- 0.56 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁶M + bFGF 100ng/ml</td>
<td>47.11 +/- 1.28 ***</td>
<td>36.22 +/- 0.37 ***</td>
<td>45.26 +/- 0.85 ***</td>
<td>31.74 +/- 0.63 ***</td>
<td>47.91 +/- 1.40 ***</td>
</tr>
<tr>
<td>BIS 10⁻⁶M</td>
<td>4.32 +/- 0.10</td>
<td>3.94 +/- 0.12</td>
<td>3.67 +/- 0.05</td>
<td>2.74 +/- 0.12</td>
<td>5.96 +/- 0.31</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁶M + BIS 10⁻⁶M</td>
<td>9.66 +/- 0.51</td>
<td>7.53 +/- 0.47</td>
<td>10.51 +/- 0.23</td>
<td>6.52 +/- 0.13</td>
<td>13.58 +/- 0.76</td>
</tr>
<tr>
<td>bFGF 100ng/ml + BIS 10⁻⁶M</td>
<td>10.55 +/- 0.25</td>
<td>8.56 +/- 0.38</td>
<td>7.14 +/- 0.31</td>
<td>6.09 +/- 0.17</td>
<td>13.70 +/- 0.58</td>
</tr>
<tr>
<td>Retinoic Acid 10⁻⁶M + bFGF 100ng/ml + BIS 10⁻⁶M</td>
<td>56.16 +/- 1.58</td>
<td>39.74 +/- 0.57</td>
<td>43.26 +/- 1.57</td>
<td>38.73 +/- 2.05</td>
<td>41.52 +/- 2.75</td>
</tr>
</tbody>
</table>
concentration of BIS also caused a small but significant increase in LDH release from the cells. It was therefore concluded that the decrease in TIMP-1 protein production in these cells in the presence of BIS was caused by cytotoxicity.

These experiments appear to show that BIS has no effect on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF and therefore that it occurs independently of protein kinase C. However, it is also possible that the pathway does in fact utilize protein kinase C and that BIS is not effectively inhibiting this enzyme due to inadequate accessibility. Since BIS is added to the cells at the same time as the other reagents and protein kinase C is known to be rapidly activated after growth factor treatment, it is possible that BIS does not penetrate the cells quickly enough to prevent transduction of the signal. In order to eliminate this possibility, it was decided to test the effect of BIS on PMA-stimulated TIMP-1 and collagenase since PMA is known to activate and operate through protein kinase C (Nishizuka, 1984).

The effect of PMA on collagenase and TIMP-1 protein secretion was therefore investigated in the absence and additional presence of 10^6 M BIS in 3 human skin cell lines (hsf 13, hsf 15 and hsf 10). The usual assay protocol was used as described above with the following test reagents.

1. control - DMEM + 1% ATFCS only
2. BIS at 10^-M
3. PMA at 10ng/ml
4. PMA at 10ng/ml + BIS at 10^-M

TIMP-1 and collagenase protein secretion by the cells after 72 hours of stimulation under these various conditions were measured as previously described.

These experiments showed that the stimulatory effect of PMA on collagenase and TIMP-1 protein production from the cells was completely or almost completely abrogated by 10^-M BIS in all cases. Figure 8.9. shows a representative experiment. Hence although BIS has no effect at all on the synergistic induction of TIMP-1 by
Figure 8.9.
The effect of BIS on collagenase induction by PMA in the human skin cell line hsf 15 (passage 3).
retinoic acid and bFGF, it has a powerful effect on PMA-stimulated TIMP-1 and collagenase. This indicates that BIS is able to gain access to and inhibit protein kinase C under the experimental conditions used. The lack of effect of BIS on the synergistic induction of TIMP-1 by retinoic acid and bFGF therefore demonstrates that the transduction of this signal is independent of protein kinase C.

8.2.4. The effect of BIS on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and PDGF-BB

In order to provide some comparison between the mechanism of TIMP-1 protein induction by retinoic acid and bFGF and that by the other polypeptide growth factors, the effect of BIS was also examined on the synergistic induction of TIMP-1 by retinoic acid and PDGF-BB. These experiments were set up in an identical manner to those described in section 8.2.3. except that 100ng/ml of PDGF-BB was used in place of 100ng/ml bFGF. Four experiments were performed using 4 different human skin fibroblast cell lines (hsf 13, hsf 15, hsf 10 and hsf 9).

In all experiments, 10^5 M retinoic acid and 100ng/ml PDGF-BB each caused an increase in TIMP-1 protein production by the cells and a synergistic increase in TIMP-1 when applied together. When 10^5 M retinoic acid and 100ng/ml PDGF-BB were applied to the cells in the additional presence of 10^5 M BIS, some degree of inhibition of TIMP-1 induction was seen in all except 1 of the experiments. The level of inhibition varied in different cell lines (41% - 100%). The results from 1 of the 4 experiments are shown in figure 8.10. and the results of all experiments are shown in table 8.5. 10^5 M BIS did not cause cytotoxicity in any of the experiments performed (data not shown).

These experiments suggest that the synergistic induction of TIMP-1 protein by retinoic acid and PDGF-BB may be partially dependent upon protein kinase C. The extent to which protein kinase C is involved appears to vary in different experiments from no dependence at all to a substantial dependence. In one experiment (hsf 10,
Figures 8.10. (top) and 8.11. (bottom)
The effect of 10^{-6} M BIS on TIMP-1 protein induction by retinoic acid and PDGF-BB (figure 8.10.) and on collagenase protein induction by PDGF-BB (figure 8.11.) in the human skin cell line hsf 9 (passage 5).
Table 8.5. The production of TIMP-1 protein by human skin fibroblasts following treatment with PDGF-BB and retinoic acid in the absence and additional presence of BIS. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and PDGF-BB are as follows: ♦ p<0.05, ♦ ♦ p<0.01, ♦ ♦ ♦ p<0.001. Statistical comparisons with the effect of retinoic acid and PDGF-BB without BIS are as follows: ♦ ♦ ♦ p<0.001.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 15</td>
<td>hsf 13</td>
<td>hsf 9</td>
<td>hsf 10</td>
</tr>
<tr>
<td></td>
<td>passage 3</td>
<td>passage 3</td>
<td>passage 5</td>
<td>passage 4</td>
</tr>
<tr>
<td>Control</td>
<td>4.35 +/- 0.16</td>
<td>6.85 +/- 0.39</td>
<td>2.92 +/- 0.059</td>
<td>8.66 +/- 0.32</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>14.91 +/- 0.95 ***</td>
<td>15.73 +/- 0.57 ***</td>
<td>5.63 +/- 0.14 ***</td>
<td>27.00 +/- 1.62 ***</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml</td>
<td>11.74 +/- 0.57 ***</td>
<td>9.47 +/- 0.56 **</td>
<td>3.43 +/- 0.09 ***</td>
<td>15.39 +/- 0.50 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-4 M PDGF-BB 100ng/ml</td>
<td>31.55 +/- 1.12 ***</td>
<td>33.95 +/- 1.84 ***</td>
<td>8.32 +/- 0.26 ***</td>
<td>40.04 +/- 2.25 *</td>
</tr>
<tr>
<td>BIS 10^-6 M</td>
<td>8.03 +/- 0.56</td>
<td>5.91 +/- 0.06</td>
<td>2.38 +/- 0.08</td>
<td>8.55 +/- 0.23</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + BIS 10^-6 M</td>
<td>9.18 +/- 0.49</td>
<td>12.92 +/- 0.41</td>
<td>5.30 +/- 0.12</td>
<td>21.15 +/- 0.88</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml + BIS 10^-6 M</td>
<td>9.54 +/- 0.70</td>
<td>8.85 +/- 0.55</td>
<td>3.08 +/- 0.15</td>
<td>15.70 +/- 0.86</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + PDGF-BB 100ng/ml + BIS 10^-6 M</td>
<td>24.69 +/- 0.90 ***</td>
<td>32.08 +/- 0.94</td>
<td>6.57 +/- 0.16 ***</td>
<td>22.20 +/- 0.72 ***</td>
</tr>
</tbody>
</table>
passage 4), a total inhibition of the synergistic response is seen although it should be noted that the level of synergy seen in this experiment is very small.

The effect of BIS at $10^{-6}$ M on collagenase induction by PDGF-BB alone was also evaluated. Using the cell supernates from the experiments described in the above paragraphs, the levels of collagenase protein produced were measured under the following test conditions:

1. control - DMEM + 1% ATFCS only
2. BIS at $10^{-6}$ M
3. PDGF-BB at 100ng/ml
4. PDGF-BB at 100ng/ml + BIS at $10^{-6}$ M.

In all experiments, PDGF-BB at 100ng/ml caused a potent stimulation of collagenase protein production. Furthermore, in all experiments this induction of collagenase was significantly although only partially blocked by the additional presence of $10^{-6}$ M BIS. Figure 8.11. shows a representative experiment and table 8.6. shows the results of all experiments. The level of inhibition varied in different experiments between 42% and 83%. These results suggest that the induction of collagenase production by PDGF-BB in skin fibroblasts is dependent upon the activity of protein kinase C but that other signal transduction pathways which do not involve protein kinase C are also contributing to the response. The level of involvement of protein kinase C in the response appears to vary in different cell lines.

8.2.5. The effect of the p38 MAP kinase inhibitor 203580 on the synergistic induction of TIMP-1 protein by all-trans-retinoic acid and bFGF

Initially, the effect of 203580 on the induction of collagenase protein by IL-1β in skin fibroblasts was investigated. Since IL-1 is believed to activate the p38 MAP kinase, this should show whether or not 203580 is active in these cells and at which concentration it is most active.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 15 passage 3</td>
<td>hsf 13 passage 3</td>
<td>hsf 9 passage 5</td>
<td>hsf 10 passage 4</td>
</tr>
<tr>
<td>Control</td>
<td>0.11 +/- 0.0077</td>
<td>0.096 +/- 0.0052</td>
<td>0.19 +/- 0.036</td>
<td>not detectable</td>
</tr>
<tr>
<td>BIS 10^-6M</td>
<td>0.15 +/- 0.015</td>
<td>0.17 +/- 0.005</td>
<td>0.15 +/- 0.02</td>
<td>not detectable</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml</td>
<td>4.17 +/- 0.22 ***</td>
<td>8.15 +/- 0.32 ***</td>
<td>5.34 +/- 0.12 ***</td>
<td>7.54 +/- 0.54 ***</td>
</tr>
<tr>
<td>PDGF-BB 100ng/ml + BIS 10^-6M</td>
<td>2.46 +/- 0.07 ***</td>
<td>4.02 +/- 0.18 ***</td>
<td>1.38 +/- 0.07 ***</td>
<td>1.42 +/- 0.06 ***</td>
</tr>
</tbody>
</table>

Table 8.6. The production of collagenase protein by human skin fibroblasts following treatment with PDGF-BB in the absence and additional presence of BIS. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of PDGF-BB alone are as follows: * p<0.05, ** p<0.01, *** p<0.001.
Cells were seeded out and prepared for stimulation according to the basic cell assay system. Five different experiments were performed using 4 different skin cell lines (hsf 10, hsf 13 and hsf 12 were each investigated once and hsf 9 was investigated twice). The following test reagents were added in triplicate and in cases in which more than 1 test reagent was added, the reagents were mixed and added simultaneously. The concentrations of 203580 used were based on previous studies in which the IC<sub>50</sub> for inhibition of TNF production from cells in response to bicyclic imidazole inhibitors was shown to vary between 5 x 10<sup>-7</sup>M (human monocytes) to 8 x 10<sup>-6</sup>M (murine macrophages) (Lee et al., 1993).

1. control - DMEM + 1% ATFCS only
2. IL-1β at 10ng/ml
3. IL-1β at 10ng/ml + 203580 at 10<sup>-7</sup>M
4. IL-1β at 10ng/ml + 203580 at 10<sup>-6</sup>M
5. IL-1β at 10ng/ml + 203580 at 10<sup>-5</sup>M.

After 72 hours, the production of collagenase protein by the cells under these various conditions was assessed by ELISA and corrected with respect to total cell protein as previously described. The release of LDH from the cells was also measured. Statistical analysis was by one way analysis of variance.

In all experiments, IL-1β at 10ng/ml caused a marked stimulation of collagenase protein production by the cells. Furthermore, in all experiments the additional presence of increasing concentrations of 203580 caused a dose-dependent downregulation of IL-1β-stimulated collagenase which was maximum in all cases with the highest dose of 203580. The maximum level of inhibition varied in different experiments between 52% and 87%. The results of a representative experiment are shown in figure 8.12. and the results of all experiments are shown in table 8.7. No cytotoxicity was seen in any experiments (data not shown).
Figure 8.12. (top)
Production of collagenase protein by the human skin cell line hsf 9 (passage 5) following treatment with IL-1β alone and in the additional presence of 203580.

Figure 8.13 (bottom)
The effect of 203580 on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF in the human skin cell line hsf 9 (passage 4).
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hsf 10</td>
<td>hsf 9</td>
<td>hsf 13</td>
<td>hsf 12</td>
<td>hsf 9</td>
</tr>
<tr>
<td>Cell Line</td>
<td>passage 3</td>
<td>passage 3</td>
<td>passage 3</td>
<td>passage 13</td>
<td>passage 5</td>
</tr>
<tr>
<td>No Additions</td>
<td>0.56 +/-</td>
<td>0.12 +/-</td>
<td>0.73 +/-</td>
<td>0.19 +/-</td>
<td>0.19 +/-</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.006</td>
<td>0.026</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-1β 10ng/ml</td>
<td>1.44 +/-</td>
<td>2.58 +/-</td>
<td>6.58 +/-</td>
<td>10.95 +/-</td>
<td>1.59 +/-</td>
</tr>
<tr>
<td></td>
<td>0.10 ***</td>
<td>0.19 ***</td>
<td>0.26 ***</td>
<td>0.40 ***</td>
<td>0.08 ***</td>
</tr>
<tr>
<td>IL-1β 10ng/ml + 203580 10^7 M</td>
<td>1.16 +/-</td>
<td>1.20 +/-</td>
<td>4.43 +/-</td>
<td>8.92 +/-</td>
<td>1.14 +/-</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.05 ***</td>
<td>0.13 ***</td>
<td>0.35 **</td>
<td>0.08 **</td>
</tr>
<tr>
<td>IL-1β 10ng/ml + 203580 10^6 M</td>
<td>1.13 +/-</td>
<td>1.37 +/-</td>
<td>3.38 +/-</td>
<td>7.06 +/-</td>
<td>0.65 +/-</td>
</tr>
<tr>
<td></td>
<td>0.03 *</td>
<td>0.13 ***</td>
<td>0.15 ***</td>
<td>0.40 ***</td>
<td>0.06 ***</td>
</tr>
<tr>
<td>IL-1β 10ng/ml + 203580 10^5 M</td>
<td>0.77 +/-</td>
<td>0.44 +/-</td>
<td>2.69 +/-</td>
<td>5.34 +/-</td>
<td>0.53 +/-</td>
</tr>
<tr>
<td></td>
<td>0.05 ***</td>
<td>0.02 ***</td>
<td>0.21 ***</td>
<td>0.24 ***</td>
<td>0.03 ***</td>
</tr>
</tbody>
</table>

Table 8.7. The production of collagenase protein by human skin fibroblasts following treatment with IL-1β in the absence and additional presence of the p38 MAP kinase inhibitor 203580. The results are expressed as collagenase in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of IL-1β are as follows: * p<0.05, ** p<0.01, *** p<0.001.
These experiments indicate that 203580 is biologically active in the experimental system used and that the maximum level of activity is seen at $10^{-4}$M. A further set of experiments were then set up to investigate the effect of $10^{-5}$M 203580 on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF. The following test conditions were used:

1. control - DMEM + 1% ATFCS only
2. recombinant bFGF at 100ng/ml
3. all-trans-retinoic acid at $10^{-5}$M
4. all-trans-retinoic acid at $10^{-5}$M + recombinant bFGF at 100ng/ml
5. 203580 at $10^{-5}$M
6. recombinant bFGF at 100ng/ml + 203580 at $10^{-5}$M
7. all-trans-retinoic acid at $10^{-5}$M + 203580 at $10^{-5}$M
8. all-trans-retinoic acid at $10^{-5}$M + recombinant bFGF at 100ng/ml + 203580 at $10^{-5}$M

In all experiments, $10^{-5}$M retinoic acid and 100ng/ml bFGF each caused a significant increase in TIMP-1 protein production by the cells and a synergistic increase in TIMP-1 when applied together. When $10^{-5}$M retinoic acid and 100ng/ml bFGF were applied to the cells in the additional presence of $10^{-5}$M 203580, a further potentiation of the stimulation of TIMP-1 protein occurred in all experiments. The magnitude of this effect varied in different experiments from being very modest in the case of hsf 12 (passage 13) to being much more potent in the experiments using hsf 9 (passage 4 and passage 8). It was also observed that $10^{-5}$M 203580 potentiated the effect of 100ng/ml bFGF alone on TIMP-1 production in all except 1 experiment. The experiment in which this did not occur was hsf 12 (passage 13). This is the cell line in which the weakest effect is seen on the potentiation of the synergistic induction of TIMP-1. This suggests that the induction of TIMP-1 by bFGF alone in the presence of 203580 is connected to the induction of TIMP-1 by retinoic acid and bFGF in the presence of the inhibitor. The results of a typical experiment are shown in figure 8.13.
results of all experiments are shown in table 8.8. The potency of 203580 in potentiating the synergistic induction of TIMP-1 by retinoic acid and bFGF in the different cell lines also seemed to correlate with its potency in inhibiting IL-1β-stimulated collagenase production in the same cell lines (compare table 8.7. with table 8.8). 10⁻⁶M 203580 did not cause cytotoxicity in any of the experiments performed (data not shown).
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>hsf 9 passage 8</td>
<td>hsf 12 passage 13</td>
<td>hsf 13 passage 3</td>
<td>hsf 9 passage 4</td>
<td>hsf 10 passage 8</td>
</tr>
<tr>
<td>Control</td>
<td>0.64 +/- 0.025</td>
<td>2.20 +/- 0.06</td>
<td>4.53 +/- 0.20</td>
<td>1.26 +/- 0.033</td>
<td>5.25 +/- 0.14</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M</td>
<td>7.15 +/- 0.33 ***</td>
<td>9.57 +/- 0.30 ***</td>
<td>12.85 +/- 0.26 ***</td>
<td>2.69 +/- 0.11 ***</td>
<td>20.65 +/- 0.39 ***</td>
</tr>
<tr>
<td>bFGF 100ng/ml</td>
<td>0.83 +/- 0.04 **</td>
<td>4.34 +/- 0.14 ***</td>
<td>7.57 +/- 0.55 ***</td>
<td>2.39 +/- 0.16 ***</td>
<td>10.98 +/- 0.58 ***</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + bFGF 100ng/ml</td>
<td>11.80 +/- 0.23 ***</td>
<td>23.13 +/- 0.59 ***</td>
<td>31.26 +/- 2.08 ***</td>
<td>22.14 +/- 0.45 ***</td>
<td>51.12 +/- 4.04 ***</td>
</tr>
<tr>
<td>203580 10^-5 M</td>
<td>1.31 +/- 0.056</td>
<td>2.10 +/- 0.26</td>
<td>7.20 +/- 0.56</td>
<td>1.39 +/- 0.044</td>
<td>5.16 +/- 0.14</td>
</tr>
<tr>
<td>Retinoic Acid 10^-5 M + 203580 10^-5 M</td>
<td>8.88 +/- 0.35</td>
<td>7.18 +/- 0.15</td>
<td>12.56 +/- 0.49</td>
<td>2.48 +/- 0.11</td>
<td>14.59 +/- 0.52</td>
</tr>
<tr>
<td>bFGF 100ng/ml + 203580 10^-5 M</td>
<td>1.69 +/- 0.10 §§§</td>
<td>4.43 +/- 0.20</td>
<td>11.97 +/- 0.43 §§§</td>
<td>4.40 +/- 0.09 §§§</td>
<td>16.90 +/- 0.58 §§§</td>
</tr>
<tr>
<td>Retinoic acid 10^-5 M + bFGF 100ng/ml + 203580 10^-5 M</td>
<td>21.96 +/- 1.04 ***</td>
<td>27.62 +/- 0.63 ***</td>
<td>45.19 +/- 0.92 ***</td>
<td>68.51 +/- 7.33 ***</td>
<td>77.09 +/- 3.55 ***</td>
</tr>
</tbody>
</table>

Table 8.8. The production of TIMP-1 protein by human skin fibroblasts following treatment with bFGF and retinoic acid in the absence and additional presence of the p38 MAP kinase inhibitor 203580. The results are expressed as TIMP-1 in ng per μg of cell protein. The mean values are shown for triplicate culture medium samples +/- standard error of the mean. Statistical comparisons with the control are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the additive effect of retinoic acid and bFGF are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of retinoic acid and bFGF without 203580 are as follows: * p<0.05, ** p<0.01, *** p<0.001. Statistical comparisons with the effect of bFGF without 203580 are as follows: § p<0.05, §§ p<0.01, §§§ p<0.001.
8.3. DISCUSSION

In this chapter, the possible signal transduction mechanisms involved in the synergistic induction of TIMP-1 protein by retinoic acid and bFGF are investigated. The effect of 2 different tyrosine kinase inhibitors, HERB A and genistein on this response was investigated first; genistein was found to be successful in inhibiting the response where as HERB A was not. The results obtained with genistein show that it inhibits TIMP-1 induction by retinoic acid and bFGF in a dose-dependent manner without causing cytotoxicity to the cells. This implies that tyrosine kinase activity is necessary for this signal transduction pathway to proceed. It is likely that genistein is inhibiting the bFGF receptor tyrosine kinases and therefore also blocking other events downstream of this. This implies that receptor tyrosine kinase autophosphorylation is the first step in the transduction of the bFGF signal to stimulate TIMP-1 production in the additional presence of retinoic acid. This may then be followed by the phosphorylation and activation of 1 or more cytoplasmic substrates such as phospholipase Cγ. However, the use of an alternative nuclear signalling pathway in which bFGF is translocated to the nucleus and elicits a signal there cannot be ruled out. Exogenously added bFGF is translocated to the nucleolus in bovine aortic endothelial cells (Bouche et al., 1987, Baldin et al., 1990) and this is correlated with increased transcription of ribosomal genes. In similar experiments, the FGF receptor flg becomes redistributed to a perinuclear location following stimulation by extracellular ligand (Mason, 1994). A number of proteins are phosphorylated on tyrosine residues early during the redistribution of flg including p80/p85 which is the murine version of chicken cortactin, an actin-associated protein (Zhan et al., 1993). Cortactin is a substrate for the Src tyrosine kinase and coprecipitation studies have identified associations between flg and Src and between Src and p80/p85 (Zhan et al., 1994). These data implicate the cytoskeleton in the translocation of the receptor from the cell surface to a perinuclear location and raise the possibility that this is also the mechanism by which exogenous ligand is delivered to the nucleus. The translocation of bFGF to the nucleus may therefore be a tyrosine kinase-dependent pathway which
could be inhibited by genistein. The operation of a nuclear signalling pathway in causing the synergistic induction of TIMP-1 protein by retinoic acid and bFGF therefore cannot be ruled out.

The reason why genistein was effective in inhibiting the synergistic induction of TIMP-1 protein by retinoic acid and bFGF, but HERB A was ineffective is not clear. The fact that HERB A inhibits tyrosine kinase activity by a different mechanism to genistein is probably relevant. The most likely explanation is that HERB A is ineffective at the concentration used in this study and that higher concentrations are required to give a substantial inhibition. This is supported by results from other workers using human keratinocytes and mouse osteoblastic cells in which a concentration of $10^{-6} \text{M} - 2 \times 10^{-6} \text{M}$ HERB A was used to achieve an effective inhibition of biological responses (Sudbeck et al., 1994, Hurley et al., 1995). The present study shows that such concentrations of HERB A are toxic to human skin fibroblasts.

Another possibility is that HERB A does not penetrate the cells and inhibit tyrosine kinase activity quickly enough to prevent transduction of the bFGF signal. This is a valid explanation since HERB A is added to the cells at the same time as the other reagents and receptor tyrosine kinase autophosphorylation is rapidly activated after growth factor treatment. This problem could have been avoided by pretreating the cells for 1 hour with HERB A before the addition of retinoic acid and bFGF.

The effect of HERB A on collagenase induction by bFGF was also investigated and some level of inhibition was seen in all experiments. These data agrees with results reported elsewhere; Hurley et al. (1995) have shown that transcriptional regulation of collagenase gene expression by bFGF is inhibited by HERB A. In this study, it appears that the induction of collagenase protein by bFGF alone is more sensitive to HERB A than the synergistic induction of TIMP-1 protein by bFGF in combination with retinoic acid. This differential sensitivity suggests that bFGF may regulate collagenase production via a different signalling pathway to the TIMP-1 induction pathway. The various intracellular substrates which associate with the activated FGF
receptor cytoplasmic domain are believed to interact with specific phosphorylated tyrosine residues on the receptor (Jaye et al., 1992). The ability of these different tyrosine residues to be phosphorylated by the receptor tyrosine kinase may show differential sensitivity to HERB A. In addition, different signalling pathways downstream of the receptor tyrosine kinase are likely to vary in the extent to which they utilize tyrosine phosphorylation, again leading to a differential sensitivity to HERB A.

Having demonstrated that the synergistic induction of TIMP-1 by retinoic acid and bFGF is tyrosine kinase-dependent, the next step was to investigate possible events occurring downstream of the receptor tyrosine kinase. The protein kinase C inhibitor BIS was found to have no effect at all on the synergistic induction of TIMP-1. However, it abolished or almost completely abolished the stimulation of collagenase or TIMP-1 by PMA, an agent which is known to activate and operate through protein kinase C (Nishizuka, 1984). These results therefore demonstrate that protein kinase C is not involved in the synergistic induction of TIMP-1 by retinoic acid and bFGF.

The data using PMA contrast with a previous report showing that collagenase stimulation in bovine chondrocytes by PMA is only minimally affected by the protein kinase C inhibitor staurosporine (Conquer et al., 1992). This discrepancy probably reflects the different cell types involved.

FGF receptors are believed to bind to phospholipase Cγ via tyrosine 766 in the C-terminal tail of the receptor leading to activation of this enzyme (Mohammadi et al., 1991, Peters et al., 1992). Interestingly, a mutant receptor in which tyrosine 766 was replaced by phenylalanine was still able to mediate a full FGF-stimulated mitogenic response although it could not direct FGF-stimulated phosphorylation of phospholipase Cγ, increased phosphoinositide turnover or calcium mobilization (Peters et al., 1992). These findings show that although phospholipase Cγ is stimulated in response to FGF, it is not necessarily required for FGF-induced cellular events. These findings correlate with the data reported in this study in which the
phospholipase Cγ-mediated pathway, or at least the protein kinase C part of it is not required for the synergistic induction of TIMP-1.

This raises the question of which signalling molecules are involved in the induction of TIMP-1 by retinoic acid and bFGF. The Ras pathway culminating in MAP kinase activation is one of the most broadly implicated avenues of cellular signalling from growth factor receptors and is a possible candidate (see figure 1.3., chapter 1). The involvement of Ras could have been investigated by using a commercially available inhibitor. The function of Ras proteins is dependent upon the attachment of a farnesyl group to a cysteine by the action of a farnesyltransferase. Inhibitors of these farnesyltransferases are available such as α-hydroxyfarnesylphosphonic acid and could have been used to test the involvement of Ras in the mechanism (Gibbs et al., 1993).

BIS caused a partial inhibition of the synergistic induction of TIMP-1 in response to PDGF-BB and retinoic acid. This suggests that the activation of protein kinase C contributes to this response. The data also imply that bFGF and PDGF-BB use different pathways rather than the same pathway to cause TIMP-1 induction in the presence of retinoic acid. In contrast to bFGF, the stimulation of phospholipase Cγ appears to play a role in PDGF-BB-induced mitogenesis (Peters et al., 1992). This correlates with the results reported here in which the protein kinase C part of the phospholipase Cγ pathway appears to be involved. However, the fact that BIS caused only a partial inhibition in most of the experiments indicates that other signalling pathways independent of protein kinase C are also contributing to the response. In addition, the proportion of the response which is sensitive to protein kinase C appears to vary in different experiments. In a similar manner, the induction of collagenase protein by PDGF-BB alone also appears to be partially dependent upon protein kinase C, with the level of protein kinase C involvement varying in different experiments. This variability suggests that individual cell lines express different levels of protein kinase C.
The effect of the MAP kinase inhibitor 203580 on the synergistic induction of TIMP-1 protein by retinoic acid and bFGF is intriguing. This effect could be caused by the relief of negative regulation of the TIMP-1 gene by 203580. Activation of stress-response MAP kinases is known to lead to increased transcription of certain genes (Han et al., 1994). It is possible that bFGF overlaps with the cytokine signalling pathway and can activate the p38 MAP kinase. This in turn could lead to negative rather than positive regulation of the TIMP-1 gene via transcription factors which are activated in response to the p38 MAP kinase. In the additional presence of 203580, the p38 MAP kinase is inhibited leading to relief of this repression and increased TIMP-1 gene expression. This theory implies that bFGF simultaneously exerts positive and negative effects on TIMP-1 gene expression in the cells. The net effect is usually positive since a stimulation of TIMP-1 protein is sometimes seen in response to bFGF alone and always in response to retinoic acid and bFGF in combination.

The data shows that 203580 potentiates the synergistic effect of retinoic acid and bFGF on TIMP-1 protein induction in all experiments and that it also potentiates the effect of bFGF alone on TIMP-1 production in 4 experiments out of 5. The experiment in which bFGF-stimulated TIMP-1 was not affected by 203580 is the same one in which a weak potentiation of the synergistic effect is seen, suggesting that these 2 events are connected. These results suggest that 203580 is interacting with the bFGF signalling pathway rather than with the retinoic acid signalling pathway and support the hypothesis presented above. This hypothesis is of course purely speculative and further investigation is required to elucidate the effect of this inhibitor on the response of cells to retinoic acid and bFGF.

The discovery of the pyridinyl-imidazole compounds was through their inhibition of lipopolysaccharide-stimulated human monocyte IL-1 and TNF-α protein production (Lee et al., 1990, Lee et al., 1993) and it is thought that the stimulation of these cytokines is regulated partly at the translational level. Bacterial endotoxin causes translational derepression which is mediated by an AUUUA region in the 3' untranslated region of TNF mRNA (Han et al., 1990). The pyridinyl-imidazole
compounds have been shown to inhibit IL-1β and TNF-α synthesis at the translational level (Lee et al., 1993, Young et al., 1993) and they may act by preventing this translational derepression. The effects of 203580 on TIMP-1 protein production may also be regulated at the level of translation rather than transcription. However the mechanism must be completely different as TIMP-1 mRNA does not contain AUUUA regions in its 3' untranslated region.

Another possibility is that bFGF induces the production of IL-1 by the cells and that IL-1 acts to suppress TIMP-1 production. The effect of 203580 would then be to prevent this induction of IL-1 and/or to block the autocrine effect of IL-1 on the cells by MAP kinase inhibition. This in turn could lead to a potentiation of TIMP-1 production. Some support for this theory is provided in chapter 5, in which it is shown that the additional presence of IL-1β causes a reduction in the synergistic induction of TIMP-1 by retinoic acid and bFGF. Hence the inhibition of IL-1 action by 203580 may lead to a potentiation of TIMP-1 production when retinoic acid and bFGF are additionally present.

This chapter also shows that 203580 also causes a dose-dependent inhibition of IL-1β-stimulated collagenase protein in human skin fibroblasts. In chapter 1 of this thesis, arguments for the role of both IL-1 and collagenase in the pathology of rheumatoid and osteoarthritis were put forward. Collagenase mRNA or protein is present in the synovium (Firestein et al., 1991), in synovial fluid (Clark et al., 1993) and at the cartilage-synovium junction (Woolley et al., 1977); it is also produced by cartilage (Martel-Pelletier et al., 1994). Furthermore, levels of collagenase expression correlate with the severity of disease (Firestein et al., 1991, Martel-Pelletier et al., 1994, Ehrlich et al., 1978, Pelletier et al., 1983, Martel-Pelletier and Pelletier 1987) and are greater in rheumatoid arthritis than in osteoarthritis (Firestein et al., 1991, Clark et al., 1993). In addition, some therapeutic drugs for arthritis reduce the expression of collagenase mRNA in rheumatoid arthritis synovium (Firestein et al., 1991, Firestein et al., 1994). Cartilage breakdown can be stimulated by IL-1 which induces cartilage collagenase production (Martel-Pelletier et al., 1994, Gowen et al., 1984, Ellis et al., 1994) and
this cytokine has been isolated from human joint effusions (Wood et al., 1983) and is produced by human synovial tissue in *vitro* (Wood et al., 1985). IL-1 also stimulates the production of collagenase from human synovial fibroblasts and human articular chondrocytes (Dayer et al., 1986, Stephenson et al., 1987).

Taken together, such evidence suggests that destruction of joint cartilage collagen is caused by collagenase, the synthesis of which may be driven at least in part by IL-1. In the light of these arguments, the inhibition of IL-1-stimulated collagenase by 203580 may be of therapeutic use in the treatment of arthritis. 203580 and its related compounds appear to show very narrow substrate specificity in their actions upon cells (Lee et al., 1994) which makes them good candidates for drug development since beneficial effects can be achieved without undesirable side-effects. It has already been shown that these compounds have anti-arthritis activity in the mouse collagen-induced arthritis model (Lee et al., 1993).

The inhibition of IL-1β-stimulated collagenase by 203580 also provides new insights into the mechanism by which this cytokine stimulates collagenase production in fibroblasts since it indicates the involvement of the p38 MAP kinase. The fact that complete inhibition of IL-1β-stimulated collagenase is not achieved in any of the experiments may suggest the operation of other pathways which do not require the p38 MAP kinase. Alternatively, 203580 may be less stable under the experimental conditions used than IL-1β, leading to some stimulation of collagenase by the latter agent after 203580 has ceased to be effective. It is also possible that the concentration of 203580 used does not give complete inhibition of p38 MAP kinase. In addition, pretreatment with the inhibitor may be required to prevent activation of the MAP kinase since the effect of IL-1 on the cells may be more rapid than the effect of 203580.
CHAPTER NINE

FINAL DISCUSSION

This study has investigated TIMP-1 gene expression in response to retinoic acid in combination with polypeptide growth factors. The findings of the thesis demonstrate that TIMP-1 production can be potently induced by retinoic acid in combination with bFGF, EGF, PDGF-BB or TGF-β as a result of synergistic interactions between retinoic acid and the growth factors. Two main aspects of this study are addressed in this final chapter - firstly, the elucidation of the synergistic mechanisms at the molecular level and secondly, the relevance of the findings (if any) to connective turnover in vivo, particularly within the clinical setting of pathological tissue destruction.

Chapters 6-8 of the thesis have started to address the possible mechanisms by which TIMP-1 is synergistically induced with particular emphasis on the effects of retinoic acid and bFGF. In the case of all 4 growth factors, the responses are at least partially secondary rather than primary effects on the cells since a minimum of 72 hours of stimulation is required to give the optimal induction of TIMP-1 protein.

In the case of bFGF, the induction of a transient, primary effect in response to growth factor alone has been demonstrated. This effect is most likely to be the induction of a new protein, a hypothesis which is supported by the finding that new protein synthesis is required for induction of TIMP-1 mRNA by retinoic acid and bFGF. A key question arising from the data is the identity of this protein. Although further experiments are required to resolve this question, it is feasible at this point to speculate on possible candidates. The induction of retinoic acid receptors by bFGF may be the mechanism by which TIMP-1 gene expression is stimulated by these factors. Previous reports show that constitutive levels of RAR mRNA are low in fibroblasts, and are readily inducible by stimulatory agents such as their own ligands (Pan and Brinckerhoff, 1994, Brinckerhoff et al., 1995). Induction of RAR mRNAs
was observed within 3 hours of treatment and peaked at 6-12 hours of treatment. These kinetics are similar to those of the transient event induced by bFGF in human skin fibroblasts. This suggests that RAR mRNAs can be upregulated by stimulatory factors in a manner which is consistent with possible participation in the synergistic induction of TIMP-1.

There is evidence to suggest that the expression of retinoic acid receptors in cells is the limiting factor in controlling the response to retinoic acid. Experiments investigating the regulation of collagenase gene expression by retinoic acid have shown that cotransfection of an RAR expression vector along with a collagenase promoter-chloramphenicol acetyltransferase (CAT) reporter construct is necessary to obtain retinoic acid-mediated repression (Pan et al., 1992). A similar situation has been reported in the case of the laminin B1 gene and the phosphoenolpyruvate carboxykinase gene, where cotransfection of RAR expression vectors and promoter-CAT constructs give an enhanced response to retinoic acid (Vasios et al., 1989, Lucas et al., 1991). Such data suggests that the induction of RARs is a potential means by which retinoid-responsive genes can be more potently regulated by retinoic acid.

The induction of 1 or more components of the retinoic acid signalling pathway by bFGF is also consistent with the lack of effect of retinoic acid when it is applied first. It is postulated that this is due to the decline of free retinoic acid levels in the cells during the time that bFGF takes to induce its effects. If bFGF is modulating a protein or proteins which interact(s) with retinoic acid such as RARs, RXRs or CRABPs, then this lack of free retinoic acid would result in the loss of the response. However, the data also fit a model in which synergistic interactions occur between RARs/RXRs and bFGF-induced transcription factors at the level of TIMP-1 gene expression. Such interactions could occur at the TIMP-1 AP-1 site(s) as a result of the binding of RARs and RXRs to proteins such as c-fos and c-jun. Such a theory requires the growth factor-induced proteins to bind first and may account for the lack of effect when retinoic acid is applied first. The induction of c-fos by bFGF probably does not contribute to the synergistic induction of TIMP-1 in the presence of retinoic
acid because this effect is too rapid and transient to correlate with the kinetics of the effect induced by bFGF. However, this does not preclude the involvement of c-jun and of additional proteins such as c-myc which could possibly bind to a non-consensus AP-1 site. Previous work shows that proteins which do not bind a consensus AP-1 site are able to interact with the TIMP-1 non-consensus motif (Edwards et al., 1992). Clearly, further investigations are required to resolve these questions and it would be particularly useful to identify and characterize the transient effect of bFGF on the cells. To this end, further experiments are being carried out in the Rheumatology Research Unit to determine the effect of bFGF on mRNA levels for RARs, RXRs, c-fos, c-jun and c-myc in human skin fibroblasts at various time points after stimulation with this factor using Northern blotting techniques.

Interestingly, chapter 6 revealed differences rather than similarities in the synergistic actions of the different growth factors combined with retinoic acid on TIMP-1 production. It was found that prolonged treatment (72 hours) with TGF-β followed by retinoic acid treatment was able to produce a synergistic response; however, in the case of retinoic acid and EGF, it was obligatory for both agents to be present together. It would be useful to characterize further these differences and to obtain more data on the action mechanisms of EGF, PDGF-BB and TGF-β in combination with retinoic acid. TGF-β, like bFGF, appears to induce an intracellular event which is responsible for the synergistic induction of TIMP-1 protein in the additional presence of retinoic acid. However, this is either a much slower or a more prolonged effect than that seen with bFGF. The kinetics of this response could be characterized by further experiments in which the incubation time with TGF-β is reduced. The sequential data suggests that TGF-β in combination with retinoic acid has the most long-term effect on TIMP-1 induction of the 4 growth factors. This is further supported by the kinetics of TIMP-1 mRNA accumulation in response to retinoic acid and TGF-β in which a maximal effect is not seen until 72 hours of stimulation.

The data shown in chapter 7 demonstrate that the synergistic effects on TIMP-1 gene expression occur at a pretranslational level but do not differentiate between
transcriptional and posttranscriptional events. In the light of other investigations, it seems more likely that these effects are of a transcriptional nature. All previous reports except for 1 have demonstrated the importance of transcriptional effects rather than changes in mRNA stability (Edwards et al., 1987, Overall and Sodek, 1990, Overall et al., 1991, Shapiro et al., 1993, Overall, 1994, 1995). In the case of retinoic acid and bFGF, the time course data showed an initial large increase in TIMP-1 mRNA by 12 hours followed by a more modest increase from 12 to 72 hours. It can be postulated that an initial burst of transcription gives the marked effect seen at 12 hours followed by a slower increase from 12 to 72 hours due to increased mRNA stability. Experiments are currently being carried out in the Rheumatology Research Unit to investigate the roles of transcription and mRNA stability in TIMP-1 induction by retinoic acid and bFGF using the transcriptional inhibitor actinomycin D. We are also examining the effect of these agents on transiently transfected TIMP-1 promoter-CAT reporter constructs in cells. The purpose of these experiments is to demonstrate whether or not the effect is transcriptional and additionally to identify sequences of the TIMP-1 promoter which are involved in mediating transcriptional changes. The role of transcription will also be investigated using nuclear run-off transcription assays and reverse transcriptase-polymerase chain reaction which measures levels of heterogeneous nuclear RNA.

The findings of chapter 8 address the role of the bFGF signalling pathway in mediating the synergistic induction of TIMP-1 by bFGF and retinoic acid. The effect appears to be tyrosine kinase-dependent suggesting the involvement of the receptor tyrosine kinase followed by the activation of downstream cytoplasmic substrates. However the involvement of a nuclear signalling pathway cannot be excluded. It would be useful to identify the intracellular substrates involved in the transduction of the growth factor signal from the cytoplasmic membrane to the nucleus for each of the 4 growth factors. The data shown in the thesis indicate that protein kinase C is partially involved in the synergistic induction of TIMP-1 protein in response to retinoic acid and PDGF-BB but is not involved at all in the effect of retinoic acid and
bFGF. It would be useful to investigate the role of Ras and MAP kinase using appropriate inhibitors as this pathway is believed to be important in the action of growth factors.

The effect of the p38 MAP kinase inhibitor on the induction of TIMP-1 by retinoic acid and bFGF is intriguing. The data imply that bFGF regulates TIMP-1 production in a negative manner through complex cross-talk with the p38 MAP kinase stress-response signalling pathway. The idea that factors can simultaneously exert both positive and negative effects on TIMP-1 gene expression is a novel one and worthy of further study. The existence of cross-talk between mitogen-stimulated pathways and stress-response or cytokine signalling pathways is also a poorly understood area which requires further investigation. There are data to indicate that IL-1 is able to activate the p42/44 mitogen-stimulated MAP kinases, thus demonstrating the existence of such cross-talk (Dunford et al., 1995). Another report has also demonstrated cross-talk between the different pathways; this study showed that activation of the stress-activated protein kinase (SAPK) pathway resulted in increased gene expression of mitogen-activated protein kinase phosphatase 1, which in turn led to the inactivation of mitogen-activated MAP kinases (Bokemeyer et al., 1996).

The pyridinyl-imidazole compounds have been shown to exert their effects at the translational level (Lee et al., 1993, Young et al., 1993) and it would be useful to establish whether or not this is the case for TIMP-1. This could be done by Northern blotting which would show whether the potentiation of TIMP-1 protein induction caused by the inhibitor is paralleled by an increase in TIMP-1 mRNA levels. The data could also be further substantiated by showing that the effect of 203580 is dose-dependent; the current data shows only the effect of a single concentration. It was also suggested in chapter 8 that the data could be explained by the induction of IL-1 by bFGF and further experiments using neutralizing antibodies to IL-1 would be helpful in testing this hypothesis.

Although many questions remain unanswered, the findings of this thesis provide important new insights into the regulation of TIMP-1 gene expression in fibroblasts.
Such information is important as a prerequisite to the use of TIMP-1 induction as a therapeutic strategy in the treatment of pathological connective tissue breakdown. In contrast to most previous investigations which have examined the effect of single growth factors or cytokines, this project has addressed possible interactions between different stimulatory agents. Since cells in vivo are simultaneously exposed to many different modulatory agents, it is important to understand such interactions. In addition, this study has utilized primary cultures of fibroblasts; these cells are believed to mediate connective tissue turnover under normal physiological conditions and it is therefore hoped that the effects seen in vitro are directly relevant to the situation in vivo.

This leads on to the question of whether the findings of this thesis have any application to clinical situations in which pathological connective tissue breakdown results in disease. Previous investigations have examined the effect of retinoids (N-(4-hydroxyphenyl)-retinamide or 13-cis-retinoic acid) on 3 different animal models of experimentally-induced arthritis. It was found that adjuvant arthritis and streptococcal cell wall-induced arthritis were both suppressed by retinoid treatment (Brinckerhoff et al., 1983, Brinckerhoff et al., 1985); collagen-induced arthritis however was exacerbated (Trentham and Brinckerhoff, 1982). It was also shown that collagenase production from synovial cells of retinoid-treated animals was decreased compared to untreated animals in all the models investigated. This suggests that retinoids may suppress disease activity by modulating collagenase activity - this may be through the inhibition of collagenase production or the inhibition of active collagenase by the induction of TIMP-1. Since all of these investigations used rodents, the collagenase activity measured would be attributable to MMP-13 rather than to MMP-1. Retinoids (etretinate) are also an effective treatment for psoriatic arthritis (Klinkhoff et al., 1989) and etretinate has additionally been used successfully in the treatment of psoriasis (Ehmann et al., 1982).

However, in direct contrast to these encouraging results are numerous experiments demonstrating that retinoids induce proteoglycan and in some cases collagen release.
from cartilage *in vitro* (Fell and Mellanby, 1952, Dingle et al., 1966, Dingle et al., 1975, Dingle and Dingle, 1980, Jubb and Fell, 1980, Hembry et al., 1982, Caputo et al., 1987, Campbell and Handley, 1987, Buttle et al., 1993). Retinoids also reduce chondrocyte glycosaminoglycan synthesis and cause dedifferentiation of chondrocytes into fibroblastic cells which no longer synthesize type II collagen (Shapiro and Poon, 1976, Benya and Padilla, 1986). Furthermore, retinoids have also been used in animal models of osteoarthritis (e.g. Lapadula et al., 1995) in which an intraarticular injection of retinol palmitate results in gross pathological changes to the chondrocytes, cartilage matrix and synovium. It is noteworthy that the vitamin A model of osteoarthritis utilizes intraarticular injection of retinoids whereas the beneficial effects in experimentally-induced arthritis occurred in animals which were fed retinoids. This suggests that retinoids may have different effects in the joint depending upon whether they are systemically or locally applied. This is analogous to the situation with TGF-β in which locally administered TGF-β exacerbates an ongoing inflammatory lesion as demonstrated in an experimental model of synovial inflammation and tissue destruction (Wahl et al., 1991). However, systemic administration of TGF-β is beneficial in prevention of disease in animal models of rheumatoid arthritis (Kuruvilla et al., 1991).

Another factor which cannot be ignored is the significant incidence of side-effects resulting from the therapeutic application of retinoids; these include hair loss, mucocutaneous effects, bone lesions, elevated serum levels of cholesterol and triglycerides, hepatotoxicity, teratogenicity, musculoskeletal effects and occasionally the onset of acute aseptic arthritis (Kaplan and Haettich, 1991, Klinkhoff et al., 1989, Matsuoka et al., 1984).

Although the detrimental effects of retinoids make any possible therapeutic use in arthritis complex and maybe impossible, another option which should be considered is the development of synthetic retinoid compounds in which beneficial effects can be enhanced and undesirable side-effects diminished. Particularly encouraging are recent reports identifying receptor-selective retinoids which selectively inhibit AP-1
activity but do not activate gene transcription (Fanjul et al., 1994). Another recent report has shown that certain synthetic retinoids can inhibit transcription from the stromelysin AP-1 motif via RAR-α without also inducing gene transcription through this receptor (Nagpal et al., 1995). This raises the possibility of designing retinoids which selectively suppress collagenase production from connective tissue cells. A novel synthetic retinoid which lacks RAR-γ binding activity has recently been shown to markedly inhibit rat type II collagen-induced arthritis (Kuwabara et al., 1996). The data suggests that this was achieved through reducing serum levels of antibodies to type II collagen, but other mechanisms including the inhibition of collagenase production may also be involved.

The effect of retinoic acid on fibroblast collagenase and TIMP-1 production in vitro which would be expected to prevent connective tissue breakdown contrasts sharply with the in vitro effect of retinoids on cartilage. This discrepancy is difficult to explain but may be due to the different cell types involved (chondrocytes instead of fibroblasts) or to the fact that retinoids are potent stimulators of plasminogen activator production which may play a role in cartilage resorption via plasmin production (Meats et al., 1985). It is also possible that the breakdown of cartilage collagen by retinoids is mediated by the induction of MMP-13 (collagenase-3); this enzyme is induced by retinoids in rodent chondrocytes and bone cells (Ballock et al., 1994, Heath et al., 1990, Connolly et al., 1994). Retinoic acid may also stimulate the production of 'aggrecanase', the putative enzyme believed to be responsible for the breakdown of cartilage proteoglycan. Clearly, the effect of retinoids on connective tissue cells is complex and further work is necessary to elucidate the full picture.

The interactions which occur between retinoids and polypeptide growth factors as demonstrated in this project may be relevant to the effect of retinoids in vivo since there is evidence to show that all 4 of the growth factors examined are present in the arthritic joint. bFGF has been detected by immunohistochemistry in rheumatoid arthritis synovial tissue associated with monocytes and macrophages and with areas of new blood vessel formation (Qu et al., 1990). EGF is probably present in synovial
fluid due to its widespread occurrence in body fluids (Carpenter and Zendegui, 1986). Other work shows that mRNA transcripts for PDGF-A and PDGF-B and immunoreactive polypeptides are present in rheumatoid arthritis synovial tissue (reviewed by Remmers et al., 1991). TGF-β is also found in synovial fluid from patients with rheumatoid arthritis and osteoarthritis; it is produced by rheumatoid synovial tissue in vitro and TGF-β mRNA is expressed by synovial fibroblasts and macrophages in vivo (Wahl et al., 1990, Fava et al., 1989, Brennan et al., 1990, Lafyatis et al., 1989). However, it must be taken into account that the dose of retinoic acid used in this project is far greater than serum retinoic acid levels under normal physiological conditions (5-10nM). It is also considerably greater than that observed during retinoic acid therapy in which a peak value of approximately 75-100nM occurs after 3-4 hours of ingestion with a corresponding level in synovial fluid of approximately 45nM (Goodman, 1984, Wolf, 1984, Goodman and Blaner, 1984, Muindi et al., 1994, Matsuoka et al., 1984).

In conclusion, therefore, it is clear that both the effect of retinoids and the regulation of TIMP-1 gene expression are important areas of investigation in furthering our understanding of connective tissue biology. The findings of this project have provided some new insights into these areas, and it is hoped that future work at the Rheumatology Research Unit and in other laboratories will lead to a more complete understanding of the full picture.
REFERENCES


Baldin, V., Roman, A.-M., Bosc-Bierne, I., Amalric, F., and Bouche, G. (1990) Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. EMBO J. 9, 1511-1517.


collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res. 50, 6184-6191.


Carpenter, G., and Cohen, S. (1976) $^{125}$I-labeled human epidermal growth factor -
binding, internalization, and degradation in human fibroblasts. J. Cell Biol. 71, 159-
171.


Carpenter, G., and Zendegui, J.G. (1986) Epidermal growth factor, its receptor and


Carpenter, G., and Wahl, M.I. (1990) The epidermal growth factor family of
mitogens. In Peptide Growth Factors and their Receptors, In Handbook of
Experimental Pharmacology, A.B. Sporn, M.B. Roberts, eds., Springer-Verlag,
Heidelberg, Vol. 95, pp. 69-171.

derived growth factor stimulates Na$^+$/H$^+$ exchange and induces cytoplasmic

Castaigne, S., Chomienne, C., Daniel, M.T., Ballerini, P., Berger, R., Fenaux, P., and
Degos, L. (1990) All-trans retinoic acid as a differentiation therapy for acute


Cawston, T.E., Murphy, G., Mercer, E., Galloway, W.A., Hazleman, B.L., and


Dawson, M.I., Chan, R.L., Derdzinski, K., Hobbs, P.D., Chao, W., and Schiff, L.J. (1983) Synthesis and pharmacological activity of 6-[E]-2-(2,6,6-trimethyl-1-cyclohexen-1-yl)ethen-1-yl]- and 6-(1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-6-naphthyl)-2-naphthalene-carboxylic acids. J. Med. Chem. 26, 1653-1656.


Li, J., Brick, P., O'Hare, M.C., Skarzynski, T., Lloyd, L.F., Curry, V.A., Clark, I.M.,
length porcine synovial collagenase reveals a C-terminal domain containing a
calcium-linked four-bladed β-propellor. Structure 3, 541-549.

Li, L., Hu, J-S., and Olson, E.N. (1990) Different members of the \textit{jun} proto-oncogene
family exhibit distinct patterns of expression in response to type β transforming
growth factor. J. Biol. Chem. 265, 1556-1562.

Liboi, E., Di Francesco, P.D., Gallinari, P., Testa, U., Rossi, G.B., and Peschle, C.
(1988) TGFβ induces a sustained c-fos expression associated with stimulation or
inhibition of cell growth in EL2 or NIH 3T3 fibroblasts. Biochem. Biophys. Res.
Commun. 151, 298-305.

Expression cloning of the TGF-β type II receptor, a functional transmembrane


López-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S., and Massagué, J.
(1991) Structure and expression of the membrane proteoglycan betaglycan, a
component of the TGF-β receptor system. Cell 67, 785-795.

to the TGFβ signaling receptor. Cell 73, 1435-1444.

can act as a dual modulator of TGF-β access to signaling receptors: mapping of ligand

inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). J.


carboxy-terminal peptide of the fibroblast growth factor receptor (flg) is a binding site for the SH2 domain of phospholipase C-γ1. Mol. Cell. Biol. 11, 5068-5078.


Olson, J.A. The biological role of vitamin A in maintaining epithelial tissues. (1972) Israel J. Med. Sci. 8, 1170-1178.


72kDa gelatinase and pump-I is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. J. Biol. Chem. 265, 21141-21151.


with low molecular weight inhibitors in culture medium of embryonic chick skin
explants. J. Biochem. 81, 261-263.

Relationship to the state of differentiation of cultured human keratinocytes. Biochem
J. 268, 371-378.

measure the effect of transforming growth factor β and epidermal growth factor on
rabbit articular chondrocytes. Cancer Res. 45, 4416-4421.

factors adherent to cell substrate are mitogenically active in situ. Nature 296, 154-156.

Smith, R.J., Justen, J.M., Sam, L.M., Rohloff, N.A., Ruppel, P.L., Brunden, M.N., and
En Chin, J. (1991) Platelet-derived growth factor potentiates cellular responses of
articular chondrocytes to interleukin-1. Arthritis Rheum. 34, 697-706.

neutral proteinase and prostanoid production in bovine nasal chondrocytes by
interleukin-1 and tumor necrosis factor α: modulation of these cellular responses by
interleukin-6 and platelet-derived growth factor. Clin. Immunol. Immunopathol. 64,
135-144.

tissues. In The Biological Mechanisms of Tooth Eruption and Root Resorption, Z.

fibroblast growth factor: protection of the angiogenic protein from proteolytic

Spizz, G., Hu, J.-S., and Olson, E.N. (1987) Inhibition of myogenic differentiation by
fibroblast growth factor or type β transforming growth factor does not require


derivatives as measured by induction of differentiation of murine F9 teratocarcinoma cells and human HL-60 promyelocytic leukemia cells. Cancer Res. 43, 5268-5272.


that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67, 1251-1266.


ADDENDUM

1) The use of the term 'cell line' throughout the thesis refers to a primary (rather than an established) culture of cells established from skin, synovium or tendon tissue for human skin, synovial and tendon fibroblasts respectively. Each cell line was established from a separate tissue specimen and therefore from a separate individual. 2) Performance of the free TIMP-1 immunoassay

The set-up and use of this assay is described in detail in previous publications from the Rheumatology Research Unit (Clark et al., 1991, Cawston et al., 1995). The detection limit is 5ng/ml and it is linear across the range 5 - 50ng/ml of TIMP-1. The intraassay and interassay variability are reported as 13.8% and 15.4% respectively (Cawston et al., 1995).

Performance of the total TIMP-1 immunoassay

This assay was performed essentially as previously described (Plumpton et al., 1995). The detection limit of the assay is 1.4ng/ml and intra and interassay coefficients of variation are between 8.8 - 9.7% and 10.4 - 13.7% respectively. The assay is linear across the range 5 - 50ng/ml of TIMP-1.

Performance of the collagenase immunoassay  This assay was again performed essentially as previously described (Clark et al., 1992, Cawston et al., 1995). The detection limit of the assay is 2ng/ml and it is linear from 2 - 100ng/ml of collagenase. The intra and interassay variabilities are 12.4% and 18.2% respectively.