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How to cite:

Harle, Jamie; Kim, Hae-Won; Mordan, Nicky; Knowles, Jonathan C. and Salih, Vehid (2006). Initial responses of human osteoblasts to sol-gel modified titanium with hydroxyapatite and titania composition. *Acta Biomaterialia*, 2(5) pp. 547-556.

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Version: Accepted Manuscript

Link(s) to article on publisher's website:

<http://dx.doi.org/doi:10.1016/j.actbio.2006.05.005>

http://www.elsevier.com/wps/find/journaldescription.cws_home/702994/description#description

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Initial responses of human osteoblasts to sol-gel modified titanium with hydroxyapatite and titania composition

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Keywords : Titania; hydroxyapatite ; Sol-gel coating; Titanium substrate ;

Osteoblast ; Cell response ; Gene expression

Abstract

Sol-gel thin films of hydroxyapatite (HA) and titania (TiO₂) have gained great attention in the areas of bioactive surface modification of titanium (Ti) implants. Herein the authors developed sol-gel coatings on Ti substrates with various compositions, including HA, TiO₂, and their composites (HA+10% TiO₂ and HA+20% TiO₂) under controlled processing conditions, and evaluated the biological properties of the coatings. All the coating layers exhibited thin and homogeneous structures and phase-pure compositions (either HA or TiO₂). Primary human osteoblast cells showed good attachment, spreading and proliferation on all the sol-gel coated surfaces, particularly with enhanced cell proliferation on the coated surfaces relative to uncoated Ti control, as observed by MTT assay and electron micrographs. Cell attachment rates were also enhanced on pure HA and HA+10% TiO₂ coatings relative to control Ti. This later composite coating furthermore supported the greatest cell proliferation of osteoblasts at 1 and 4 days. Moreover, the gene expression level of several osteogenic markers including bone sialoprotein and osteopontin, as measured by RT-PCR at 24 h, was shown to vary according to coating composition. These findings suggest that human bone cells show marked and rapid functional changes in response to HA and TiO₂ sol-gel coatings on Ti. Gene mechanisms activated in the first 24 h on the Ti surface by the sol-gel HA and TiO₂ coatings may play a role in mediating the cell function at prolonged periods as previously reported in literature.

1. Introduction

The technique of sol-gel processing can be applied in a uniform and well controlled surface layer onto bulk titanium (Ti) implants. This approach aims to enhance the surface characteristics of the Ti implant, and improve host tissue bonding strength and corrosive resistance, while still maintaining the overall favourable mechanical properties of the bulk Ti substrate, such as high strength and fracture toughness [1]. Hydroxyapatite (HA; $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and titania (TiO_2) represent two promising coating compositions on Ti surface that have been clinically applied.

HA has been widely investigated as a coating material due to its biological and chemical similarity to the inorganic phases of bones and teeth [2], with HA coatings on Ti resulting in enhanced bone formation and apposition [3], and improved fixation to adjacent bone in comparison to uncoated Ti [4]. However, unsatisfactory bonding strength of the HA layer with respect to Ti substrate, particularly in the case of plasma-sprayed thick coatings, has led to biological concerns, such as HA particulate debris and inflammation reaction [5,6].

The parallel development of TiO_2 coatings on such implants has seen promise. A variety of methods, such as anodization, sol-gel process and thermal oxidation, have been applied and have shown improved corrosion resistance of Ti and enhanced biological properties [7,8]. Moreover, TiO_2 coatings have been reported to improve the biocompatibility of Ti by allowing the formation of an O-H bond in TiO_2 under moist conditions [9].

The sol-gel technique produces a surface coating of high chemical homogeneity and purity, with the potential to mix the molecular percentages of HA and TiO_2 within the coating in order to optimise the Ti implant surface in terms of mechanical and

biological performance. Sol-gel methods additionally have a practical advantage of being applicable at mass-production level and of being a simple, relatively low temperature means of surface modification for complex implant shapes [10]. Moreover, materials prepared by the sol-gel process have shown to be more bioactive than those of the same composition prepared by other methods, with improved calcium and phosphate precipitation onto the implant surface [11,12].

In previous work, this group has manufactured, characterized and mechanically tested both pure and composite HA and TiO₂ sol-gel coatings on Ti [13]. The HOS osteoblast-like cell line was then used to assess biological response to various coating compositions in terms of proliferation over 5 days and alkaline phosphatase (ALP) production at 7 days. While no significant change was found in proliferation, marked changes in ALP activity were found with such HA/TiO₂ coated surfaces [13]. Ramires et al. (2001) [14] also found altered expression of other differentiation markers at 7 days using the MG63 osteoblast-like cell line, and confirmed these findings with rat primary osteoblasts [15], although different HA/TiO₂ compositions and manufacture methods were utilized. An absence of cytotoxic effects for HA/TiO₂ sol-gel coatings, assessed by viability assays in material extracts at 3 days, was also reported [15]. Furthermore, *in vivo* data from an HA/TiO₂ sol-gel composite coated implant in rabbits produced greater torque removal values and bone-to-implant contact than an uncoated Ti equivalent [16].

However, despite such promise for the HA/TiO₂ coatings, and in particular those developed by a sol-gel approach, knowledge of the early biological response of bone to such surface treatments is very limited. Sol-gel derived thin HA films manufactured on Ti showed reduced attachment characteristics for HOS cells [17] while TiO₂ implant coatings, applied by plasma spray methods, altered attachment

characteristics and early morphology of MG63 cells [18]. Variation in the attachment of bone cells to different HA/TiO₂ sol-gel coatings could be an important consideration in choosing optimal surface characteristics for future *in vivo* studies. Thus this study aimed to assess, for the first time, the response of primary human bone cells to Ti substrates coated with pure and composite HA/TiO₂ sol-gel coatings. Attention to early cellular responses, including the expression of several bone-associated genes known to play key roles in the growth and function of bone, was examined.

2. Materials and methods

2.1. Preparation of HA, TiO₂ and their composite coatings

The preparation of the sol-gel coatings was slightly modified from our previous works [13,19]. As sol-gel precursors for HA, calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; Sigma-Aldrich, Dorset, UK) and triethyl phosphite ($\text{P}(\text{OC}_2\text{H}_5)_3$, Sigma-Aldrich) of 3M were used. Both were hydrolyzed separately in ethanol and distilled water (DW) for 24 h, and then mixed at a Ca/P ratio of 1.67 and stirred for 2 h. Ammonium hydroxide (NH_4OH ; BDH, Poole, UK) was added at 5 % to the mixture, in order to improve the gelation and polymerization process [19]. The sol was aged for 7 days to produce a clear HA sol. To produce a TiO₂ sol, titanium propoxide ($\text{Ti}(\text{OCH}_2\text{CH}_2\text{CH}_3)_4$, Sigma-Aldrich) of 1.5M was hydrolysed within an ethanol-water solution, containing diethanolamine ($[(\text{HOCH}_2\text{CH}_2)_2\text{NH}]$, Sigma-Aldrich), and stirred for 5 days. The ratios of the diethanolamine/Ti and water/Ti were determined 1.00 and 2.00, respectively. To obtain HA+TiO₂ composite sols, the HA and TiO₂ pure sols were mixed together at ratios of 10 and 20 mol% with continuous stirring for 24 h.

Commercially pure Ti (cp Ti, grade II) was used as a substrate for the coating after polishing with SiC paper (# 2000 grit) and cleaning in acetone / ethanol. The Ti substrate was dipped into the prepared sols and spin-coated at 3000 rpm for 20 s. After drying, samples were heat-treated at a temperature of 550 °C for 2 h in air at a heating rate of 2 °C/min.

The morphology of the sol-gel coatings was observed with scanning electron microscopy (SEM; JEOL, Peabody, MA, USA). The phase of the coatings was characterised with X-ray diffraction (XRD; PW 3710 Philips, Eindhoven, Holland).

Roughness of the coating layer was measured by means of scanning the surface with a 3D laser profiler (Proscan 1000; Scantron Industrial Products Ltd, Taunton, UK) [13]. Average roughness was measured on 8-10 difference sections for each sample.

2.2. *Culture of alveolar bone (AB) cells*

Overall, four Ti surface coatings were manufactured for further investigation, with ground commercially pure Ti surface ("cp Ti") acting as a non-coated control. The surface coatings consisted of a pure HA coating ("HA"), a HA composite coating with 10 mol % titania ("HA+10% TiO₂"), a HA with 20 mol % titania ("HA+20% TiO₂"), and a pure titania coating ("TiO₂"). Prior to tests, the coated surfaces were rinsed with distilled water and sterilised with UV light (Steristrom 2537Å, Coast-Air, London, UK) for 1 h.

Fragments of AB were obtained from a male patient aged 26 undergoing routine molar extraction, following a protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital (London, UK). The fragments were immediately placed into alpha-minimal essential medium (α -MEM) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10% fetal calf serum (FCS), and 25 μ g/ml fungizone (all from Gibco Life Technologies, Paisley, UK). After mincing into small pieces (1–2 mm³), they were washed with phosphate-buffered saline (PBS) (Gibco), placed into 6-well culture plates (Becton Dickinson, Cowley, UK) in Dulbecco's minimal essential medium (DMEM), supplemented as with α -MEM but without fungizone, and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells migrated from the fragments onto tissue culture plastic after approximately 7 to 15 days and were grown to confluence before detaching with 0.25% trypsin-1 mM EDTA (Gibco) for 5 min at

37°C. The resulting cell suspensions were centrifuged, washed with fresh medium and seeded into 25-cm² culture flasks, and maintained in supplemented DMEM.

The osteoblastic phenotype was confirmed by flow cytometry analysis of the expression of characteristic bone antigens (osteonectin, osteopontin, bone sialoprotein, and alkaline phosphatase) and the formation of mineralized nodules *in vitro* by von Kossa staining, as previously described [20]. The cells were used up to passage 5.

2.3. Cell attachment

Cells were radiolabelled with [³H]-thymidine ([³H]-TdR) to measure the percentage attachment of AB cells to the five different surfaces in replicate experiments. Cultures of AB cells were incubated in culture flasks at 37°C. At 18 h prior to seeding onto each coating, cell cultures exhibiting exponential growth phase were incubated with supplemented DMEM containing radioactive precursor of 2.5 µCi ml⁻¹ [³H]-thymidine (51.0 Ci per mmol; Amersham Biomedical, Little Chalfont, UK) in order to incorporate radioactive label into the cellular DNA.

For the attachment assay, the cell flasks were washed three times with PBS and thereafter harvested, resuspended in full medium, and replicate aliquots removed to count the total number of cells and also determine the level of isotope incorporated per cell. The specific activity was calculated as the disintegrations per minute (dpm) per 1000 cells. 3 x 10⁴ cells were seeded onto each of the Ti surfaces, comprising 10 mm squares, and incubated at 37°C for 3 h in full culture medium, then washed three times with PBS to remove the non-adherent cells. Ice-cold 10% trichloroacetic acid

(Sigma-Aldrich) was added to precipitate the DNA, thereafter removed, and the cells solubilized in 1 ml of 1% sodium dodecyl sulphate (Sigma-Aldrich), transferred to vials containing 10 ml scintillation fluid (ACS, Amerham Biomedical), and the level of radioactivity determined using a WALLAC 1409 liquid scintillation counter (PerkinElmer, Wellesley, USA). The number of attached cells was calculated from the amount of isotope, using the dpm per 1000 cells value determined previously. Results were repeated four times using separate 1 cm² squares for each surface under investigation.

2.4. Cell proliferation

The effects of the surface coatings on the growth characteristics of the AB cells were thereafter studied. 3 x 10⁴ cells in 0.5 ml of supplemented medium were plated onto 3 replicate 1 cm² samples for each Ti coating and for the control cp Ti surface, and material samples then placed in individual wells of a 6-well plate. Cells were left on the sample surface for 3 h to allow attachment, and then 3 ml of culture medium was added to each well, a volume sufficient to cover the surface of the discs. The cultures were then incubated at 37°C in supplemented DMEM media for a further 1 and 4 days. Cell proliferation was measured at these times using the MTT assay according to the manufacturer's instructions (Chemicon, Temecula, CA, USA).

The reagent, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is enzymatically cleaved within the mitochondria of living cells to form a dark blue/purple formazan product. The intensity of the colour produced is therefore directly related to the number of viable cells, and thus to their proliferation *in vitro*. Each of the Ti samples was removed from its 6-well plate and placed individually into

the wells of a 24-well plate (Becton Dickinson) with 0.5 ml medium. This ensured that no residual cells growing on the plastic surface of the original culture well could contribute to assay results. Then 0.25 mg of MTT reagent, in 50 μ l PBS, was added to each 24-well plate. After 4 h incubation at 37°C, the blue formazan product was solubilised with 500 μ l of isopropanol-HCl, and a 100 μ l aliquot of each sample removed three times for assay, which was performed in a 96-well plate.

Absorbance was measured at 570 nm (A_{570}) using a Titertek Multiskan Plus spectrophotometer (Labsystems, Helsinki, Finland) and the average A_{570} of the three replicate wells was calculated. The growth of the cells is shown as the average A_{570} of 3 separate experiments $\pm$ standard error ($\pm$ SE).

2.5. *Scanning electron microscopy (SEM)*

AB cells were seeded onto 1 cm² samples of each Ti surface treatment, and additionally onto control cp Ti squares, at 3×10^4 cells per sample as with previous attachment and proliferation work. They were incubated at 37°C for 1 and 4 days, and thereafter fixed in 2.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) (both Sigma-Aldrich) at 4°C overnight then dehydrated in a graded series of alcohols (50%, 70%, 90%, and two changes of 100% ethanol), washed with hexamethyldisilazane (TAAB Laboratories, Aldermaston, UK) for 5 min and placed in a desiccator overnight. After a further 24 h, the Ti samples containing fixed cells were mounted onto stubs using Araldite (Devcon, Wellingborough, UK) and Liquid Dag (Neubauer Chemikalen, Munster, Germany) and left to air-dry overnight. Specimens were then sputter-coated with gold/palladium using a Polaron E5100 coating device (Polaron CVT, Milton Keynes, UK) and observed using a Cambridge Stereoscan S90B (Cambridge Instruments, Crawley, UK). The same treatment was

carried out on all surfaces without cells in order to depict the unseeded material surfaces.

2.6. *Gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR)*

Semi-quantitative RT-PCR was used to assess the gene expression level of several well known osteogenic markers in AB cells at 24 h cell culture on each Ti surface coating and cp Ti control. 1.2×10^5 AB cells were seeded onto six 4 cm^2 samples for each Ti surface, an identical seeding density to earlier work described herein. At 24 h incubation, cells were detached using trypsin-EDTA as previously described, and centrifuged into a pellet that was resuspended in 5 ml of RNeasy (Ambion, Huntingdon, UK), an RNA stabilization and preservative agent, that was thereafter stored at -20°C .

RNA was extracted from the frozen and thawed RNeasy cell suspensions using RNeasy (Qiagen, Germantown, NY, USA), in accordance with manufacturer's instructions. A total of 2.5 μg of cellular RNA was used for reverse transcription with 500 ng oligo-dT (Promega, Madison, WI, USA) in 40 μl of water containing 0.1% DEPC (Sigma-Aldrich). After 5 min at 65°C , the first strand of cDNA was synthesized in a total volume of 50 μl , containing 50 U of M-MuLV reverse transcriptase, 1x M-MuLV buffer, 10 pmol of each dNTP, and 40 U of RNase block (all Stratagene, La Jolla, CA, USA). After incubation at 37°C for 60 min, the enzyme was heat inactivated by 5 min at 90°C . Subsequently, 5 μl of each cDNA sample was added to a 50 μl PCR reaction mix containing 1.5 U REDTaq DNA polymerase, 1x REDTaq PCR buffer, 10 pmol of each dNTP (all Sigma-Aldrich), and 12.5 pmol of the respective sense and antisense primer pair sequences (Sigma-Genosys,

Pampisford, UK) for osteopontin (OP), osteonectin (ON), bone sialoprotein (BSP) and procollagen type I (COL I) as outlined in Table 1. Replicate reaction tubes containing the primer pairs for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also used for each cDNA sample as an internal control. PCR was carried out using a PCT-100 thermal cycler (MJ Research, Boston, MA, USA) with an initial 3 min 94°C denaturation step, followed by cycles of denaturation, annealing and extension of 30 s at 94°C, 120 s at 60°C (51°C for BSP, 55°C for COL I) and 120 s at 72°C, respectively, with a terminal 10 min 72°C step. 36 cycles were used for all osteogenic genes and 32 cycles for GAPDH, which were found to be within the linear range of PCR amplification for all primer pairs (data not shown). The amplified products were separated using agarose gel electrophoresis and visualized by ethidium bromide staining with ultraviolet illumination image capture (MultiImage Light Cabinet; Alpha Innotech, San Leandro, CA, USA)

After electrophoresis, image analysis of the gels was carried out using Scion Image software (Scion Corporation, Frederick, MD, USA) to measure optical intensities of each gene transcript band. The ratio of gene / GAPDH band intensity was used to determine expression level for each gene, normalised to the ratio for the control cp Ti surface which was deemed 100% expression for each gene.

2.7. Statistical analysis

Data was analysed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and a two-tailed Students' t-test used to compare results from the four Ti surface coatings to the cp Ti surface. Data was presented as the mean $\pm$ SE. P values <0.05 and <0.01 were considered as significant and highly significant, respectively.

Results

3.1. Characteristics of HA and TiO₂ coatings

The phase of the sol-gel derived coatings was characterized by XRD. Data on HA+10%TiO₂ composite coating are shown representatively in Fig. 1. For pure HA and TiO₂ coatings, the crystallization of apatite and TiO₂ started to appear at ~450 °C. In the composite coatings, the crystallization occurred at slightly higher temperature (~500 °C). Of special note was that only apatite and TiO₂ phases were developed without the formation of other phases at 550 °C, suggesting high thermal stability of both apatite and TiO₂ structures in the composite coatings developed by the sol-gel method. This was in marked contrast to results of HA+TiO₂ composite coatings obtained by other methods, wherein the reaction compounds, such as tricalcium phosphate, calcium titanium oxides and calcium oxide, were normally observed.

The morphology of the sol-gel coatings was evaluated with SEM. As a representative example, morphologies of HA+10%TiO₂ are shown in Fig. 2. The coating surface was very dense and uniform on the microscale (Fig. 2(a)) for all Ti coatings. A closer examination (cross-section view, Fig. 2(b)) showed somewhat rough morphology on the nanoscale. The coating layer adhered well onto the Ti substrate without the formation of cracks or delaminations for all four Ti coatings. Moreover, all coating layers were dense and uniform with a thickness of approximately 1 μm. The roughness of all the coatings was measured to be in a similar range (~0.5-0.7 μm on average), as analyzed by a surface profiler.

3.2. Responses of AB cells to HA and TiO₂ coatings

Fig. 3. shows a panel of representative SEM images showing AB cell morphology on the four kinds of Ti coated surfaces and uncoated cp Ti control at one (a) and four (b) days of cell culture. SEM images of cells at day one showed variation in the number of cells, and also their cell morphology and orientation relative to surface scoring for each Ti surface. For uncoated cp Ti control, cells of both a rounded and spread morphology were visible, mainly aligned in the orientation of surface marking. A greater number of cells were seen on the HA and TiO₂ surfaces, with improved spreading characteristics relative to control cp Ti, and similar alignment relative to scoring with fewer rounded cells. A similar morphology and cell orientation was observed on the HA+10% TiO₂ surface, although more cells per unit area were observed, while on HA+20% TiO₂ there was a notably lower cell density although such attached cells exhibited the most spreading.

At four days incubation, cell numbers had increased on all Ti surfaces and AB cells exhibited an elongated shape as seen normally on tissue culture plastic. A confluent monolayer of cells was seen on the HA+10% TiO₂ surface, with cells on the HA surface also showing high numbers, although not full confluence. Less cell numbers were seen on the HA+20% TiO₂ and TiO₂ coatings, but cell densities were still greater than on cp Ti control. On all surfaces with the exception of HA with its network of defects, cells appeared to align in the direction of surface scoring.

To further examine the surface-dependant difference in AB cell densities, specific cell attachment and proliferation studies were performed. Fig. 4(a) shows that the percentage of cells attached at 3 h was 65-70% of cells for control cp Ti, similar to percentages for the HA+20% TiO₂ and TiO₂ coatings. However, significantly greater attachment was found for the pure HA surface and, to a lesser extent, the HA+10%

TiO₂ composite surface. Proliferation was examined by using the MTT assay at 24 and 96 h, as shown in Fig. 4(b). At 24 h, cell numbers were already significantly greater on all four control surfaces than on cp Ti, with the HA+10% TiO₂ showing greatest cell numbers. By 96 h, the HA and, most notably, the HA+10% TiO₂ composite coating showed a marked increase in cell population over other coated Ti surfaces, consistent with SEM findings. Again all coated surfaces had greater cell numbers than uncoated cp Ti.

Fig. 5. shows representative electrophoresis gels of the expression of several osteogenic genes at 24 h of cell culture on each Ti surface. While the intensity of the GAPDH band was observed to be approximately constant for cells on each Ti surface, notable variation in the expression of the examined genes was observed for each Ti surface. For OP, down-regulation was found on all pure and composite coated surfaces relative to cp Ti control, with down-regulation more apparent as surface TiO₂ increased. The same pattern of expression was found for COL I, with very low expression on the pure TiO₂ coating. For ON, high levels of expression were found on the pure HA, pure TiO₂ and cp Ti surfaces while decreased expression was apparent on composite coatings. BSP showed optimal gene expression at 24 h on the HA+10% TiO₂ and pure TiO₂ surfaces.

4. Discussion

The initial biological response of hard tissue to orthopaedic and dental implants is of key importance in the establishment of a stable, direct and functional interface between bone and implant material. Various Ti implant modifications including plasma-spraying, ion-beam assisted deposition and coating with calcium phosphate or bioactive glass have been developed to assist in improving this osseointegration process [10]. In this work, sol-gel processing was used to manufacture four different HA/TiO₂ surface coatings on Ti, which had previously shown promise over untreated Ti as regards physical and biological properties. Previous studies on such surfaces have used osteoblast-like cell lines or non-human primary cells to assess bioactivity by proliferation and differentiation marker production. This study used primary cultures of early passage and established human osteoblastic phenotype [20], thus eliminating the use of transformed or animal cells. Early cell responses such as ability to attach, spread and proliferate were examined, together with gene expression of specific osteogenic markers known to play key roles in mediating processes of bone growth, repair and differentiation.

The sol-gel coatings used herein showed uniform coating thickness and similar ranges of roughness. Previous works have shown that the sol-gel derived coatings are thin, highly homogeneous and dense, as well as have good bonding strength to Ti substrate, which improves with increased TiO₂ composition [13]. However, as high TiO₂ content within the composite HA/TiO₂ coatings (above 30% composition) was previously found to render the surface porous, composites used in this study had a bias towards high HA and low TiO₂ content.

In comparison to uncoated cp Ti, all the pure and composite HA and TiO₂ sol-gel coatings resulted in improved osteoblast proliferation, and improved or similar cell attachment. Attachment rates were found to be significantly increased on the pure HA coating, as may be expected given its natural abundance in bone matrix, while rates comparable to uncoated Ti were present above 10% TiO₂ coating composition. Notably for the HA+10% TiO₂ coating, a small increase in attachment above control uncoated Ti at 3 h was complimented by a very significant increase in cell numbers both at 24 and 96 h, apparent by both MTT spectrophotometric and SEM pictographic techniques. SEM images generally confirmed proliferation findings from the MTT assay, and showed that marked differences in cell function were being driven by chemical coating properties rather than physical topography, as all surfaces were shown to have similar roughness value. Results also supported the previous finding that such sol-gel surfaces were not cytotoxic [15], with dividing, well spread and anchored cells clearly apparent at numbers equal to or in excess of non-coated cp Ti.

For all sol-gel coatings, relative proliferation findings at 24 h were mirrored at 96 h, possibly suggesting that a consistent long term factor, which is not leached out or dissolved from the surface within 4 days, was driving such biological response. Cell numbers near to double those of untreated control were measured on the HA+10% TiO₂ coating, with also greatly enhanced proliferation on the pure HA coating. Previous work on such sol-gel composites has suggested optimum compositions of HA+20% TiO₂ [13] and HA+50% TiO₂ [14], as determined by differentiation marker production in immortal or animal cells. This work, assessing earlier cell functions and behaviour using human primary cells, proposes that a lower composition of TiO₂ may be more biologically optimal in such composite coatings, although decreased

TiO₂ composite levels may not be practical *in vivo* due to their adverse affect on coating adhesion strength [13]

The early expression of osteogenic genes by all surfaces was additionally studied. Both OP and COL I gene expression were shown to enhance with increased HA coating composition, consistent with literature suggesting that there may be a group of HA-responsive genes in osteoblasts that are evident within 24 h of first surface contact [21]. Only BSP exhibited greatest gene expression at the HA+10%TiO₂ optimal surface for cell proliferation. As well as HA-responsive genes, recent microanalysis of MG63 cells on anodised titanium oxide surfaces has highlighted a further group of genes, including heat shock protein-90, that show response to varied Ti oxide layer conditions [22]. Thus, gene expression from composite HA/TiO₂ coatings may be hard to interpret due to gene responses to both HA and the TiO₂ oxide layer. Nevertheless, surface-specific changes in osteogenic bone-associated gene expression were observed in human osteoblasts, which may mediate, at least partly, the later changes in cell proliferation and differentiation observed in this work and in literature [13-15].

5. Concluding remarks

Ti surfaces were modified using sol-gel coating techniques to produce both pure and composite HA and TiO₂ surfaces on Ti. Phase-pure apatite or / and TiO₂ were developed well on all the sol-gel derived coating layers. Primary human osteoblast cells of established phenotype exhibited enhanced cell proliferation on all such sol-gel coatings compared to untreated Ti, as measured by MTT assay and SEM observation. Initial cell attachment was also enhanced on pure HA and the HA+10%TiO₂ composite coatings, on which the optimal cell proliferation was also measured. The expression of bone-associated genes such as osteopontin and bone sialoprotein was further modified by coating composition at 24 h, suggesting that early cell responses to the coated surfaces may play a role in the overall biocompatibility of such HA and TiO₂ sol-gel modified Ti implants.

Acknowledgements

This work was supported by grants from the Korea Research Foundation (R08-2004-000-10192-0).

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Figure Captions

Fig. 1. XRD phase analysis of the sol-gel derived coating on Ti following heat treatment at 550 °C. Result on HA+10%TiO₂ is representatively shown.

Fig. 2. Electron micrographs of the HA+10%TiO₂ coating on Ti, typically showing (a) surface and (b) cross-section image (ultrahigh magnification).

Fig. 3. Electron micrographs of the sol-gel modified titanium surfaces with cell attachment after (b) 1 day of incubation and (c) 4 days of incubation with AB cells. The polished cp Ti serves as a control surface to the four surface treatments, as described in materials and methods.

Fig. 4. (a) Attachment and (b) proliferation of AB cells on each of the Ti surface treatments, compared to cells on the polished titanium control, cp Ti. Radiolabeling techniques were used to measure cell attachment at 3 h while the MTT assay was used to determine cell numbers on each surface at 1 and 4 days of incubation. Statistical significance was assessed relative to cp Ti for each surface coating, with * and ** denoting $p < 0.05$ and $p < 0.01$ respectively.

Fig. 5. Agarose electrophoresis gels showing the expression of several osteogenic genes, compared to approximately constant GAPDH 'housekeeping gene' expression, for AB cells grown for 24 h on each modified Ti surface.

Table 1.
Oligonucleotide primers for PCR amplification

Target cDNA		Primer Sequence (5' - 3')	Product Size (bp)	Reference
OP	Sense:	CCAAGTAAGTCCAACGAAAG	347	23
	Antisense:	GGTGATGTCCTCGTCTGTA		
ON	Sense:	ACATGGGTGGACACGG	405	24
	Antisense:	CCAACAGCCTAATGTGAA		
BSP	Sense:	ATTGAAAACGAAAGCGAAG	450	25
	Antisense:	ATCATAGCCATCGTAGCCTTGT		
COL1 procollagen	Sense:	TGACGAGACCAAGAACTG	599	23
	Antisense:	CAATCCAAACCACTGAAACC		
GAPDH	Sense:	CCACCCATGGCAAATTCCTCATGGCA	600	26
	Antisense:	CTGGACGGCAGGTCAGGTCCACC

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