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Expression of chemokines on the surface of different human endothelia

Running title: Endothelial presentation of chemokines

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Abstract

Expression of chemokines at the endothelial surface depends on their rate of synthesis, the capacity of the endothelium to bind chemokines and the rate of clearance from the surface. The aim of this study was to establish how these factors depend on the chemokine and the tissue of origin of the endothelium. Human lung and dermal microvascular endothelium, saphenous and umbilical vein endothelium and a bone marrow endothelial line, were assayed in vitro. Chemokine expression, localisation and transport was measured by immunoassay and confocal microscopy. All endothelia bound CCL3 (MIP-1α), CCL5 (RANTES), and CXCL10 (IP-10) less strongly. However, the profile of chemokine-expression varied between endothelia and different chemokines were shown to bind to the endothelial surface by distinct mechanisms. The half-life of CCL3 and CCL5 at the cell surface was approximately 30 minutes and chemokines were cleared primarily by endocytosis into caveolae.

Endothelia from different tissues synthesise distinctive sets of chemokines but the profile of surface-expressed chemokines also depends on the distinctive characteristics of each endothelia. These two mechanisms may contribute to the differential recruitment of leukocyte subsets to different tissues.

Keywords: Human endothelium, Chemokines, Glycosaminoglycans, Caveolae, Endocytosis
Introduction

Leukocyte migration is a key step in inflammation, involving attachment, activation, arrest and extravasation of the leukocytes\textsuperscript{1,2}. The cells are initially slowed by binding to endothelial selectins, at which stage they may be triggered to migrate across the endothelium by chemokines expressed on the endothelial surface\textsuperscript{3}. The population of leukocytes that enters a site of inflammation depends both on the inflammatory stimulus and on the tissue involved. This process is thought to be determined by the set of chemokines present on the endothelial surface and studies in situ suggest that endothelia are selective in their chemokine-binding properties\textsuperscript{4}. To date, more than 40 different human chemokines have been discovered, acting on a diverse family of chemokine receptors\textsuperscript{5,6}. We recently reported that human endothelia from different vascular beds have distinctive patterns of chemokine production, both in the resting state and following activation with the cytokines TNF-\(\alpha\), IFN-\(\gamma\) and TGF-\(\beta\)\textsuperscript{7}. However, the ability to express chemokines also depends on the surface characteristics of the endothelium, and the rate at which chemokines are cleared from the surface. In this study we have examined how these factors affect chemokine expression.

Many chemokines have a heparin-binding site, by which they bind to proteoglycans\textsuperscript{8-12}. Endothelial proteoglycans consist of a core protein that has been modified post-translationally by the addition of unbranched glycosaminoglycan (GAG) side chains\textsuperscript{13}. There are more than 20 different core proteins, including the syndecans, the glypicans and CD44. The GAGs that are commonly encountered in human tissues include chondroitin sulphate, dermatan sulphate, heparan sulphate and keratan sulphate, of which the most abundant is the polyanionic species heparan sulphate. Chemokines with a heparin binding site have been shown to bind with high affinity to heparan sulphate \textit{in vitro}. Binding of chemokines to heparan sulphate proteoglycans (HSPG) assists in orientation of the chemokine, which is thought to enhance leukocyte responses to chemokines\textsuperscript{10,12,14}. Binding to GAGs also allows high concentrations of
chemokines to accumulate in the correct location as well as aiding presentation to specific chemokine receptors on leukocytes and providing some protection from damage by enzymes. It is notable that specific chemokines bind different types of GAGs with different affinities\textsuperscript{11,15}. Moreover, endothelia express different GAGs depending on their tissue of origin\textsuperscript{16,17}. One can therefore reasonably hypothesise that each endothelium will have a different chemokine-binding capacity. Varied chemokine expression could thus act as a method of recruiting particular leukocyte subsets to specific tissues.

Variations in chemokine synthesis and GAG expression, are just two facets of the functional heterogeneity of endothelia from different anatomical sites, which also affects chemokine receptor expression and adhesion molecule expression\textsuperscript{7,18,19}. The functional significance of the numerous endothelial chemokine receptors is still under investigation, and one possibility is that they are involved in clearance of chemokines from the cell surface. Although chemokine clearance is very important for the control of inflammation and for ensuring that leukocytes do not become desensitised, the mechanisms for clearing chemokines have received less attention than analysis of their secretion and expression.

The aim of this study was to investigate whether chemokine expression depends on the tissue of origin of the endothelium and to examine factors controlling the presentation of inflammatory chemokines.
Materials and Methods

Endothelial cells

Endothelial cells were isolated from human umbilical veins (HUVEC) and human adult saphenous veins (SVEC) as described previously. Dermal and lung microvascular endothelium (DMVEC and LMVEC) were purchased from Clonetics/Biowhittaker (Wokingham, Berks. UK). The human bone marrow endothelial cell line (BMEC) was kindly donated by Babette Weksler of Cornell University. For the distinctive culture conditions of these cells, the details of their chemokine production profiles, and their expression of chemokine receptors and adhesion molecules, see reference 7. All experiments were performed on subcultures of confluent cells between the second and seventh in vitro passage. At this stage the cells all retained their distinctive morphology, expressed von-Willebrand factor and had distinctive patterns of GAG expression as determined using a panel of 21 different lectins. All cells were cultured in M199 medium, containing 10% foetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin for 24 hours beforehand and throughout the assays – ie culture conditions in the assays, including the substratum, were identical for each cell type.

Chemokine binding assay

Confluent monolayers of cells on 96 well microplates were washed four times in Hanks balanced salt solution (HBSS). 65 µl of recombinant human chemokine, CXCL8, CXCL10, CCL2, CCL3, or CCL5 (R & D systems) was added in triplicates at 0, 250 and 500 ng/ml in maintenance medium and incubated at 37°C for 1 hour. (Preliminary studies had determined that saturation binding was achieved after 30 mins). At the end of the period, chemokines were removed, the cells were washed three times in HBSS at 37°C, and surface bound chemokines were then detected by in situ ELISA. The expression of MHC
class-I molecules was used as an internal standard. MHC class-I was detected using 0.5µg/ml antibody to the human MHC class I framework (clone W6/32, Lab Vision) in the ELISA. (Previous studies have shown that MHC class-I surface density varies between the different endothelial types by less than 20%, when assayed in parallel). To measure the rate of decay of chemokines from the cell surface, binding assays were set up as above, but following removal of the chemokines, cells were cultured in maintenance medium for up to 150mins, before washing and fixation. All assays were carried out in triplicate and each experiment was repeated independently 2-3 times to show consistent patterns of binding.

**Digestion of GAGs**

Confluent monolayers were washed three times in HBSS. Then enzymes were added prior to commencing the chemokine binding assay. 65 µl of 10 U/ml heparinase III, 1 U/ml chondroitinase ABC or 1 U/ml neuraminidase (diluted in HBSS, all from Sigma) was added to triplicate wells for 1 hour at 37°C before being washed off. We have previously used this methodology and the conditions stated to remove individual GAGs from brain endothelium, an endothelial cell type that expresses high levels of GAGs.

**Immunofluorescence**

Localisation of chemokines within the cells, was done using double immunofluorescence labelling with anti-chemokine and anti-caveolin antibodies. Cells were grown as confluent monolayers on spots of NH3-vapour-fixed type-I collagen on glass cover slips. The cells were then treated with chemokines, according to the protocols given above. They were washed three times in HBSS (37°C) fixed with 0.25% glutaraldehyde in PBS for 10 minutes, blocked with 0.05M Tris/HCl (pH 7.5) for 20 minutes and permeabilised with 0.1% Triton X100 in PBS for 10 minutes. The cells were stained with a primary
combination of 1:25 rabbit anti-caveolin and 1:50 biotinylated mouse anti-chemokine, for 1hr, and a secondary combination of 1:50 fluorescein-anti-rabbit-Ig and 1:100 texas red-streptavidin for 1hr. All antibodies were diluted in PBS, containing 5mg/ml bovine serum albumin, and all wash stages were carried out using 3 washes in PBS.
Results

Chemokine binding to endothelial monolayers

The levels of chemokine binding to five different endothelial types, SVEC, BMEC, DMVEC, LMVEC and HUVEC were determined by *in situ* ELISA. Figure 1 shows the binding of four different inflammatory chemokines (CCL2, CCL3, CCL5 and CXCL10) to these endothelial cells. (We also tested CXCL8 binding, but found that at this level, the chemokine activated and severely disrupted the endothelial monolayers.) CCL3 and CCL5 both bound at significant but differing levels to all five endothelial types, while the two microvascular endothelia also bound CXCL10. CCL2 did not bind significantly to any of the endothelia tested.

The pattern of chemokine binding depended on the source of the endothelium. For example, the BMECs bound similar amounts of CCL5 and CCL3, whereas using the same conditions, CCL5 bound more strongly to SVEC than did CCL3, but the converse was true for DMVEC, LMVEC and HUVEC.

Figure 1 also indicates that CCL3 and CCL5 bind to the endothelia by different mechanisms. On each endothelia, doubling of the applied CCL3 concentration from 250 ng/ml to 500 ng/ml caused a much greater proportionate increase in CCL3 binding. Typically, the binding at 500 ng/ml was 5 to 20 times that at 250 ng/ml suggesting that CCL3 uses a co-operative binding mechanism whereas CCL5 does not.

To investigate the mechanism of binding of CCL3 and CCL5, BMECs were pre-digested with glycosidases before chemokine binding. The enzymes used were heparinase III which cleaves heparan sulphate proteoglycan (HSPG), chondroitinase ABC lyase, which cleaves chondroitin sulphate/dermatan sulphate proteoglycans and neuraminidase which cleaves sialic acid from other glycoproteins. Digestion of HSPG by heparinase III significantly reduced the binding of CCL5, but not of CCL3 (Fig 2). Chondroitinase and neuraminidase had no effect on the binding of either chemokine. The results
confirm that CCL5 and CCL3 differ in their binding mechanisms and show that CCL5-binding is dependent on heparan-sulphate.

To determine whether the chemokines were binding to the same sites on the endothelial cell surface, we used a competition assay, attempting to block the binding of 500ng/ml CCL5 with 0-1000 ng/ml CCL3 or vice versa (ie a maximum 2-fold molar excess). We found that CCL3 was unable to block CCL5 binding, but CCL5 caused a small (approx. 10%) but significant (p<0.05) inhibition of CCL3 binding, at doses 250-1000 ng/ml. This suggests that the two chemokines bind at essentially separate sites on the endothelium. In accordance with these findings we found that CCL5 but not CCL3 (or CCL2) bound to immobilised purified HSPG. Hence we conclude that CCL5 binds to the endothelia via HSPG, whereas CCL3 binds to a separate site using a cooperative binding mechanism. The apparent low level of surface-expressed CXCL10 was unexpected, particularly since CXCL10 also binds to purified HSPG (data not shown) - later experiments suggested that CXCL10 was being rapidly cleared from the cell surface.

**Loss of chemokines from the endothelial surface**

In order to determine how long chemokines persist on the endothelial surface, we loaded BMEC with 500 ng/ml CCL3 or CCL5 or CXCL10 for 90 minutes in culture, and then removed the chemokines, washed the cells and incubated the cultures for 0-150 minutes to allow bound chemokines to detach or be internalised. At the end of the incubation, cultures were washed, fixed and the surface-bound chemokines were detected by ELISA. Figure 3 shows examples of decay curves. From three independent experiments, we calculated the median half-life of chemokines on BMEC as 26 minutes for CCL3 and 27 minutes for CCL5. Identical experiments using DMVEC gave very similar surface half-lives for CCL3 and CCL5. Because of its apparent low level of surface expression, it was not possible to obtain accurate clearance curves for CXCL10.
The loss of chemokines from the endothelial surface could be due to endocytosis, or dissociation from the cell surface. Initial experiments showed that reducing the temperature of the cells to 4°C or 25°C during the incubation with chemokines resulted in a significant increase in surface expression of CCL32 and CCL5 (p<0.05), suggesting that the endothelial surface was cleared more effectively at 37°C. To investigate this further, we carried out identical experiments to those above, but with the inclusion of inhibitors of endocytosis during the decay phase. The inhibitors used were chlorpromazine (targetting clathrin-coated endosomes), nystatin (caveolae), nocodazole (microtubules) and cytochalasin-D (microfilaments). In preliminary experiments, we tested a range of doses for each inhibitor and established that the levels used in the experiments did not cause disruption of the endothelial monolayers, during a 2 hour incubation period. Figure 4 shows the combined results from three independent experiments, each with duplicate determinations, using a 40 minute time-point and CCL3. At this point there was 53% loss of detectable chemokine, which was substantially inhibited by 25µg/ml nystatin. The data was analysed by ANOVA (p < 0.0005), followed by Dunnett’s multiple comparison test. Only the higher dose of nystatin was significantly different from the untreated control (p<0.001). Similar results were observed in two experiments using CCL5. In parallel we assayed the supernatants at the end of the incubation for the presence of dissociated chemokines, and detected only low levels (2-10ng/ml) in each case.

Since nystatin substantially inhibits loss of surface chemokine and since little chemokine can be detected in the supernatants, this implies that the major proportion of surface chemokine is removed by endocytosis into caveolae. To confirm this observation, we carried out double label immunofluorescence with confocal microscopy, to establish whether applied chemokines and caveolin colocalise within the cells. Figures 5a and 5b show the distribution of CCL5 and CXCL10 in the cytoplasm of monolayers of BMEC, 45 minutes after removal of applied chemokine. Most cells have internalised chemokine and show streams of vesicles converging on the centre of the cell, which was most easily seen on large cells with extensive cytoplasm. There is also strong staining in the perinuclear
region. This perinuclear staining corresponds to the expected size and location of the caveosome, an endosomal compartment that has been described as a trafficking point for caveolae in other cell types. At higher magnification and using dual staining (Figures 5c and 5d) the vesicles containing chemokines were seen to co-stain with anti-caveolin, confirming that the chemokines were taken up by caveolae, which appeared, at this stage, to be attached to the microtubule network. Many caveolae did not contain chemokine, but all small vesicles containing chemokine also stained for caveolin. In addition to the 50nm caveolae many cells had a number of larger vesicles that stained for chemokine. Some local caveolin staining at the plasma membrane and strong perinuclear staining was also seen although the localisation of caveolin in the perinuclear region, and localisation of chemokines is different (compare figures 5c and 5d). The pattern of staining was similar with CCL3, CCL5 and CXCL10, although the proportion of chemokine in the larger vesicles appeared greater with CCL3. DMVECs and LMVECs showed colocalisation of chemokines and caveolin, although the network of caveolar vesicles was generally less extensive in these microvascular endothelia, (these cells have less cytoplasm and are less heterogeneous than BMECs). The data confirm that chemokines are taken up into caveolae and suggest that they may be routed to an endosomal compartment. Because CXCL10 is clearly being internalised by the cells, we considered the possibility that the apparent low level of surface CXCL10 expression is due to rapid clearance by receptor-mediated endocytosis. Hence, we attempted to block the internalisation of CXCL10 using either 5µg/ml blocking antibody to CXCR3 or 1µg/ml CXCL11 (I-TAC), a chemokine which also binds CXCR3 with similar affinity to CXCL10. The blocking antibody or chemokine were applied 15 minutes before loading the endothelium with 500ng/ml CXCL10 and were maintained in the culture for the remainder of the assay. CXCL11 produced a slight increase (~15%) in detectable surface CXCL10, but this was not significant in any one assay (by ANOVA). The blocking antibody had no effect. Removal of CXCL10 from the cell surface does not therefore appear to be receptor-mediated, in the conditions used here.
**Discussion**

In this study, we investigated the ability of endothelia from different tissues to present chemokines at the cell surface. It has previously been shown that a subset of chemokines can bind to endothelium via GAGs present in the proteoglycan layer and it is also known that endothelial cells from different tissues and different parts of the vascular tree vary in their expressed proteoglycans\textsuperscript{18} and level of sulphation\textsuperscript{25} both *in vivo* and *in vitro*. These observations lead to the hypothesis that the ability to present chemokines would depend on the endothelial type. This was confirmed for CCL3, CCL5 and CXCL10. Taken together with our previous data on chemokine production\textsuperscript{7}, this study shows that endothelia from different areas of the vasculature, synthesise distinct subsets of chemokines and present some chemokines more effectively than others. Numerous studies have highlighted the importance of specific chemokine expression by the high endothelial cells of lymphoid tissue, which direct the migration of lymphocyte populations into those tissues. This study shows that selective chemokine expression also occurs in non-lymphoid tissues, and may underlie the distinct patterns of leukocyte traffic which are seen in different tissues during inflammation.

This study also highlighted a distinction between the ability of endothelium to synthesise chemokines and the ability to present them. For example, these endothelia all synthesise CCL2 when stimulated with inflammatory cytokines\textsuperscript{7}, but do not bind CCL2 at the cell surface, although it does attach to extracellular matrix components, laminin, collagen and fibronectin.

The data in figures 1 and 2 confirm that the mechanism of presentation depends on the chemokine, with CCL5 being bound via HSPG. Other researchers, using HUVEC, found that CCL5-binding was reduced by 10-40\% by digestion with heparinase\textsuperscript{15}, which suggests that HSPG is a common binding site for CCL5 on different endothelia. We had expected that CCL3 would also bind via heparan sulphate proteoglycans since it too has a heparin-binding site\textsuperscript{26} and soluble GAGs inhibit endothelial-binding\textsuperscript{15}.
The failure of heparinase to prevent CCL3-binding and the limited capacity of CCL5 to block CCL3-binding to endothelium suggests that the two chemokines bind to different sites on the endothelia. Although it is possible that the chemokines bind to different subsets of GAGs, the data suggests that GAG-binding is less important for CCL3 than its attachment to extracellular matrix proteins. This would explain how endothelia with high CCL5 binding can have low CCL3 binding and vice-versa.

Of the other chemokines tested, CXCL10 also has a heparin-binding site for endothelium. We were therefore surprised at the comparatively low level of CXCL10 detectable on the endothelial surface, however figure 5b shows that CXCL10 is efficiently removed by endocytosis, which may account for its low surface expression.

In these studies it is important to distinguish chemokine binding to cell surface GAGs from binding to chemokine receptors. GAGs are abundant on the cell surface, but the chemokine binding is of moderate affinity: conversely, individual chemokine receptors are present at much lower levels than the GAGs, but chemokines bind with higher affinity to these receptors. The experiments reported here use a relatively high concentration of chemokine (500ng/ml) which detects binding to GAGs, whereas studies that use low chemokine concentrations provide information on chemokine-receptor binding. This explains the apparent discrepancies between some of the studies in this area. For example, we were unable to detect CCL3 binding to dermal endothelium, whereas others have been able to detect binding in situ using 1-2ng/ml radioabelled CCL3. The results can be reconciled by stating that CCL2 does bind to dermal endothelium via the chemokine receptor (presumably CCR5, which is present on these cells), but does not bind significantly via GAGs.

In this study, we also investigated how chemokines are lost from the cell surface. CCL3, CCL5 and CXCL10 all appear to be removed by caveolar endocytosis with the majority cleared within 60 minutes. The rates of clearance were similar for CCL3 and CCL5, but we were unable to establish the rate of clearance of CXCL10. Interestingly the clearance rate of CCL3 and CCL5 was very similar on the
different endothelia in the culture conditions reported here, although we had found in preliminary experiments that cultures that had been confluent in vitro for several days showed somewhat slower chemokine clearance. It has been debated whether chemokine clearance is effected by receptor-mediated endocytosis, and it has been suggested that non-signalling chemokine receptors, including the Duffy antigen receptor for chemokines (DARC), the receptors D6 and CCX-CKR could each act as scavengers to remove extraneous chemokines from the blood, and thus prevent desensitisation of circulating leukocytes. The endothelia in this study express a wide variety of specific chemokine receptors, including CCR3, CCR5 and CXCR3, which bind to the chemokines used (CCL3, CCL5, CXCL10). Various functions have been proposed for specific endothelial chemokine receptors, including promoting cell migration, enhancing or suppressing angiogenesis or mediating chemokine transcytosis, however we have not been able to consistently show these actions on these endothelia.

We considered the possibility therefore, that a primary function of endothelial chemokine-receptors is to clear the cell surface of excess chemokines. Interestingly, in accordance with this theory, the level of expression of the chemokine receptor CXCR3 on different endothelia is inversely correlated with the level of its ligand CXCL10, detectable on the endothelial surface (data not shown). However we were unable to block CXCL10 endocytosis using a blocking anti-CXCR3 antibody, and produced only marginal (non-significant) blocking with CXCL11. Therefore, at the levels used here, it does not appear that receptor-mediated endocytosis contributes significantly to chemokine clearance, although it might do so when lower levels of chemokine are present. The results are consistent with direct fluid-phase endocytosis into caveolae and/or caveolar uptake of chemokines attached to surface GAGs/glycoproteins.

In conclusion, endothelia from different tissues selectively bind particular chemokines but only a subset of endothelium-produced chemokines can be effectively presented on the endothelial surface. Caveolae-mediated endocytosis is important in clearing the cell surface of chemokines. This process is rapid and seen in all types of endothelia studied. The data highlight the important role that endothelium
plays in determining selective chemokine-expression in different tissues and hence its potential for controlling the type of inflammatory response and leukocyte migration that occurs in those tissues.

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Figure legends

Figure 1  **Binding of chemokines to endothelia from different tissues.**
The chemokines CCL3, CCL5, CCL2 and CXCL10 were applied at 0, 250 and 500ng/ml to 5 different human endothelia for 1 hour at 37°C and binding assayed by ELISA. The results are the mean ±sd of triplicate determinations, from at least two experiments, expressed as a percentage of MHC class-I expression (=100%). (* = p<0.05).

Figure 2  **Chemokine binding after enzyme digestion of proteoglycan.**
BMEC monolayers were digested with heparinase, chondroitinase, neuraminidase or no enzyme for 1 hour at 37°C. 500ng/ml CCL3 or CCL5 was added for 1 hour at 37°C and surface-bound chemokines detected by ELISA. The results are mean and standard deviation of triplicate determinations. Significance was tested by two way ANOVA with a post hoc t-test (* = p<0.05).

Figure 3  **Decay of chemokines from endothelial surface.**
BMEC were loaded with 500ng/ml CCL3 or CCL5 for 90 minutes. The cells were cultured for a further 0-150 minutes at 37°C before assay of surface-bound chemokines by ELISA. Each data point shows the mean and standard deviation of triplicate determinations. Each curve is representative of 3 experiments with similar results.

Figure 4  **Inhibition of chemokine endocytosis.**
BMEC were loaded with 500ng/ml CCL3 for 90 minutes, before removal (t=0). Cells were cultured for a further 40 minutes in the presence of the stated concentrations of antibiotics (µg/ml), at 37°C before assay of bound chemokines by ELISA. Nystatin (Nyst.), nocodazole (Noc.), cytochalasin-D (cyt.),
chlorpromazine (Chl.) or no antibiotic (Neg.). Results are mean values and standard deviations from 3 independent experiments, expressed as the percentage detectable at $t=0$.

Figure 5 **Localisation of endocytosed chemokines.**

Micrographs of endocytosed CCL5 (A) and CXCL10 (B) in a monolayer of BMEC. The z-plane is a $1\mu$ thick composite section derived by merging two $0.5\mu$ confocal sections, running through the cytoplasm of the cells (Cells are typically 2-3$\mu$ in thickness). Dual label micrographs show caveolin staining of a single cell (C) and CXCL10 localisation (D) Dual label photomicrographs of lung endothelium show caveolin staining of a single cell (E) and and CCL5 localisation (F). Vertical arrow (C) indicates a caveolar vesicle stained for caveolin, which does not contain chemokine. Horizontal arrows indicate examples of vesicles costained for chemokine and caveolin.
Figure 1
Figure 2

CCL3 CCL5

Negative
Heparinase
Chondroitinase
Neuraminidase

Percent of control

CCL3   CCL5

*
Figure 3

CCL3 (MIP-1α)

Percent binding vs. minutes

CCL5 (RANTES)

Percent binding vs. minutes
Figure 4
Fig 5c
Fig 5d
Fig 5f