Tissue-specific differences between endothelia : expression and presentation of chemokines and their receptors

Thesis

Tissue - specific differences between endothelia: expression and presentation of chemokines and their receptors

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

The recruitment of different leukocyte subpopulations during an inflammatory response is regulated by various molecules expressed by the endothelium. This study examines how chemokine production, the expression of chemokine receptors and the binding of chemokines to the proteoglycan layer vary between endothelia from different vascular beds.

Human dermal, lung and liver endothelia were compared with endothelia derived from saphenous and umbilical veins and with a transformed bone marrow cell line. All endothelia produced MCP-1 (CCL2) and IL-8 (CXCL8) constitutively with both these chemokines and IP-10 (CXCL10) and RANTES (CCL5) being secreted after TNF-α or IFN-γ stimulation, whereas MIP-1α (CCL3) was not produced under any conditions tested. A combination of TNF-α and IFN-γ stimulation increased RANTES and IP-10 production, but reduced IL-8 production. The addition of TGF-β reduced secretion of all chemokines. Differences were found in production levels of each of these chemokines depending on the tissue that the endothelial cells were derived from. MIP-1α, and RANTES bound to the cell surface of each endothelium tested at differing levels, but IP-10 bound only to lung endothelium. RANTES binding was found to be dependent on the presence of Heparan Sulphate Proteoglycan (HSPG), but MIP-1α appeared to bind via a different method. Chemokine receptors CXCR1, CXCR3 and CCR3 were expressed highly at the endothelial cell surface, with lower levels of CXCR2, CXCR4, CXCR5, CCR5 and CCR6. Expression of CCR4 varied widely. Using lectins to identify specific oligosaccharides, differences in the composition of the proteoglycan layer were probed. Small variations were shown, but these cannot account for the differences in chemokine binding.

These results demonstrate that endothelial cells could be important in the selective recruitment of leukocytes to different tissues, in particular in their expression of chemokines in response to inflammatory stimuli and their presentation of chemokines to the leukocytes in the blood stream.

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Abbreviations

bFGF	Basic fibroblast growth factor
BMEC	Bone marrow endothelial cells
BSA	Bovine serum albumin
CAEC	Coronary artery endothelial cells
C5a	Complement component 5a
DARC	Duffy receptor for chemokines
DC	Dendritic cells
DMVEC	Dermal microvascular endothelial cells
DTH	Delayed type hypersensitivity
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENA-78	Epithelial derived neutrophil attractant 78 (CXCL5)
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
G-CSF	Granulocyte-colony stimulating factor
GlyCAM	Glycosylation-dependent cell adhesion molecule
GM-CSF	Granulocyte macrophage-colony stimulating factor
G-protein	Guanine nucleotide binding protein
Gro	Growth related oncogene
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus
HSPG	Heparan sulphate proteoglycan
HUVEC	Human umbilical vein endothelial cells
IAEC	Iliac artery endothelial cells
ICAM-1	Intercellular adhesion molecule 1
ICAM-2	Intercellular adhesion molecule 2

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LFA-1	Leukocyte functional antigen-1
LMVEC	Lung microvascular endothelial cells
LPAM	Leukocyte peyer's patch adhesion molecule
LPS	Lipopolysaccharide
M199	Medium 199
MAdCAM	Mucosal addressin cell adhesion molecule
M-CSF	Macrophage colony stimulating factor
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1 alpha (CCL3)
MIP-1β	Macrophage inflammatory protein 1 beta (CCL4)
NO	Nitric oxide
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PF-4	Platelet factor 4 (CXCL4)
PSGL	P-selectin glycoprotein ligand
RANTES	Regulated upon activation, normal T-cell expressed and secreted (CCL5)
SDF-1	Stromal derived factor 1 (CXCL12)
SLC	Secondary lymphoid chemokine (CCL21)
SVEC	Saphenous vein endothelial cells
TGF	Transforming growth factor
TGFβRI	Transforming growth factor beta receptor 1
T_H1	T helper 1 cells
T_H2	T helper 2 cells
TNF	Tumour necrosis factor
TNFRI	Tumour necrosis factor receptor 1
TNFRII	Tumour necrosis factor receptor 2
UEA-1	Ulex europeaus agglutinin 1
VCAM-1	Vascular cell adhesion molecule 1

VEGF	Vascular endothelial growth factor
VLA	Very late antigen
vWF	von Willebrand factor (factor VIII)

Chapter 1
Introduction

1. Introduction

1.1 The Endothelium

1.1.1 The functions of endothelium

For many years endothelium was considered to be a quiescent lining layer on the inside of blood vessels. It is now evident that endothelial cells are important in haemostasis and during immune responses, particularly because of their strategic position as an interface between the circulating blood and the tissues of the body (Fishman 1982; Simionescu and Simionescu 1986). The endothelium is made up of a continuous monolayer of cells joined together by junctional adhesion molecules and acts as a physical barrier between the blood and the tissues. It is now known to control transport of molecules into tissues, vascular tone, cell migration, angiogenesis and coagulation. Leukocyte migration from the blood stream through the vessel wall and into the tissues occurs constitutively as part of the normal immune surveillance, but increases at sites of inflammation.

Endothelial cells are able to control the vascular tone and blood flow through the production of dilators and constrictors, which can act on the vessel wall. The constant production of nitric oxide and prostaglandins, which act on adjacent smooth muscle cells, ensures that the patency of the vessel is maintained. This production is also antithrombotic and as such prevents aggregation of platelets and leukocyte adhesion. When the endothelium becomes activated, it can produce endothelin-1 and thromboxane which act as vasoconstrictors. Histamine receptors are also present on the cell surface (Simionescu and Simionescu 1986).

Leukocytes are required to extravasate during both innate and adaptive immune responses. Endothelial cells contribute to the local accumulation of leukocytes during an immune response by upregulating the expression of adhesion molecules on their surface and by increasing the production of chemokines and other mediators in response to cytokines (Pober and Cotran 1990b; Springer 1994). They can also contract to regulate capillary permeability to various molecules and cells. Since only a limited subset of circulating cells will respond to each chemokine, regulation of chemokine production can contribute to the selectivity of leukocytes recruited.

1.1.2 The immune response

Inflammation is a defence reaction caused by tissue damage or injury, characterised by redness, heat, swelling and pain. The objective of inflammation is to remove or neutralise the irritant and repair the damaged tissue. The body does this by increasing the blood supply to the affected area, increasing the capillary permeability in the area and finally by the migration of cells of the immune system out of the blood stream and into the tissue.

In the early stages of the immune response, the proteins and cells of the innate immune system are the most important, these are non-specific. They include viral and microbial protein receptors on cells as well as plasma proteins such as the complement system. The cells involved in this early response are natural killer (NK) cells and phagocytic cells. Phagocytes include the granulocytes (eosinophils, neutrophils and basophils) as well as macrophages. Signals are then generated at the inflammatory site that activate the local leukocytes and adjacent endothelium.

Many mediators are involved including the kinin system, which causes vasodilation and increased permeability and the complement system that has many functions including triggering mast cell degranulation. One component of the complement system, C5a, has chemotactic properties for neutrophils and macrophages. The plasmin and fibrinolytic systems also act in a cascade during acute inflammation to finally allow fibroblasts to begin the repair process and restore the area to normal function. What follows is a series of events that forms the body's inflammatory response - one of its most important defence mechanisms. It is the physiological response to injury, whether the injury is microbial or if it results from physical, chemical, thermal, toxic or immunogenic damage. This response is a well co-ordinated series of vascular and cellular reactions (Levy 1996). The earliest events involve the production of chemical mediators such as prostaglandins, C5a, histamine, thrombin, leukotrienes and cytokines including interleukins (IL) -1 and -6 and tumour necrosis factor (TNF-α). These mediators produced by phagocytes or by complement activation, cause vasodilation, increase vascular permeability and also initiate the recruitment of more effector cells.

1.1.3 Effects of cytokines on endothelium

Cytokines include the interleukins (IL-1 - 26), interferons (IFN-α, IFN-β, IFN-γ), tumour necrosis factors (TNF-α, TNF-β), transforming growth factors (TGF-α, TGF-β) and colony stimulating factors

(GM-CSF, G-CSF, M-CSF); they function by binding to specific protein receptors on the target cells and then act by altering the behaviour of the target cell. Individual cytokines often modulate the effects of other cytokines on the same target cell - they can act in synergy causing a greater effect or a new effect that neither can produce alone. One cytokine will often cause the release of a second cytokine by its target cell - known as a cytokine cascade (e.g. TNF-α stimulates IL-1 production by endothelium). Cytokines are not always secreted, but may be expressed on the surface of the stimulated cell (e.g. the transmembrane form of TNF-α or the chemokine fractalkine).

TNF-α and IL-1 are pro-inflammatory cytokines and the intracellular pathways which mediate their effects have been extensively researched, however less is known about how signals are integrated within the cell for instance to show an antagonistic or synergistic effect, though some details are beginning to emerge. Since more than one cytokine is likely to be found at any one site of inflammation, this is an important and developing area of research.

The endothelial layer lining blood vessels has also been shown to be an important focus of cytokine interactions (see table 1.1 and review Pober and Cotran 1990a). TNF-α and IL-1 cause endothelial cells to upregulate the expression of several cell surface and secreted molecules, including the adhesion molecules (Mantovani *et al.* 1997; Meager 1999). This process is known as endothelial activation. These changes can take varying lengths of time and can be the result of *de novo* synthesis or of release of vesicular molecules. The molecular mechanisms involved in the cytokine regulation of genes expressed by endothelial cells are now better understood (Foletta *et al.* 1998; Mantovani *et al.* 1998; Denk *et al.* 2001). In particular, NFκB is a crucial element for the cytokine-mediated inducibility of adhesion molecules and cytokines in endothelial cells (Collins *et al.* 1995; May and Ghosh 1998; Richmond 2002). Inflammatory cytokines promote leukocyte extravasation by altering the dynamics of microcirculation via nitric oxide (NO) and prostaglandins and by inducing adhesion molecules and chemotactic cytokines.

Table 1.1 Molecules induced by cytokines acting on endothelium

MOLECULE	INDUCED BY	REFERENCES
MHC-I	IFN-γ, TNF-α	Wedgwood et al. 1988
MHC-II	IFN-γ	Wedgwood et al. 1988
ICAM-1	TNF-α	Pober 1987, Detmar et al. 1990
	IFN-γ, IL-1	Dustin et al. 1986
VCAM-1	TNF-α, IL-1	Osborn et al. 1989
E-selectin	TNF-α, IL-1	Bevilacqua et al. 1987 & 1989
iNOS	TNF-α, IL-1	Kanno et al. 1994, Pellegatta et al. 1994
IL-8	TNF-α, IL-1	Sica et al. 1990a
MCP-1	TNF-α, IL-1	Strieter et al. 1989b
RANTES	TNF-α & IFN-γ	Marfaing Koka et al. 1995
IP-10	IFN-γ	Luster and Ravetch 1987a
Gro-α	TNF-α, IL-1	Wen et al. 1989

1.1.3.1 Tumour necrosis factor (TNF)

TNF is the principal mediator of the response to gram negative bacteria and may also play a role in innate immune responses to other infectious organisms (reviewed in Vassalli 1992). It was initially described in serum of endotoxin-treated mice as the mediator of necrosis of some transplantable tumours (Old 1985).

Mononuclear phagocytes are the predominant cellular source of TNF-α, although many other cell types, such as T cells, B cells, activated NK cells, activated mast cells and some tumour cells, may make TNF-α. TNF-β is produced solely by cytotoxic T cells. The genes for TNF-α and TNF-β (previously known as lymphotoxin) are located in tandem within the major histocompatibility complex (MHC) - on chromosome 6 (Aggarwal *et al.* 1985). TNF-α is synthesised by the conventional secretory pathway through the endoplasmic reticulum and golgi apparatus as a non-glycosylated transmembrane 25 kDa precursor polypeptide, which is processed to yield a large extracellular carboxy terminus derived 17 kDa product with a transmembrane leader sequence. This precursor subunit can be proteolytically cleaved off the membrane to produce the secreted form - a stable 51 kDa homotrimer in the shape of a pyramid (Arakawa and Yphantis 1987; Jones *et al.* 1989).

TNF-α exerts its biological effects by binding of the soluble trimer to one of two cell surface receptors (Bazzoni and Beutler 1996). These are of 55 and 75 kDa respectively, each encoded by

a separate gene, but both present on most cell types. The affinity of TNF-α for its receptors is low for a cytokine, however it is synthesised in very large quantities and can easily saturate the receptors. Most biological effects have been shown to be mediated through TNFRI (p55) (Wiegmann *et al.* 1992; Mackay *et al.* 1993; Pfeffer *et al.* 1993; Rothe *et al.* 1993). Endothelial cells express both the p55 and p75 TNFR, with the p75 form being the most abundant on the cell surface. The p55R is more abundant overall, but is expressed at much lower levels on the membrane itself, and is found mainly in the golgi apparatus and in cytoplasmic vacuoles. The effect of p75 is best observed at low TNF-α concentrations, which is in accordance with the concept that this molecule can present TNF-α at low concentrations to p55 (known as ligand passing) (Leeuwenberg *et al.* 1995). p75 also binds to the transmembrane form of TNF-α and is thought to be important in juxtacrine interactions between endothelial cells and leukocytes (Lukacs *et al.* 1995). Activated cells shed their TNF-α receptors and these soluble receptors may act as competitive inhibitors of the cell surface receptor (Higuchi and Aggarwal 1994; Black *et al.* 1997; Hooper *et al.* 1997).

Many TNF-α responses involve increased rates of expression of particular target genes, often through activation of NFκB or AP-1 transcription factors (May and Ghosh 1998). However, the signalling pathways are very complex and differ depending on the cell type. TNF-α acts by phosphorylating IκBα, allowing its rapid degradation (Beg *et al.* 1993). This releases NFκB which migrates to the nucleus and binds specific sites on promotor regions of target genes which are then transcribed and protein synthesis occurs – producing cytokines, chemokines and adhesion molecules as required by the cell (Ebnet *et al.* 1997; Martin *et al.* 1997).

TNF-α has varying biological effects at different concentrations. At low concentrations TNF-α can act on leukocytes and endothelial cells as a paracrine and autocrine regulator, in this way, it is a potent mediator of the inflammatory response (Pober and Cotran 1990a; Mantovani *et al.* 1997). It increases adhesion molecule expression on endothelial cells and leukocytes, stimulates mononuclear phagocytes and endothelium to secrete chemokines while inflammatory leukocytes such as neutrophils, eosinophils and mononuclear phagocytes are activated to kill microbes. It also has other effects on endothelium, including stimulation of prostaglandin and nitric oxide production and induction of platelet activating factor expression. Chronic low production of TNF-α can cause tissue remodelling including angiogenesis and fibroblast growth factor production which allows connective tissue deposition. This may be through indirect actions dependent on leukocytes,

although there is also some *in vitro* data that suggests a direct angiogenic activity for this cytokine. At sufficiently high levels, TNF-α acts on the hypothalmic region of the brain to induce fever and on hepatocytes to increase synthesis of serum amyloid proteins that are one component of the acute phase reaction (Baumann and Gauldie 1994). The actions of TNF-α on mononuclear phagocytes and endothelial cells initiate secretion of IL-1 and IL-6 into the circulation and induce activation of the coagulation system by altering pro and anti coagulant properties of the endothelium.

Although many TNF-α activities are indistinguishable from those of IL-1, TNF-α and IL-1 activities are often additive and always appear to be independent (i.e. not mediated through IL-1 synthesis). Through its effect on MHC I antigen expression, TNF-α may promote lymphocyte dependent inflammation (an action which is not shared by IL-1). IFN-γ, produced by T cells augments TNF-α synthesis by lipopolysaccharide (LPS) stimulated mononuclear phagocytes (Doukas and Pober 1990). Thus TNF-α is an important link between specific immune responses and acute inflammation.

1.1.3.2 Interferon (IFN)

Interferons are a family of proteins encoded by different genes all sharing a capacity to inhibit viral infections, though they have many additional biological activities. They have been subdivided into IFN-α, IFN-β, and IFN-γ. IFN-α consists of several (over 20) homologous 18 kDa proteins produced by mononuclear phagocytes, IFN-β is a 21 kDa glycoprotein which is derived from tissue cells and IFN-γ which is a 20 or 25 kDa glycoprotein which in its native form is a dimer. They are only related to each other by their induction of an antiviral state. IFN-γ is synthesised only by T_H1 cells and NK cells in response to antigen.

IFN-γ acts via specific interferon receptors and has many effects both on leukocytes and on endothelium (Novick *et al.* 1987; Fischer *et al.* 1988; Cofano *et al.* 1990). It stimulates B cells to mature and switch isotype and also acts as a maturation factor for cytotoxic T cells. It is an important activator of macrophages and is able to stimulate the alternative complement pathway, which leads to phagocytosis of infected cells. In addition to these effects it has many important consequences for endothelium. Knockout mice demonstrate an impaired response to microbial pathogens, but exhibit no problems in the absence of pathogens (Flynn *et al.* 1993).

IFN-γ induces endothelial cell expression of ICAM-1, MHC II antigens and angiostatic chemokines including IP-10, Mig and I-TAC (see Table 1.1). It enhances expression of MHC class I and CD40 and acts in synergy with TNF-α to increase the levels of adhesion molecules expressed on the endothelial cell surface. It has also been shown to have a similar effect on chemokine production as well as augmenting LPS induced production of IL-1.

1.1.3.3 Transforming growth factor (TGF)

TGF-β was originally described as a component of tumour cell conditioned medium that when combined with TGF-α could cause cell growth (Todaro *et al.* 1980). It has since been shown that TGF-α promotes cell growth while TGF-β has cell modulatory functions. Many cell types produce TGF-β, including activated T cells, macrophages and cytokine stimulated endothelial cells. The protein is formed of two separate, but closely related 14 kDa polypeptides from different genes making two different forms TGF-β1 and TGF-β2 which then normally homodimerise, although heterodimers do exist. These dimers then reassociate with the precursor to create a latent form. This can be activated by several different factors. These include acidic conditions, thrombospondin, plasmin and enzymes such as transglutaminase. TGF-β binds to specialised receptors TGF-βR type I and TGF-βR type II as well as to proteins of the extracellular matrix including fibronectin, collagen and heparin, it is thought to exert some of its effects through heparin binding.

Effects of TGF-β include the inhibition of endothelial proliferation and migration (Heimark *et al.* 1986; Bell and Madri 1989; Pepper *et al.* 1993; Pepper 1997), this is associated with increased extracellular matrix and proteoglycan synthesis. It also stimulates the synthesis of VEGF and PDGF-B (platelet derived growth factor-B) and allows the release of bFGF into the subendothelial matrix. In this way it exerts much control over angiogenesis, in particular it appears to be important in the later stages of reducing proliferation and remodelling as it has been found to cause tubule formation in matrix embedded EC. Treatment of endothelial cells with TGF-β hinders leukocyte migration, this is associated with decreased expression of E-selectin on the cell surface and lowered secretion levels of IL-8 (Gamble and Vadas 1988; Gamble *et al.* 1993).

1.2 Heterogeneity of endothelium

Endothelial cells throughout the vasculature share certain common functions, but heterogeneity exists structurally and functionally both along the vascular tree and in the microvascular beds of various organs (Gerritsen 1987; Turner *et al.* 1987; Page *et al.* 1992; Swerlick *et al.* 1992a; Risau 1995). The differences observed are thought to be adaptations to the different functions of each tissue and the pressures of the local microenvironment, so the needs of a particular tissue will determine what is required of the endothelial cells in its blood vessels (Garlanda and Dejana 1997). Endothelium can be classified into three general types; continuous, discontinuous and fenestrated. Continuous endothelium forms a tight monolayer as in the brain or the lungs, while discontinuous and fenestrated endothelium form a less tight surface because of the transport functions requiring it to allow cells or molecules to pass more freely between different compartments (e.g. kidney, bone marrow, capillaries).

Endothelial cells share several common markers, which can aid identification *in vitro* and *in vivo*. These include the presence of von Willebrand Factor (vWF or factor VIII) and binding to the lectin Ulex europeaus –1 (UEA-1) (Holthofer *et al.* 1982). Many of the specialised characteristics of endothelium are induced during differentiation, whereas adult endothelium is not so susceptible to proliferation and differentiation. It however, does react to the pressures of the different microenvironments that it might be exposed to. It is in this way that differences between organs are maintained. This makes it difficult to maintain the phenotype of the specific endothelium *in vitro*, in particular for a cell type as specific as bone marrow endothelium (Masek and Sweetenham 1994; Candal *et al.* 1996; Rood *et al.* 1999). This is why the production of a cell line for this cell type is so important and culture conditions have to be carefully maintained (Schweitzer *et al.* 1997).

The earliest studies identifying heterogeneity of endothelium used tracers to assess the surface charge (Simionescu *et al.* 1982; Vorbrodt *et al.* 1986), then lectins were found to bind to endothelium and via conjugation to radioactive molecules or biotin were used to detect endothelial surface glycoconjugates. There have been several recent developments in techniques, which have allowed more selective identification of markers on the endothelial cell surface, although as yet only one molecular marker for specific endothelium has been identified (a zinc metalloproteinase is found only on lung endothelium).

Phage display peptide libraries can be used to probe for defined peptide sequences which interact with a particular molecule (reviewed in Ruoslahti and Rajotte 2000). In this method up to a biilion permutations of different peptides are expressed on the coat of phages. The desired peptides are chosen by their ability to bind a specific target molecule. The advantage of this is that it allows selective binding to only the exposed region of the molecule without being hampered by conformational problems. Phage libraries can be used to select for proteins, carbohydrates and peptides on cultured cells. Rajotte *et al.* (1998) have studied organ specific differences in the vasculature and have been able to identify phages that homed to specific organs. They then identified the peptide sequences, but the identity of the molecules recognised by these peptides is only beginning to be addressed. One distinct marker has been detected so far by this method, that is membrane dipeptidase, found only on lung endothelium, it is a cell surface zinc metalloprotease involved in the metabolism of glutathione, leukotriene D4 and certain antibiotics. A more recent study has identified several more potential molecules that show differential expression throughout the vasculature (Arap *et al.* 2002).

Recent studies have demonstrated that it is possible to isolate and purify just the blood front of the endothelial cell surface (Ghitescu and Robert 2002). Patterns of 2D electrophoresis staining showed that while there were similarities in some proteins expressed, there were also some interesting differences between endothelium derived from brain, heart and lung of adult male Sprague Dawley rats. Differences between organs were also observed in the binding profiles of various lectins to the membrane preparations, with similarities observed between the heart and the lung, neither of which shared any similarities with the patterns of staining of the brain endothelium. Although differences between organs have been detected using this technique, the specific molecules which are different from organ to organ have not yet been identified. Further development of this technique couid prove useful in the identification of specific markers for endothelium.

Because chemokines and their receptors are fundamental to leukocyte recruitment, their varied expression by different endothelial cells, both during normal function and during inflammatory reactions could help determine which particular cell types will be recruited into which tissues. Adhesion molecules and the type of molecules in the proteoglycan layer are also important in recruitment, so specificity seems to be determined by a combination of factors. The fact that chemokines are produced during so many immunological events suggests that they could be very

useful targets for drug intervention and other therapies (Saunders and Tarby 1999; Schwarz and Wells 1999).

Despite the realisation that there may be physiological differences between various vascular beds, the majority of *in vitro* investigations into chemokine expression by endothelium have been performed using Human Umbilical Vein Endothelial Cells (HUVEC) because of the relative ease with which they can be obtained. HUVEC have over the last couple of decades been put to good use in identifying many of the functions of endothelium during the inflammatory response. Much of the data shown in Table 1.1 has been described in HUVEC. However, the principle site of neutrophil adhesion and emigration is not at the level of the large veins such as HUVEC and Saphenous Vein Endothelial Cells (SVEC), but at the level of the postcapillary venule. Since the isolation of microvascular endothelium is becoming easier, more researchers are investigating other types of endothelium. I will now discuss in further detail some of the major differences found so far between the different endothelial cell types used in this study.

1.2.1 Human Umbilical Vein Endothelial Cells

HUVEC isolated by collagenase digestion of umbilical veins have been used for many years as a standard endothelial cell type (Jaffe *et al.* 1973). They have a familiar cobblestone appearance, bind UEA-1 on the cell surface and VWF in vesicles. HUVEC are formed during pregnancy and are hence considered to be foetal in origin – not adult. The function of the vessel is simply to transport waste substances away from the foetus and back to the mother for breakdown or disposal. However there is a huge amount of information available on these cells – including analysis of all the molecules shown in table 1.1.

1.2.2 Saphenous Vein Endothellum

The enzymatic isolation of human SVEC was first described by Watkins *et al.* (1984). The saphenous veins are large vessels taken from the leg, they are commonly used in bypass surgery, so knowledge of their distinct phenotype is important. In culture, they demonstrate a slightly more elongated form than HUVEC, but still express similar amounts of VWF in their Weibel-Palade bodies. In some early experiments, before it was realised that there are differences between endothelium, these were used interchangeably with and in mixed cultures with HUVECs.

1.2.3 Dermal Microvascular Endothelium

The skin is a barrier and protective structure for the body, and as such forms one of the first lines of defence against toxins and infectious agents. The skin is formed of an epidermis (outermost layer) and the underlying dermis.

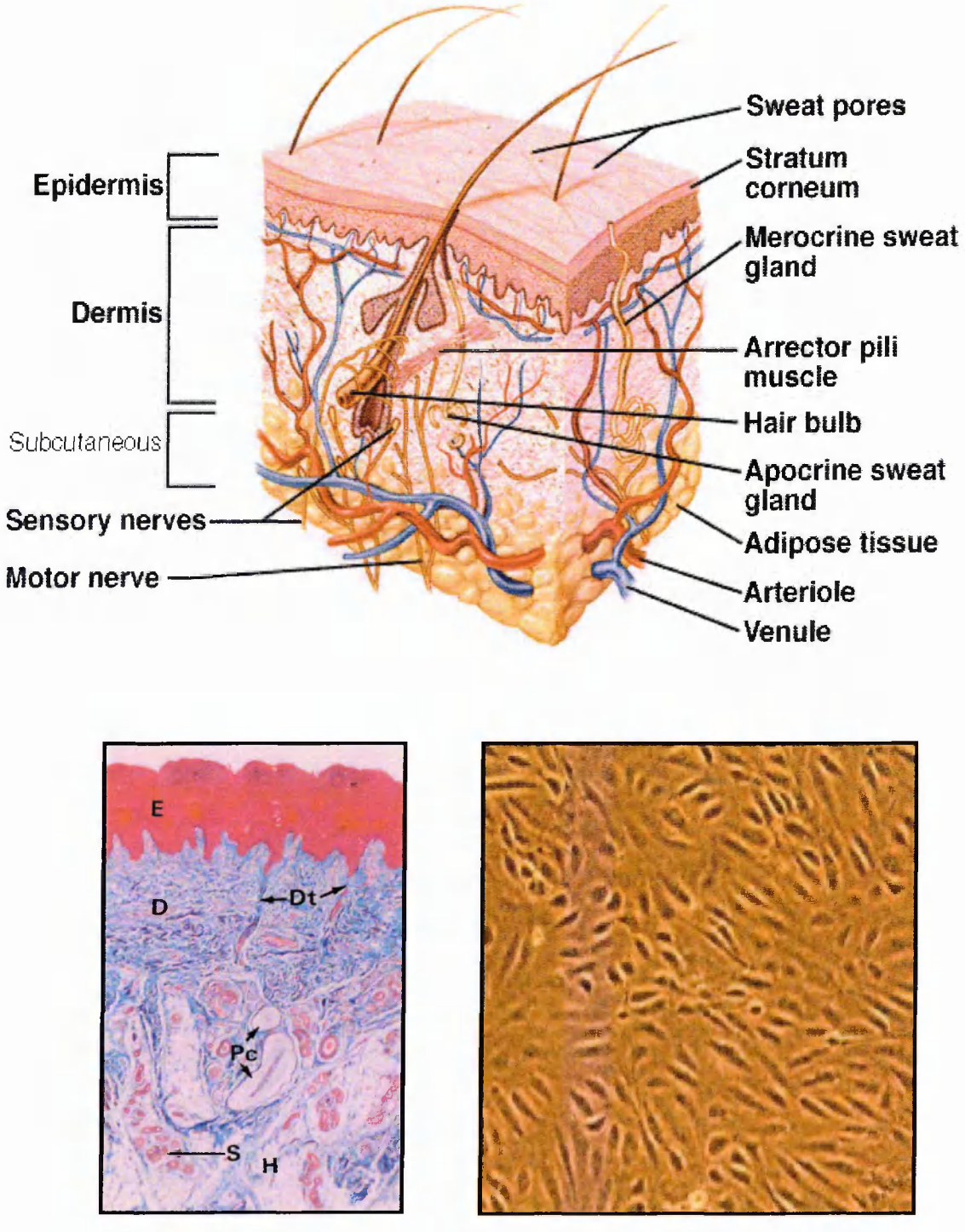


Figure 1.1 Structure of the skin

The overall structure of the skin (A.), a histology section of the skin (B.) showing the layers of epidermis (E), dermis (D) and hypodermis (H). Sweat glands (S), sweat ducts (Dt), and a pacinian corpuscle (Pc) are also shown (from Wheater *et al.* 1985). Figure C. shows the morphology of confluent DMVEC (from Clonetics).

The dermal microvascuiar endothelial cells (DMVEC) are isolated from the dermal layer of the skin (shown in fig. 1.1 a and b). This is the protective layer, and consists of a strong mesh like structure that is created by fibroblasts. Most of the fibres in the dermis are collagen and elastin fibres, which maintain the strength and elasticity of the skin. It is also in this layer that the blood microvessels are found. These capillaries function to regulate body temperature as well as to supply nutrients to the avascular epidermis. DMVEC are extracted first by dissection, then enzymatic digestion and selection either on magnetic beads coated with UEA-1 or by cell sorter. When these cells are contact inhibited, they exhibit the cobblestone type morphology (fig. 1.1c), however at lower densities, they have a much larger, rounder morphology (see fig. 2.1).

Common processes in the skin in which endothelial cells play an important role include wound healing and allergic reactions. In wound healing, the physical damage to the skin must be repaired and infectious agents neutralised. The first cell type to appear after wounding is the neutrophil, at one day after injury, these cells can form as much as 50% of the cells at the injured site. Monocytes are also recruited to the wound site at the same time as neutrophils, and become activated, they are important in the later stage of the reaction and in the production of growth factors and chemokines for the remodelling of the tissue. Lymphocytes and mast cells are also recruited during this type of reaction, lymphocytes not only because they act as effector cells, but because of their cytokine producing properties.

1.2.4 Lung Microvascular Endothelium

Lung capillaries are a highly selective conduit for the exchange of gases between the blood system and the alveolar air space. To aid in this exchange, there is very low blood pressure, very thin vascular walls and a high capillary density with low levels of smooth muscle cells. In this way, the vessels within the lungs are very different from the rest of the vasculature (see fig. 1.2).

Lung microvascuiar endothelial cells (LMVEC) are isolated in the same way as dermal microvascular cells, and demonstrate a very similar morphology, cobblestone at confluence, but bigger and flatter sub-confluence (Carley *et al.* 1992). vWF staining in the lung has been found to be more diffuse and cytoplasmic than seen in other cell types, it does not appear to be attached to the Weibel Palade bodies in any way (fig. 2.1).

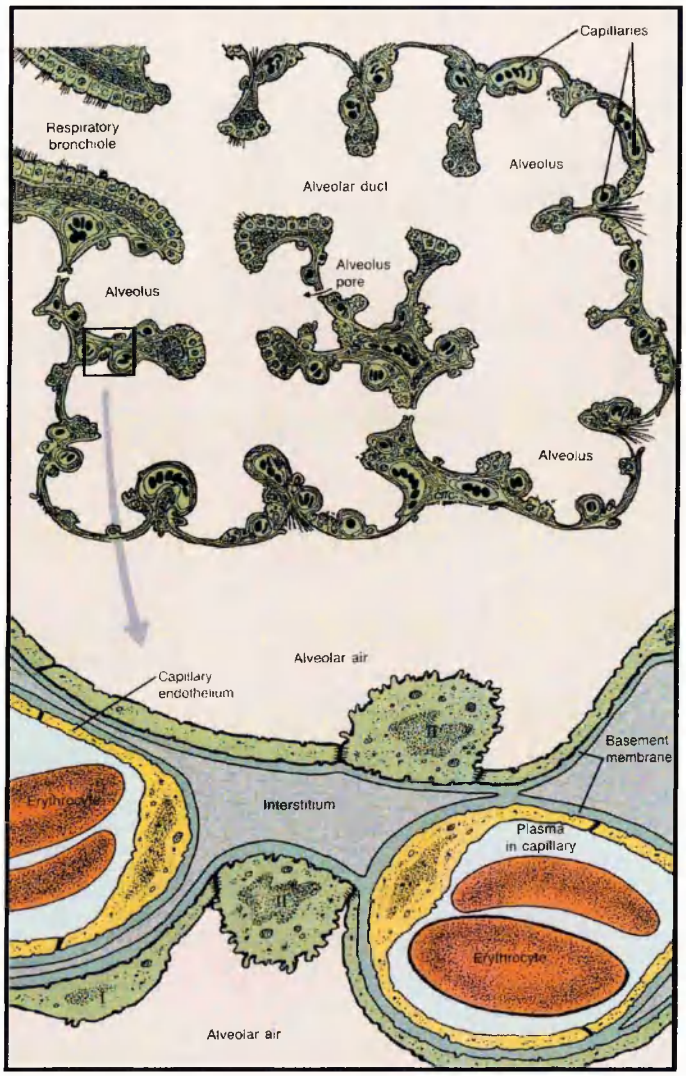


Figure 1.2 Structure of the lungs

A. is a cross section through an area of the lungs showing the structure of the alveoli, B. shows an enlargement of one portion of an alveolus showing the structure of the capillaries in relation to the air spaces modified from Vander *et al.* (1994).

The lungs are an important filter for pathogens, the types of infection often found here include bacteria and viruses. The lungs are also a site for hypersensitivity reactions such as asthma. During allergic inflammation, T_H2 cells recognise the allergen, then produce cytokines such as IL-4, IL-5, IL-10 and IL-13, which involve IgE producing B cells, eosinophils and mast cells. They have various effects including stimulating production of chemokines and other cytokines, inducing adhesion to endothelium and stimulating mast cell degranulation.

1.2.5 Bone Marrow Endothelium

Bone marrow is a primary lymphoid tissue and is the site of origin of all leukocytes, platelets and erythrocytes in the adult human. Bone marrow stroma is composed of monocytes, reticular cells, adipocytes, osteoblasts and fibroblasts as well as endothelial cells (fig. 1.3).

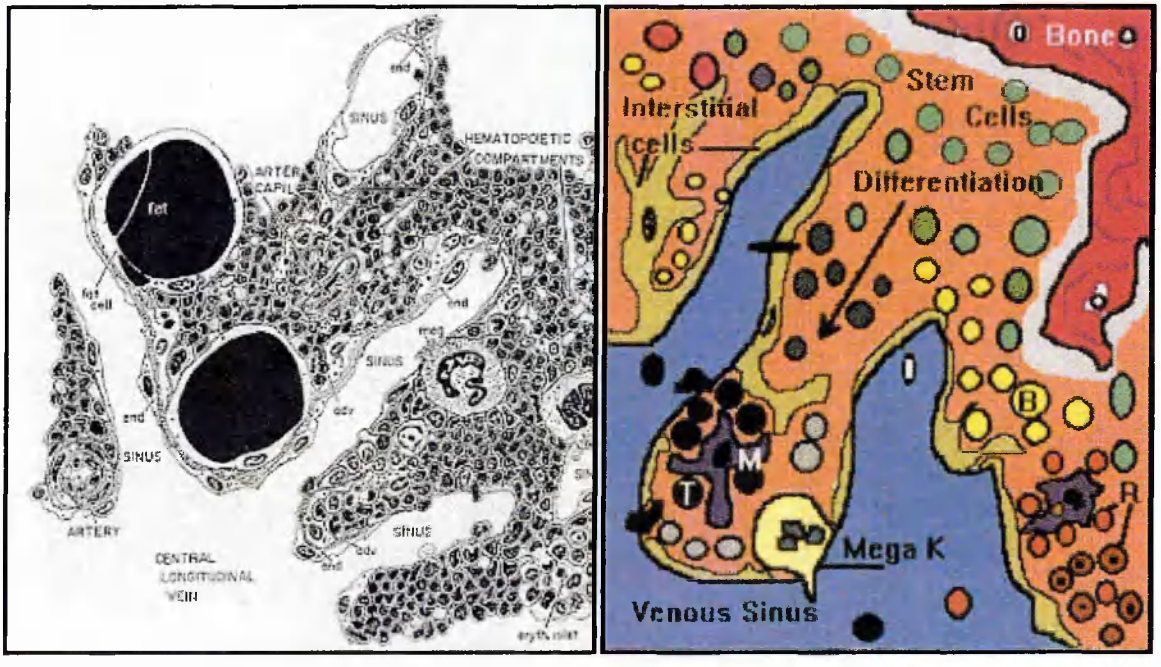


Figure 1.3 The structure of bone marrow

The bone marrow microenvironment, showing the position of the endothelial cells lining the sinuses (A. modified from Weiss and Greep 1977) . B. shows the movement of haematopoietic progenitor cells during differentiation and maturation. Mature cells migrate across the endothelium (light green) into the blood stream.

The stromal cells produce cytokines and matrix proteins forming a complex microenvironment in which haematopoietic cells can proliferate, differentiate and mature. The endothelium situated at the interface between the blood and bone marrow microenvironment can regulate the transport of naïve and mature blood cells into the circulation. *In vivo*, bone marrow endothelium has fenestrated sinusoids, which facilitate this transport.

Because of the scarcity of bone marrow, it was only recently that bone marrow endothelial cells (BMEC) were first isolated and characterised (Masek and Sweetenham 1994; Rafii *et al.* 1994; Rafii *et al.* 1995). It has been found that G-CSF, GM-CSF and IL-6 are all constitutively produced by BMEC. They also express CD34, PECAM (CD 31) and thrombospondin. One of the major differences between bone marrow endothelium and endothelium from the rest of the vasculature is that VCAM-1 and E-selectin are continually expressed both *in vivo* and *in vitro*. It appears that this

is a property of the local microenvironment as after several passages in culture, this phenomenon is lost.

It is because of the difficulty of obtaining BMEC, that recently several different cell lines have been described, each with slightly differing characteristics. The cell line that we have used in this study was first produced by Schweitzer *et al.* (1997). Although it highly expresses CD34, it appears that the VCAM-1 and E-selectin are not expressed unless the cells are cytokine stimulated. Both vWF and UEA-1 are present in these cells – confirming the endothelial phenotype. vWF is present in large granules. Bone marrow is not normally the site of inflammation, but is part of the normal recirculation of leukocytes.

1.2.6 Liver Endothelium

The human liver has many different functions, it acts as a secretory organ for bile and is important in metabolism, but it also has important functions in secretion of hormones, plasma clotting factors, acute phase proteins, binding proteins for steroid hormones as well as many other molecules. The liver consists of a large number of lobules, which usually in section look hexagonal, each lobule contains a central vein that eventually drains to the hepatic vein (fig. 1.4). The cells of the lobule are parenchymal or epithelial glandular cell (hepatocytes) which are arranged in plates, with each plate only being one cell thick and radiating from the centre of the lobule towards the periphery. These plates are separated by sinusoids that are lined by sinusoidal endothelial cells and Kupffer cells (phagocytes).

There are many different types of liver EC – portal vein and sinusoidal being the most common. Those that we are using have been shown to have ICAM-1 and MHC I expressed on their cell surface when resting, but not VCAM-1 or MHC II (see table 1.2). High ICAM-1 is indicative of sinusoidal endothelium.

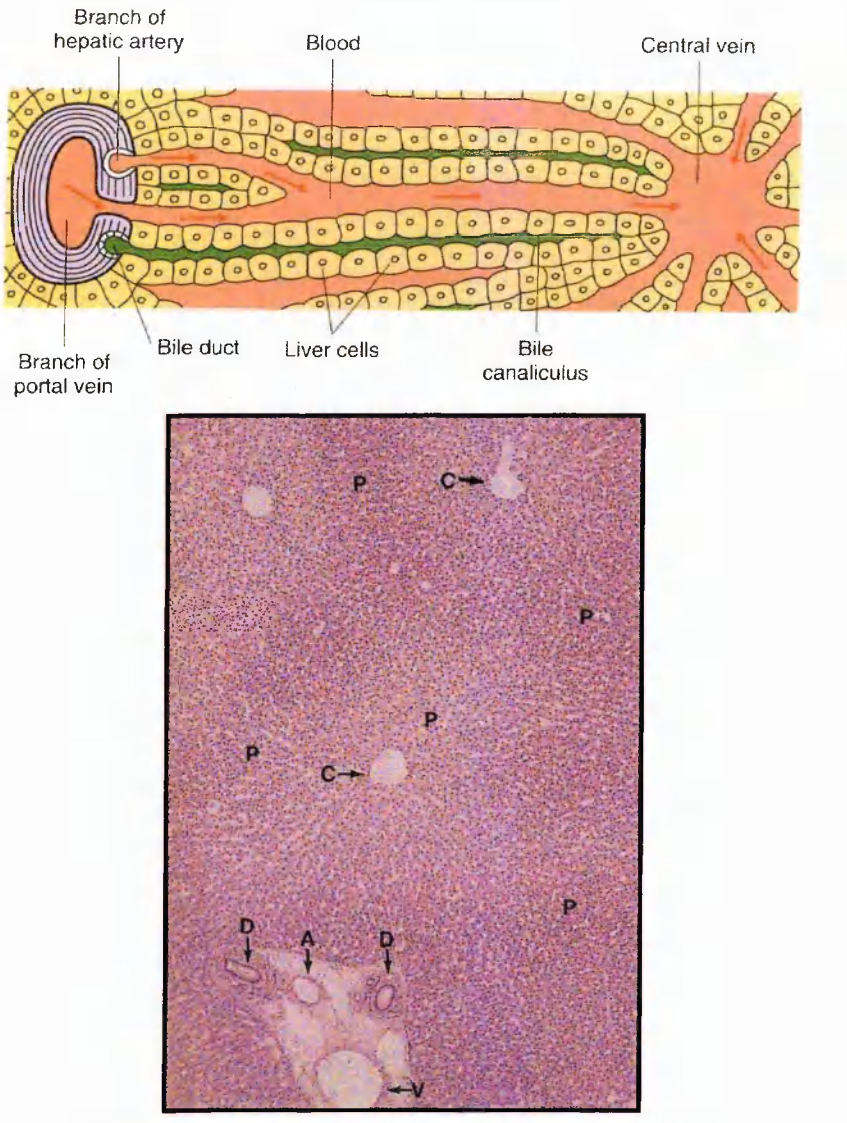


Figure 1.4 Structure of a lobule of human liver

A. diagrammatic representation of the structure of the human liver (taken from Vander *et al.* 1994), B. a liver lobule stained with haematoxylin and eosin showing the portal triad including the portal vein (V), the bile duct (D), and a branch of the hepatic artery (A). Also shown are the hepatocytes of the parenchyma (P) and the central vein (C) picture from Berman *et al.* (1993).

1.3 Leukocyte migration

A crucial step in an effective inflammatory response is the promotion of leukocyte adhesion to the vascular endothelium (Springer 1994; Imhof and Dunon 1995). Leukocytes usually pass through the blood stream as non-adherent cells, but they become adherent in order to migrate into the tissues during normal leukocyte recirculation or during an inflammatory response. Endothelial cells lining the postcapillary venules are the primary site of leukocyte migration.

At sites of inflammation, leukocyte migration is mediated by a sequence of adhesive interactions between endothelial cells and the emigrating leukocyte (summarised in fig. 1.5 and table 1.2). This adhesive cascade allows the circulating immune cell to slow down and finally stop on the vessel wall at the point where the target tissue requires it. Specific stimulatory signals are presented to the recruited leukocyte to enable the cell to generate shear-resistant adhesion to the vessel wall.

1.3.1 Capture and rolling on Selectins

Localisation of leukocytes to specific sites of tissue injury is aided by at least three families of adhesion molecules expressed by the cells involved. These are the selectins, the integrins and the Immunoglobulin (Ig) superfamily (Springer 1995). The first step in this process is margination, where leukocytes leave the central stream of fast flowing cells in the blood vessel and begin interactions with the endothelium. The selectins mediate the initiation of cell contact between leukocytes and endothelial cells, forming weak interactions with the leukocyte which leads to a rolling of the cell along the vessel wall (Lawrence and Springer 1991).

The selectin family consists of three molecules, L-selectin (on leukocytes), P-seiectin (on activated endothelium and platelets) and E-selectin (on activated endothelial cells). They are ail single chain transmembrane glycoproteins, which project up above the surrounding glycocalyx. They recognise structures containing sialylated carbohydrate residues via their C-type lectin domain found at the N-terminus of the molecule. The ligands of selectins include GlyCAM-1, CD34, MAdCAM-1 and PSGL-1, which are all composed of a protein backbone that is then glycosylated. This means that not only is the specific molecule that selectins bind to required, but the correct enzymes need to be present in the environment of the cell for glycosylation.

1.3.2 Activation of the leukocyte

Once rolling along the wall of the vessel, the leukocytes are able to sense signals from the endothelium, the longer a celi is held rolling along the vessel wall; the more likely it is to receive migration signals. If it is not activated, then it will return to the circulation immediately, however, interactions with chemokines, leukotriene B4 or complement components can activate leukocyte integrins and trigger firm adhesion. Chemokines are produced by inflammatory cells and endothelial cells and have been shown to have binding sites for glycosaminoglycans (GAGs) in addition to those for their specific receptors (Webb *et al.* 1993; Tanaka *et al.* 1993a; Witt and Lander 1994). It is thought that they can pass onto the lumenal side of the endothelium where they bind to the proteoglycan layer from which point they can be presented to rolling leukocytes (Tanaka *et al.* 1993b; Middleton *et al.* 1997).

1.3.3 Firm adhesion

Binding then proceeds to a second class of adhesion molecule – the integrins, which via their interactions with cell adhesion molecules of the Immunoglobulin superfamily, mediate firm adhesion. Integrins are heterodimers, which are formed by a combination of one of 17α and 8β chains in humans. Integrins contain large (α) and small (β) subunits of sizes 120 - 170 kDa and 90 - 100 kDa, respectively. They fall into three major families depending on which β chain they have.

The integrins $\alpha_4\beta_1$ (VLA-1), $\alpha_4\beta_7$ (LPAM-1), $\alpha_x\beta_2$ (p150,95) $\alpha_L\beta_2$ (LFA-1), and Mac-1($\alpha_M\beta_2$) have major functions in regulating leukocyte – endothelial interactions. Firm adhesion of leukocytes to endothelium via integrins can occur by two possible mechanisms. The first is increased affinity, of integrins for their ligands which is mediated by the binding of divalent cations such as Mg^{2+} and Ca^{2+} resulting in rapid conformational changes. The second is an increase in avidity through integrin clustering (Stewart *et al.* 1996). This has been shown to strengthen adhesion to immobilised integrin ligands, it works by an enzyme dependent mechanism which releases the integrins from the cytoskeleton of the leukocyte so they can move together.

Chemokines were first suspected of signalling integrin activation, because it was found that adding pertussis toxin (a G-protein signalling inhibitor) could block leukocyte arrest. Several studies have since confirmed this finding for different cell types (Tanaka *et al.* 1993a; Campbell *et al.* 1998; Kitayama *et al.* 1998). A similar effect has also been observed by cross-linking selectin molecules.

The activation, movement and conformational change of the integrins leads to firm arrest of cells on the vessel wall and then transendothelial migration.

The ligands for integrins on endothelial cells are five cell adhesion molecules of the Ig superfamily whose expression on the endothelial cell surface can be regulated by cytokines and also by shear forces (reviewed in Wang and Springer 1998; Yang *et al.* 2002). To be included in this family, one or more immunoglobulin domains should be present, each domain is usually encoded by a discrete exon and consists of a primary sequence of 60 - 70 amino acids with a disulphide bridge spanning 50 - 70 residues. This sequence permits folding into particular tertiary structures consisting of a sandwich arrangement of β pleated sheets known as an antibody fold. The Ig superfamily adhesion molecules involved in migration are all single chain glycoproteins with differing numbers of extracellular Ig-like domains, followed by a transmembrane sequence and a short cytoplasmic domain.

The α4 integrins involved in leukocyte migration bind to endotheliai VCAM-1 and MAdCAM-1 with α4β1 preferentially binding VCAM-1 while α4β7 preferentially binds MAdCAM-1. β2 integrins recognise the Intercellular adhesion molecules (ICAMs), αLβ2 will bind ICAM-1,2 and 3, while Mac-1 will bind ICAM-1. ICAM-2 is constitutively expressed on endothelium and is involved in binding to resting endothelium and normal leukocyte recirculation, while ICAM-1, VCAM-1 and MAdCAM-1 are all inducible with the pro-inflammatory cytokines TNF-α, IL-1 as well as LPS. IFN-γ is only a weak inducer of ICAM-1 alone, however it shows strong synergy with TNF-α or IL-1.

1.3.4 Transendothelial migration

Once activated, integrins interact with members of the Ig superfamily (Ruoslahti, 1991; Hynes, 1992), these interactions allow the leukocytes to squeeze through or between the endothelial cells and cross the vessel wall in a process known as diapedesis. This is the final step in leukocyte transmigration. It is at this stage that adherent cells can extend pseudopodia into the interceilular junctions between endothelial cells, then squeeze through the gap, digest the basement membrane and migrate out into the tissues.

Much less is known about the precise mechanisms of this stage than is known about the first three steps in this cascade. Chemokines have been discovered to be important in this step (Randolph and Furie 1995) and have been found to drive neutrophils to migrate. Other chemokines involved in

recruitment are important for later steps – such as guiding leukocytes through the interstitium to the site of infection etc. There is also evidence for many of the junctional proteins and proteolytic enzymes playing an important role.

It is still controversial as to exactly how the leukocyte transmigrates and whether it passes through the cell or through the intercellular junctions. It is likely that different leukocytes migrate by different methods, which also depend on the type of endothelium and whether it is continuous or discontinuous.

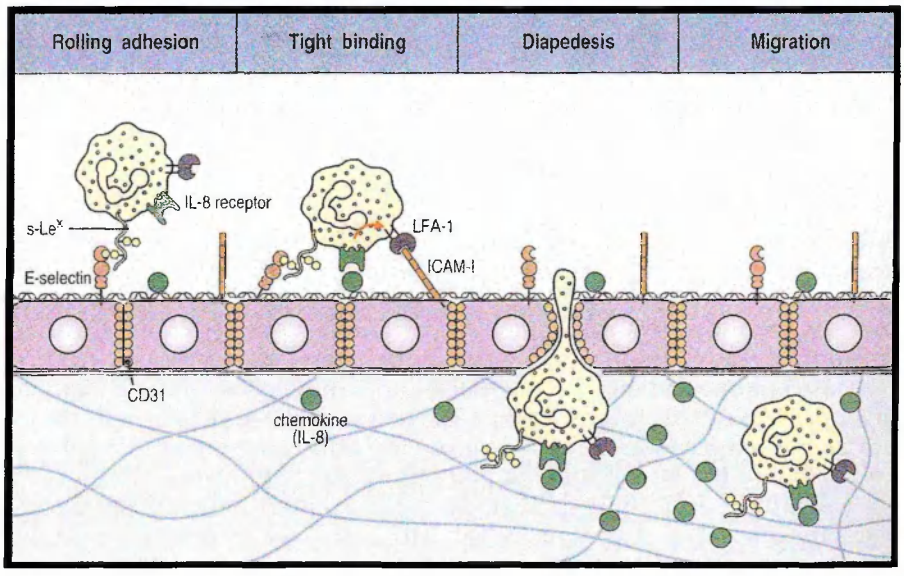


Figure 1.5 Leukocyte migration

Showing the different stages of leukocyte migration; 1. Capture and rolling on selectins, 2. Activation of the leukocyte and tight binding by integrins, 3. Transendothelial migration, 4. Migration through the tissues and ECM to the injury site. (from Janeway and Travers 1997)

Table 1.2 Adhesion Molecules involved in Leukocyte Recruitment

ADHESION MOLECULE	EXPRESSION	LIGAND	EXPRESSION OF LIGAND
E-selectin	Cytokine activated endothelium	Sialylated Lewis X and similar structures, E selectin ligand	Neutrophil, Monocyte, Eosinophil, Basophil, some lymphocytes, NK cells
L-selectin	Monocytes, Neutrophils, Eosinophils, some Lymphocytes	Sialylated, fucosylated, sulphated carbohydrates found on: GlyCAM-1, CD34, MAdCAM-1	HEV, Activated endothelium
P-selectin	Activated Endothelium, platelets	Sialomucin side chains of PSGL	Neutrophils, Monocyte, Eosinophil, Basophil, NK cells, some lymphocytes
αLβ2 (LFA-1)	All lymphocytes, monocytes, neutrophils,	ICAM-1, 2	Endothelium and other cell types (constitutive and cytokine inducible)
αMβ2 (Mac-1)	Monocytes, neutrophils	ICAM-1, C3b, fibronectin, factor X	Endothelium (and others)
αXβ2 (p150,95)	Monocytes, Neutrophils, Eosinophils	C3b, fibronectin	
α4β1 (VLA-4)	Lymphocytes, monocytes, basophils, eosinophils	VCAM-1, fibronectin,	Endothelium, macrophages and dendritic cells (constitutive and cytokine inducible)
α4β7 (LPAM-1)	Lymphocyte subpopulations	MAdCAM-1, VCAM-1, fibronectin	Mucosal endothelium

1.4 Chemokines

Chemokines are a family of small molecular weight proteins (8 – 14 kDa) with 20 – 70% homology in amino acid sequences. In 1977, Platelet factor 4 (PF-4) was first described, but it was a further 10 years before IL-8 was isolated from culture supernatants of stimulated human blood monocytes and identified as a chemoattractant for leukocytes (Walz *et al.* 1977; Walz *et al.* 1987; Yoshimura *et al.* 1987). Since then many more chemokines have been identified and have been found to function as regulatory molecules in immunosurveillance, homeostasis, traffic and homing of lymphocytes and the development and differentiation of naïve cells of the immune system. To date, more than 40 different human chemokines have been discovered (Baggiolini *et al.* 1994; Baggiolini *et al.* 1997; Zlotnik *et al.* 1999). They are classified into four distinct groups, CXC, CC, C and CX3C chemokines, which depend on the number and positioning of the cysteine residues at the N-terminus. The CXC and CC are the largest families and the most characterised. The CXC group has one amino acid between the first and second cysteines while in the CC group the cysteines are next to each other. There are two other chemokines that do not fit into these classes and map to different loci, so each forms their own class. Lymphotactin (C class) has lost the first and third cysteines, and hence has only one cysteine at the N-terminus (Kelner *et al.* 1994) but fractalkine (CX3C) is a membrane spanning protein that has 3 amino acids between the first and second cysteine (Bazan *et al.* 1997). The CXC chemokines can be further classified into ELR+ or ELR- chemokines according to the presence or absence of the glutamate - leucine – arginine motif between the N-terminus and the first cysteine. ELR+ chemokines are specific for monocytes and granulocytes, while ELR- attract a variety of leukocytes. It has been shown that IL-8 loses its activity when the ELR motif is removed or mutated. PF4, which is normally ELR-, can induce degranulation and chemotaxis of neutrophils when the ELR motif is inserted. So it appears that this motif is particularly important in the binding of neutrophils through the CXCR1 and 2 receptors. The nomenclature has recently been updated to reduce problems with the numerous names that had come into being for each different chemokine (Zlotnik and Yoshie 2000). This is shown in table 1.3.

Table 1.3 Chemokine classification and nomenclature

FAMILY	SYSTEMATIC NAME	COMMON NAME	RECEPTOR	ELR STATUS
CXC	CXCL1	Gro-α / MGSA-α	CXCR1, 2	ELR+
	CXCL2	Gro-β / MGSA-β	CXCR2	ELR+
	CXCL3	Gro-γ / MGSA-γ	CXCR2	ELR+
	CXCL4	PF-4	Unknown	ELR+
	CXCL5	ENA-78	CXCR2	ELR+
	CXCL6	GCP-2	CXCR1, 2	ELR+
	CXCL7	NAP-2	CXCR1, 2	ELR+
	CXCL8	IL-8	CXCR1, 2	ELR+
	CXCL9	Mig	CXCR3	ELR-
	CXCL10	IP-10	CXCR3	ELR-
	CXCL11	I-TAC	CXCR3	ELR-
	CXCL12	SDF-1α / -1β	CXCR4	ELR-
	CXCL13	BLC / BCA-1	CXCR5	ELR-
	CXCL14	BRAK / bolekine	Unknown	ELR-
XC	XCL1	Lymphotactin / SCM-1α	XCR1	
	XCL2	SCM-1β	XCR1	
CX_3C	CX3CL1	Fractalkine	CX3CR1	
CC	CCL1	I-309	CCR8	
	CCL2	MCP-1 / MCAF	CCR2	
	CCL3	MIP-1α	CCR1, 5	
	CCL4	MIP-1β	CCR5	
	CCL5	RANTES	CCR1, 3, 5	
	(CCL6)	Mouse C10 / MRP-1	Unknown	
	CCL7	MCP-3	CCR1, 2, 3	
	CCL8	MCP-2	CCR3	
	(CCL9)	reserved	-	
	(CCL10)	reserved	-	
	CCL11	Eotaxin	CCR3	
	(CCL12)	Mouse MCP-5	CCR2	
	CCL13	MCP-4	CCR2, 3	
	CCL14	HCC-1	CCR1	
	CCL15	HCC-2 / Lkn-1 / MIP-1δ	CCR1, 3	
	CCL16	HCC-4 / LEC / LCC-1	CCR1	
	CCL17	TARC	CCR4	
	CCL18	DC-CK-1 / PARC / AMAC-1	Unknown	
	CCL19	MIP-3β / ELC / Exodus-3	CCR7	
	CCL20	MIP-3α / LARC / Exodus-1	CCR6	
	CCL21	6Ckine / SLC / Exodus-2	CCR7	
	CCL22	MDC / STCP-1	CCR4	
	CCL23	MPIF-1 / MIP-3	CCR1	
	CCL24	MPIF-2 / Eotaxin-2	CCR3	
	CCL25	TECK	CCR9	
	CCL26	Eotaxin-3	CCR3	
	CCL27	CTACK	CCR10	

Processing from a longer protein of 92 – 125 amino acids to a shorter version of 70 – 100 amino acids is common to many chemokines. For example, the open reading frame of IL-8 cDNA codes for 99 amino acids, while the mature form has been processed to a 72 amino acid protein.

In general, CC chemokines exhibit attraction for monocytes, lymphocytes, eosinophils and basophils while CXC chemokines are chemoattractant for neutrophils and the CXCR3 binding

chemokines are specifically attractant for activated T cells. The effects of chemokines are mediated by their specific G-protein coupled receptors and cellular responses depend on the presence of the relevant receptor, which determines the spectrum of action of the chemokines (Horuk 2001). Chemokines not only mediate chemotaxis, but also have many other functions including mediator release by triggering degranulation of intracellular vesicles or upregulation of integrins. Until recently, leukocytes were thought to be the only sites of chemokine action, but there is increasing evidence that other cell types are capable of binding and responding to various chemokines.

As I have previously described, chemokines are a key element in the multistep process of leukocyte recruitment (Springer 1994; Baggiolini 1998; Olson and Ley 2002) and are produced by endothelial cells in response to molecules involved in inflammatory reactions, immunity and thrombosis. The chemokine repertoire of the EC includes members of both the CXC (IL-8, IP-10, ENA-78 and gro-α) and CC (MCP-1, MCP-3 and RANTES) families of chemokines. Most studies so far have focussed on IL-8 and MCP-1.

Chemokines have also been found to have a separate and very distinct heparin-binding site, which allows them to bind to heparan sulphate present on the proteoglycan layer (Lortat-Jacob *et al.* 2002). They are highly basic molecules and can be immobilised by interacting with the negatively charged GAGs. Binding of chemokines to HSPG is thought to assist in dimerization and orientation of the chemokine (Hoogewerf *et al.* 1997). Chemokines have been found to have enhanced activities when bound to GAGs. Specific chemokines have been found to bind different types of GAGs with different affinities (Kuschert *et al.* 1999). Since GAG type can vary with cell type, location and inflammatory status, the regulation of GAG expression by different endothelial cell types could be important within the cascade of leukocyte migration. The involvement of both adhesion molecules and chemokines in leukocyte extravasation means that selectivity for a tissue is determined combinatorially, with selectins, integrins, chemokines and GAGs all taking part (Mackay 2001).

1.5 Chemokine receptors

The specific effects of chemokines on their target cells are mediated by seven-transmembrane-spanning, G-protein coupled receptors (specifically Gαi coupled receptors)(Gerard and Gerard 1994; Murphy *et al.* 2000). The extracellular loops and NH_2-terminus are responsible for ligand binding, while the intracellular loops and carboxy-terminal tail are involved in G-protein interactions and signal transduction (see fig. 1.6). Chemokine receptors are defined by their ability to signal on binding one or more chemokines. These receptors are part of a much larger family of G-protein coupled receptors which can react to many other molecules including hormones, neurotransmitters, paracrine substances and other inflammatory mediators. Although each of these chemokine receptors binds only a single class of chemokines, they can bind to several different members of the same class with high affinity. For example CXCR3 binds to IP-10, I-TAC and Mig with high affinity, though it has been shown to bind I-TAC better than the other two. Only the promiscuous chemokine binding proteins DARC and D6 (not true chemokine receptors as they do not signal) have been shown to bind both CC and CXC chemokines.

Chemokine receptor binding initiates a cascade of intracellular events which culminates in specific biological effects. When the chemokine binds to its receptor, it induces a conformational change in the G-protein and splits off the α subunit off from the βγ subunit. These subunits can act as second messengers and activate several enzyme cascades, which induce calcium mobilisation and protein kinase activation. These reactions can have many different cellular functions depending on the particular cell type that they take place in, including increased adhesion molecule expression, stimulation of the respiratory burst, degranulation or phagocytosis.

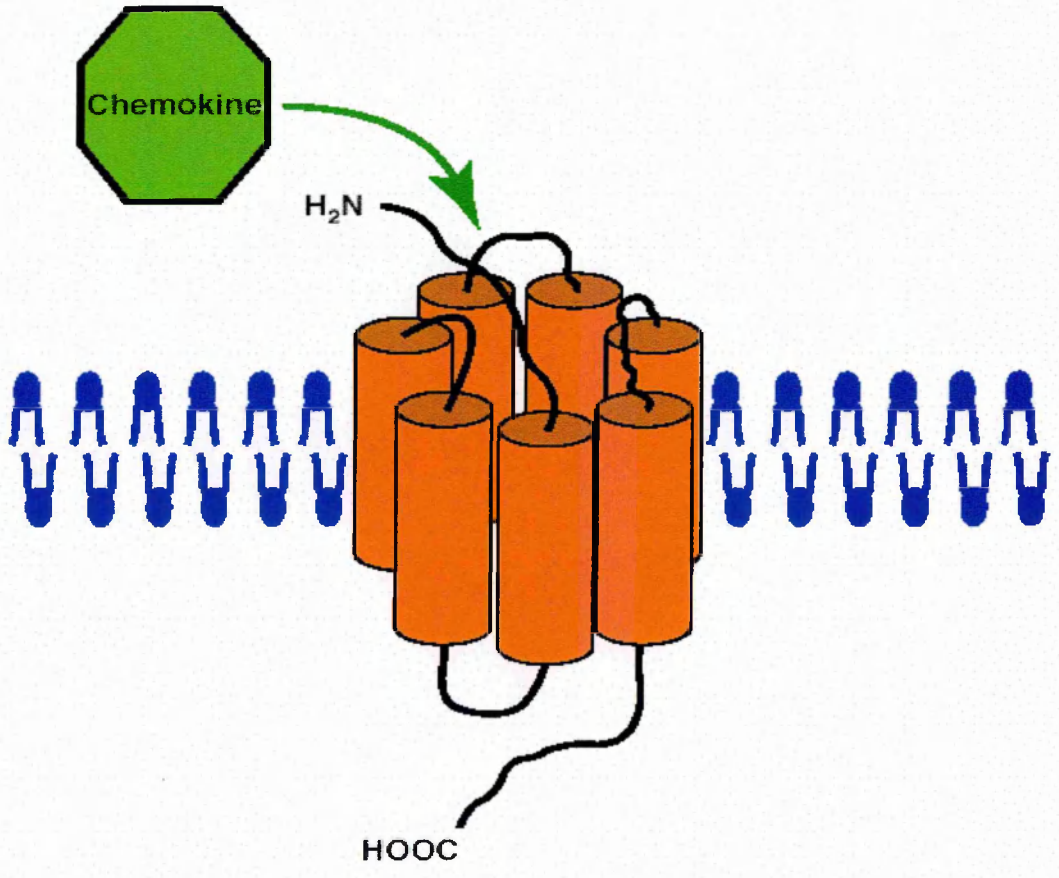


Figure 1.6 Chemokine receptor structure

Showing the seven transmembrane domains of the receptor and the position in which the chemokine binds. (modified from Murdoch and Finn 2000 with thanks to J.L. Wood for help with this image).

The first chemokine receptor to be cloned was the IL-8 receptor in 1991 (Beckmann *et al.*), to date a total of 18 chemokine receptors have been discovered (Table 1.4). Five receptors selectively bind certain CXC chemokines (CXCR1-6), whilst the CC receptor family consists of a further eleven receptors (CCR1-10). There is one receptor (XCR1) for lymphotactin – the one representative of the C family and one receptor for fractalkine (CX3CR1). A further two chemokine binding proteins have also been identified, the first is known as the Duffy antigen receptor for chemokines (DARC) which has been shown to bind promiscuously to both CXC and CC chemokines. The second is known as D6 and has been shown to bind several CC chemokines (Nibbs *et al.* 1997). DARC has a possible role as a sink for some chemokines to maintain gradients of more important chemokines (Horuk *et al.* 1994). These two binding proteins apparently do not signal and so are excluded from the standard nomenclature.

Table 1.4 Characteristics of chemokine receptors currentiy known

RECEPTOR	HIGH AFFINITY LIGANDS	CELLULAR DISTRIBUTION
CXCR1	IL-8, GCP-2,	Neutrophils, Monocytes, Macrophages, Mast cells
CXCR2	IL-8, Gro-α,Gro-β, Gro-γ, NAP-2, ENA-78, GCP-2	Neutrophils, Monocytes, Macrophages, Eosinophils, Mast cells
CXCR3	IP-10, ITAC, Mig	Activated T cells, B cells, NK cells
CXCR4	SDF-1	Neutrophils, Monocytes, Macrophages, Dendritic cells, Resting T cells, B cells, Progenitor cells
CXCR5	BCA-1	B cells, memory T cells
CXCR6	SexCKine	Dendritic cells, T cells
CCR1	RANTES, MIP-1α, MCP-3, HCC-1, Lkn-1, MCP-2, MPIF-1	Neutrophils, Macrophages, Eosinophils, Basophils, Immature dendritic cells, Memory T cells, NK cells
CCR2	MCP-1 - 4	Neutrophils, Macrophages, Activated T cells, B cells, NK cells
CCR3	Eotaxin, Eotaxin-2, MCP-2-4, Lkn-1, RANTES, Eotaxin-3	Eosinophils, Basophils, Th2 cells
CCR4	TARC, MDC	Immature dendritic cells, Th2 cells, NK cells
CCR5	MIP-1α, MIP-1β, RANTES	Monocytes, Macrophages, Dendritic cells, T cells (Th0, Th1)
CCR6	LARC	Immature dendritic cells, Memory T cells, B cells
CCR7	ELC, SLC	Mature dendritic cells, T cells, B cells
CCR8	I-309	Monocytes, Macrophages, Th2 cells, Thymus
CCR9	TECK	α4β7+ T cells, Thymus
CCR10	Eotaxin-3, CTACK, MEC	T cells, Langerhans cells
CX3CR1	Fractalkine	Monocytes, Macrophages, T cells, NK cells
XCR1	Lymphotactin	T cells, NK cells
DARC	IL-8, Gro-α, RANTES, MCP-1, NAP-2	Erythrocytes
D6	MCP-1-4, MIP-1β, RANTES, HCC-1	T cells

1.6 The Proteoglycan Layer

The endothelial cell surface is coated with a thick glycocalyx (15 – 40 nm thick); this is a carbohydrate rich zone, which on endothelial cells is composed of glycoproteins (Simionescu *et al.* 1982; Simionescu and Simionescu 1986). Most of the carbohydrates present are attached to membrane proteins, but a small proportion are secreted glycoproteins which have been adsorbed onto the cell surface.

Proteoglycans are a specific class of glycosylated proteins, which consist of a core protein with unbranched glycosaminoglycan (GAG) chains attached to it (reviewed in Kjellen and Lindahl 1991). There are more than 20 different core proteins, but those present on human endothelium, include the syndecans, the glypicans and CD44. GAGs are sulphated polysaccharide chains made up of repeating disaccharide subunits (usually 40 to 100 repeats). The GAGs that are commonly encountered in human tissues include chondroitin sulphate, dermatan sulphate, heparan sulphate and keratan sulphate, they each consist of different disaccharides with different sulphation patterns.

Table 1.5 Composition of glycosaminoglycans

DISACCHARIDE	GLYCOSAMINOGLYCAN	SULPHATION PATTERN
Glucuronic acid / iduronic acid-N-acetylgalactosamine	Dermatan sulphate	Irregular spaced pattern
	Chondroitin sulphate	Regular pattern
Glucuronic / iduronic acid-N-acetylglucosamine	Heparan sulphate	Irregular spaced pattern
	Heparin	Regular pattern
Galactose-N-acetylglucosamine	Keratan sulphate	

1.6.1 Proteoglycan synthesis

The core protein is made on membrane bound ribosomes and is then threaded into the lumen of the endoplasmic reticulum, but it is turned into a proteoglycan when the polysaccharide side chains are attached in the golgi. The synthesis of GAGs in the golgi is quite complex, but basically a special link tetrasaccharide is attached to serine residues on the core protein. This serves as a primer for polysaccharide growth as glycosyl transferases add one sugar residue at a time. While

still in the golgi, the molecule is modified by sulphation (which gives the molecule its negative charge) and epimerisation, which alters the configuration of the molecule around individual carbon atoms.

Proteoglycans have potentially unlimited heterogeneity. The core proteins can range from 10 to 600 kDa and have a great variety of numbers and types of GAG chains, which can be modified by different amounts of sulphation. This makes it difficult to accurately identify the proteoglycans in particular in terms of their sugars although the core proteins can be identified. There have been no particular structural motifs found which could aid in identification.

1.6.2 Biological effects of proteoglycans

Proteoglycans can be attached either to the basement membrane or to the surface of cells – via a transmembrane protein core (e.g. syndecans) or via covalent binding to phospholipids (e.g. glypicans). They have many functions in cells, where they perform a major role in cell to cell signalling and can also bind to and regulate the activity of secreted proteins including growth factors, proteases and protease inhibitors. They are also hydrophilic and can trap water, hence providing hydrated space around and between cells.

Heparan sulphate is the major GAG expressed on cell surfaces and is usually present as heparan sulphate proteoglycan (HSPG). In this form, it is important in cell growth and adhesion and also maintains a non-thrombogenic surface on the lumen of blood vessels (Gallagher 1995). Other important effects include aiding the dimerisation of certain protein mediators.

One of the most important roles of HSPG on the endothelial surface is in the regulation of anti-thrombin – an inhibitor of pro-coagulant proteases in blood and vascular tissues. HSPG accelerates the inhibition of thrombin and activated factor X by anti-thrombin, hence exerting effects on the coagulation pathway. Binding of heparan sulphate to anti-thrombin causes a conformational change, and exposes the reactive site for thrombin.

Heparan sulphate can also be involved in the activation of bFGF. By binding to heparan sulphate, binding to its specific FGF receptor is mediated. Similar HSPG binding properties also exist for other cytokines including TGF-β, IFN-γ, IL-3, GM-CSF and VEGF.

1.7 Chemokines involved in inflammation

The major role of chemokines is in the control of leukocyte migration. Today – fifteen years after the discovery of IL-8, numerous chemokines have been identified as attractants of different types of blood leukocytes to sites of infection and inflammation. They are produced locally in the tissues, by many cell types, and act on leukocytes and other cell types through selective receptors.

Many peptide chemoattractants may be produced in one place – for example if a bacterial infection occurs. It is thought that leukocytes can follow complex chemokine gradients in a step by step manner - from one gradient to the next and can integrate many signals. Chemokines in this situation act in an overlapping and sequential fashion on each cell (Foxman *et al.* 1997), and this may explain how receptor promiscuity and specificity are combined *in vivo*.

The effects of chemokines on cells are very rapid, the most impressive effect being the immediate shape change caused by the polymerisation and breakdown of actin. This leads to the formation and retraction of lammellipodia, whose function is in moving the cells during migration. Another rapid effect is the upregulation and activation of integrins on the leukocyte which enable the cell to tightly adhere to the blood vessel wall and subsequently migrate through the wall of the vessel. Adhesion via integrins has recently been shown to require a higher threshold concentration of chemokine present than that required for chemotaxis, therefore receptor expression may not necessarily mean that adhesion triggering will occur. There are several other effects of chemokines which can be observed on different celi types – including a rise in intracellular calcium levels, the production of microbicidal oxygen radicals and the release of the contents of certain cytoplasmic storage granules.

Many chemokines are produced under conditions of inflammation or infection by tissue cells and infiltrating leukocytes, however some chemokines are constitutively expressed by certain cell types and seem to fulfil housekeeping functions. Some chemokines (e.g. SDF-1α, SLC, MIP-3β) are capable of triggering adhesion of most lymphocytes, others are more specialised and only activate particular subsets (e.g. MIP-3α - memory CD4+ T cells). This suggests that only certain cell types will be activated – that are specific to the process in hand.

The effector phase of an immune response involves the secretion of cytokines by antigen stimulated leukocytes, which serve to recruit and activate the various responsive cell populations

that comprise the inflammatory infiltrate, among them the mononuclear phagocytes which in turn secrete additional cytokines that influence the reaction. Under certain circumstances, the immune response can be activated inappropriately and targeted at normal healthy tissue. Chemokines are associated with these problems as much as with a normal immune response for example in allergic diseases such as asthma or dermatitis and in autoinflammatory diseases such as multiple sclerosis or rheumatoid arthritis. In recent years, there has been much interest in the therapeutic potential of chemokines and chemokine antagonists (Saunders and Tarby 1999; Schwarz and Wells 1999; Cascieri and Springer 2000; Power and Proudfoot 2001; Ajuebor *et al.* 2002).

In this thesis I will be concentrating specifically on differences between cell types in chemokine activities during inflammation. To do this, I have chosen a panel of pro-inflammatory chemokines to examine, which will now be discussed in greater detail.

1.7.1 IL-8 (CXCL8)

IL-8 is a member of the CXC chemokine family and was purified as a monocyte derived factor that attracts neutrophils, but not monocytes (Yoshimura *et al.* 1987). It acts via two receptors, CXCR1 and CXCR2 and is the most potent neutrophil chemotactic agent known. The ELR motif is particularly important in neutrophil chemotaxis. Many other cell types produce IL-8 as well as monocytes – in particular T cells, neutrophils, fibroblasts, endothelial and epithelial cells. It triggers β2 integrin mediated firm adhesion of monocytes to endothelium, stimulates the respiratory burst in neutrophils, induces degranulation and promotes attachment and transendothelial migration of neutrophils (Huber *et al.* 1991). IL-8 also induces histamine release from basophils (White *et al.* 1989), has chemotactic activity for lymphocytes and eosinophils and has important functions in angiogenesis.

IL-8 in its final form can be either 72 or 77 amino acids in length depending on which cell type produced it and exactly which enzymes are used in processing (Hebert *et al.* 1990). The 72-amino acid form is predominant in cultures of monocytes and macrophages, while the longer form is common in supernatants from tissue cells such as endothelial cells. The longer form is considerably less potent as a neutrophil chemoattractant *in vitro*, but *in vivo* it shows similar chemotactic activity to the shorter form, perhaps due to processing mechanisms which are present *in vivo*, but not *in vitro*. IL-8 is expressed early in many types of infection and wounding, bringing neutrophils to the damaged site.

1.7.2 MIP-1α (CCL3)

Macrophage inflammatory protein - 1α (MIP-1α) is a 69 amino acid CC chemokine produced by fibroblasts, monocytes, lymphocytes, neutrophils, eosinophils, smooth muscle cells and mast cells. It was first purified from LPS treated monocytic cell lines (Wolpe *et al.* 1988). It binds to CCR1 (Gao et al. 1993; Neote et al. 1993b) and CCR5 (Raport *et al.* 1995; Raport *et al.* 1996) and has been found to have effects on most leukocytes with the exception of B cells, but the predominant cell type that it chemoattracts is $CD8^+$ T cells. MIP-1α production is inducible in many cell types by TNF-α, IL-1 and LPS and on neutrophils by IFN-γ. TGF-β appears to downregulate the production of MIP-1α by macrophages (Sherry *et al.* 1998). Its effects, apart from chemotaxis of most cells of the immune system, include stimulating histamine release by basophils and mast cells, triggering β2 integrins on monocytes, stimulating degranulation of eosinophils, the oxidative burst in neutrophils and enzyme release by macrophages. It also increases cytokine production and proliferation of monocytes, however it has opposing effects on T cells – decreasing levels of cytokines produced and inhibiting proliferation, but increasing adhesion.

MIP-1α is expressed in bronchoalveolar lavage fluid harvested from patients with lung diseases or hypersensitivity reactions (Alam *et al.* 1996; Capelli *et al.* 1999) and is found at increased levels in the cerebrospinal fluid of patients with multiple sclerosis (Miyagishi *et al.* 1995).

1.7.3 RANTES (CCL5)

RANTES (regulated upon activation, normal T-cell expressed, and secreted) is 68 amino acids long and induces leukocyte migration by binding CCR1, CCR3 and CCR5 (Schall 1991; Appay and Rowland-Jones 2001). RANTES is specifically chemotactic for T cells of the memory subtype, monocytes and eosinophils (Schall *et al.* 1990; Alam *et al.* 1993). It is produced *in vivo* during allergic reactions and is thought to play an important role in accumulation of CD4+ memory T cells during delayed type hypersensitivity (DTH) reactions. Induction of RANTES gene expression and production of RANTES protein has been shown to be optimally produced by a combination of TNF-α and IFN-γ (Marfaing Koka *et al.* 1995). Gene induction by TNF-α or IFN-γ has also been reported in other cell types – IFN-γ stimulates RANTES production in monocytes (Devergne *et al.* 1994), while TNF-α and IL-1β stimulate RANTES gene induction in fibroblasts and renal tubular epithelial cells (Rathanaswami *et al.* 1993; Pattison *et al.* 1994). RANTES induces calcium mobilisation and

migration in dendritic cells and mediates the trafficking of many different cell types including T cells, monocytes, basophils, NK cells, DC and mast cells. It is produced by CD8+ T cells, epithelial cells, fibroblasts and platelets.

RANTES has been implicated in a wide range of diseases and disorders – usually by the promotion of infiltration of leukocytes e.g. in arthritis, atopic dermatitis, asthma and DTH reactions (Rathanaswami *et al.* 1993; Alam *et al.* 1996; Thornton *et al.* 1999). It is also important in the response to viral infection as it is degranulated from activated cytotoxic T cells along with perforin and granzyme A. It is of particular interest for researchers because of its ability to suppress replication of HIV and because of its binding to CCR5 which is now know to be an HIV co-receptor (Lehner 2002). The precise role of RANTES In suppression of HIV is still not known, but it appears to be critically dependent on the GAGs present on the surface of the infected cells.

RANTES is known to form dimers, but this does not appear to be essential for receptor binding. However the binding to GAGs plays a central role in trafficking and migration of leukocytes as this appears to be the way in which dimers and concentration gradients are formed.

1.7.4 MCP-1 (CCL2)

Monocyte chemoattractant protein – 1 (MCP-1) was one of the first discovered and is now the most extensively studied of the CC chemokines. Human MCP-1 was purified from culture supernatants of blood mononuclear cells, glioma and myelomonocytic cell lines as a monocyte (but not neutrophil) chemoattractant (Matsushima *et al.* 1989). It has now been found to be active on several other leukocyte subsets. MCP-1 is produced by many different cell types including leukocytes, endothelial cells and fibroblasts after stimulation. The mature protein is 76 kDa (once its signal sequence has been cleaved) and can be glycosylated with a slight reduction in chemotactic activity as a result (Proost *et al.* 1998). MCP-1 has several closely related family members (MCP 2-4 and 3 eotaxins) with 56 – 71% sequence homology. MCP-1 binds strongly to CCR2, which is widely expressed in different cells of the immune system such as basophils, monocytes, activated memory T cells, NK cells and blood derived dendritic cells. CCR2 receptors are upregulated on the surface of T cells after stimulation with IL-2, but are constitutively expressed on the monocyte cell surface. LPS and IFNγ have been found to downregulate the CCR2 receptor on monocytes – hence reducing the effects of MCP-1. At physiological concentrations MCP-1 is present as a monomer, but will form dimers at high (100 μM) concentrations. CCR2 receptor

triggering may require dimerisation (Rodriguez-Frade *et al.* 1999), which is thought to be mediated by binding to heparan sulphate.

As its name suggests, the most important function of this chemokine is as a mononuclear cell chemoattractant. MCP-1 chemoattracts T cells and monocytes at very low concentrations (0.1 - 0.3 nM and 0.01 - 0.1 nM respectively) (Van Damme *et al.* 1992; Taub *et al.* 1995a; Uguccioni *et al.* 1995). At higher concentrations (>3 nM) it can attract basophils, stimulated NK cells and DC (Taub *et al.* 1995b). MCP-1 also induces enzyme release by T cells, monocytes and NK cells which allows digestion of the ECM as part of migration toward an injury or infection. It also stimulates basophils to release histamine and leukotrienes and monocytes to upregulate integrins CD11b and CD11c.

In vivo, transgenic mice with promoters that allow selective expression of MCP-1 in different organs all showed similar accumulation of mononuclear cells in the particular organ that was over expressing this chemokine. Knockout models have shown defects in monocyte recruitment in response to inflammatory stimuli or antigen challenge. CCR2 -/- mice had defects in DTH responses and in IFN-γ production (Boring *et al.* 1997).

Increased expression of MCP-1 is evident in many disease processes in humans; atherosclerosis is the best studied, where it is thought to be crucial in disease initiation, but it is also found in psoriasis and several types of hypersensitivity reactions in the skin. MCP-1 is increased in the synovial fluid of those patients with rheumatoid arthritis and is found in many lung diseases particularly allergic reactions where it stimulates release of histamine. It also is implicated in MS and the response to microbial infection.

The control of MCP-1 expression has been extensively studied unlike other chemokines and has proven quite complicated due to the number of different inputs that stimulate and repress its expression. There is one responsive element that appears to regulate basal promoter activity, another that responds to shear stress, a GC box that regulates basal transcriptional activity (Ueda *et al.* 1994; Shyy *et al.* 1995; Ueda *et al.* 1997). Several NFκB elements as well as an AP-1 sequence have been found at different sites in different cell types, which respond specifically to TNF-α and IL-1β. Some cells can respond to IFN-γ which induces the MCP-1 promotor via a gamma activated sequence (Majumder *et al.* 1996). Only the NFκB and AP-1 sites have been shown to be implicated in endothelial production of MCP-1 so far (Martin *et al.* 1997).

1.7.5 IP-10 (CXCL10)

IP-10 was identified by Luster *et al.* (1985) as an interferon inducible gene. They subsequently characterised its production by HUVEC (Luster and Ravetch 1987a; Luster and Ravetch 1987b). It lacks the ELR motif and hence has angiostatic effects, which are mediated by its sole receptor CXCR3 (Strieter *et al.* 1995a). Other functions of this molecule include acting as a chemoattractant for monocytes, NK cells and IL-2 activated T cells. It is synthesised in monocytes, endothelium, fibroblasts and keratinocytes as a 98 amino acid precursor protein, which is then cleaved to the 77 amino acid mature form. The expression of IP-10 is distinct from the other chemokines that we are investigating in that it does not respond to either TNF-α or IL-1 on their own, but when either or both of these are used in combination with IFN-γ, then a massive response is observed. Angiostatic chemokines and cytokines are thought to be required in the later stages of wound healing as an 'off' signal for angiogenesis (Belperio *et al.* 2000).

1.7.6 Gro-α (CXCL1)

Gro-α was originally described as a product of a gene differentially expressed in transformed hamster cells that had suffered loss of growth control (Anisowicz *et al.* 1987) and in human cells as melanoma growth stimulatory activity (Richmond *et al.* 1988; Richmond and Thomas 1988). It was later shown to activate human neutrophils (Moser *et al.* 1990; Schroder *et al.* 1990) and to have a similar neutrophil chemoattractant potency to IL-8. Gro-β and Gro-γ are structurally very similar (approx. 90% homologous) and show very similar properties. All three Gro chemokines are ELR+ and bind to CXCR2, which is found on monocytes and macrophages, eosinophils, mast cells and lymphocytes. A major function of these chemokines is in angiogenesis where they stimulate the proliferation of endothelial cells. Gro-α is produced by monocytes, neutrophils, endothelial cells and macrophages and its production is stimulated by IL-1, TNF-α and LPS as well as thrombin.

1.7.7 Eotaxin (CCL11)

Eotaxin was first found in guinea pig and mouse as an eosinophil chemoattractant before it was cloned in humans (Garcia-Zepeda *et al.* 1996; Kitaura *et al.* 1996; Ponath *et al.* 1996). It Is a 97 amino acid protein which when the signal protein of 23 residues is cleaved forms a mature protein of 74 amino acids. This mature protein is 66% identical to MCP-1 and belongs to the same sub family of chemokines. It is present both as a monomer and as a dimer at physiological

concentrations. Eotaxin binds only CCR3, however many other chemokines have also been found to bind to this receptor (table 1.5). CCR3 is expressed on eosinophils, basophils, T cells and DC. Eotaxin is produced by connective tissue cells as well as by subsets of leukocytes. The expression of eotaxin by epithelial cells, endothelial cells and fibroblasts has been demonstrated, results indicate that induction occurs after stimulation with pro-inflammatory cytokines such as IL-1, TNF-α, IFN-γ and IL-4 or by combinations of more than one of these. Monocytes and T cells are also sources of eotaxin, but it is not known what induces them.

Eotaxin functions in the directional migration of eosinophils and basophils at 0.1 – 1 nM. It stimulates calcium mobilisation, the respiratory burst and upregulates CD11b in eosinophils as well as stimulating leukotriene C4 release from both eosinophils and basophils (reviewed in Van Coillie *et al.* 1999). It has also been shown to upregulate ICAM-1 and 2 levels on endothelium. Eotaxin -/- mice showed reduced numbers of eosinophils in the jejunum and thymus, which suggests that eotaxin is required for normal recirculation in a healthy individual (Matthews *et al.* 1998). It has also been shown that when a mouse is challenged with antigen in a model for asthma eotaxin is important in the eariy response to challenge (Rothenberg *et al.* 1997).

Eotaxin has been implicated in asthma and other lung diseases such as allergic rhinitis as well as in diseases of the digestive tract such as Crohns disease and ulcerative colitis. It contributes to the inflammatory infiltrate and also activates basophils and mast cells to degranulate and release histamine (Nickel *et al.* 1999; Broide and Sriramarao 2001; Lloyd 2002; Romagnani 2002).

1.8 Aim

An effective inflammatory response requires cells of the immune system such as leukocytes to migrate across the endothelial cell layer into damaged and diseased tissues. This involves molecular mechanisms, which enable leukocytes to recognise sites of injury and infection from within the vasculature and form contact with the endothelium in order to exit the blood stream and migrate through the blood vessel wall and interstitium. Chemokines are necessary for many of these steps, and our research is concerned with how they might exert control over the process of leukocyte migration in different tissues.

The aim of this study is to investigate how leukocyte migration might differ between vascular beds. To do this we particularly want to concentrate on the chemokines as these are thought to be the major controlling molecules in the selective recruitment of different subsets of leukocytes to different tissues during inflammation. Several studies have demonstrated chemokine production and receptor expression by different endothelial cell types and under differing conditions. In this study we provide a comparison between many different endothelial cell types from different vascular beds under the same conditions. We investigate how they vary in their ability to produce and express different chemokines and receptors as the signalling involved in leukocyte recruitment and homing is thought to vary between different tissues. We will also investigate the role of the proteoglycan layer in presentation of chemokines on the surface of different endothelia.

1.8.1 Hypotheses

1. Endothelial chemokine production will depend on the origin of the endothelium
2. The binding of inflammatory chemokines to endothelium depends on the tissue of origin of the endothelium
3. The resting expression of chemokine receptors will be different depending on the type of endothelium
4. The composition of the proteoglycan layer differs between endothelial cell types
5. The effects of chemokines on endothelial functions will depend on the vascular bed from which the cells are derived

Chapter 2
Methods

2. Methods

2.1 Isolation and culture of cells

Endothelial cells were isolated from human umbilical veins and human adult saphenous veins by digestion with 0.1% collagenase (Sigma, Poole, England) as described previously (Jaffe *et al.* 1973; Marin *et al.* 2001). Isolated cells were expanded in Medium 199 with 2 mM glutamine (Gibco, Paisley, Scotland) supplemented with 10% Foetal Calf Serum (FCS, Gibco), 100 U/ml Penicillin, 100 µg/ml streptomycin (Gibco), 2.5 µg/ml amphotericin-B (fungizone, Gibco) and 30-50 µg/ml endothelial cell growth supplement (Sigma). Note that endothelial cells are particularly sensitive to amphotericin-B and the concentration in these cultures is lower than generally used on other cell types.

Dermal and lung endothelium were purchased from Clonetics/Biowhittaker (Wokingham, Berks. England) and expanded according to recommendation of the supplier in EGM-2-MV medium (Clonetics).

The human BMEC line was kindly donated by Babette Weksler of Cornell University (Schweitzer *et al.* 1997). To produce this cell line, primary human bone marrow endothelial cells were transfected using a construct containing simian virus 40 (SV40) large T antigen under the control of a portion of the human vimentin gene promotor. The resulting transformed cells were cultured in Dulbeccos modified eagle medium (Gibco) with low glucose (1 mg/ml) and 2 mM glutamine, supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin-B.

Liver endothelial cells were kindly donated by Adam Linke (Kings College, London) and were maintained in Medium 199 with 2 mM glutamine supplemented with 10% Foetal Calf Serum (FCS), 100 U/ml Penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin-B until assayed for chemokine production. There are several different types of endothelial cell found in the liver, which can be distinguished by phenotype, those we are using appear to show a sinusoidal phenotype. There were two distinct populations of EC in our cultures. They can be distinguished by size as shown in fig. 2.3 but all express CD31 and ICAM-1 (also shown in fig. 2.3, data courtesy of Adam Linke). As sinusoidal endothelial cells do not have Weibel-Palade bodies, they do not express P-

selectin. They also show reduced E-selectin (Daneker *et al.* 1998) perhaps because the slowing of leukocytes is less important in the already slow-flowing environment of the liver.

All endothelial cells were grown to near-confluence and passaged by exposure to trypsin 0.025%/EDTA 0.01% in calcium and magnesium free HBSS (Gibco or Clonetics) and were then cultured in 75 cm^2 tissue culture flasks. BMEC and HUVEC were grown on 0.2% gelatin. Note that although the cells were expanded using protocols and media that had been optimised and recommended for growth of that cell type, they were rested and switched into assay medium before the start of each experiment. All experiments were performed on subcultures of confluent cells between the second and seventh *in vitro* passage with the exception of the immunocytochemistry, which was performed on subconfluent cells. All the cells tested (liver EC were not examined) expressed von Willebrand factor (figs. 2.1 and 2.2) and retained their distinctive morphology. At confluence, SVEC were predominately bipolar, umbilical vein endothelium had a cobblestone appearance while lung, dermal and liver endothelium appeared intermediate. All cells showed contact inhibition, but the bone marrow endothelial cell line grew to higher densities than the primary cultures.

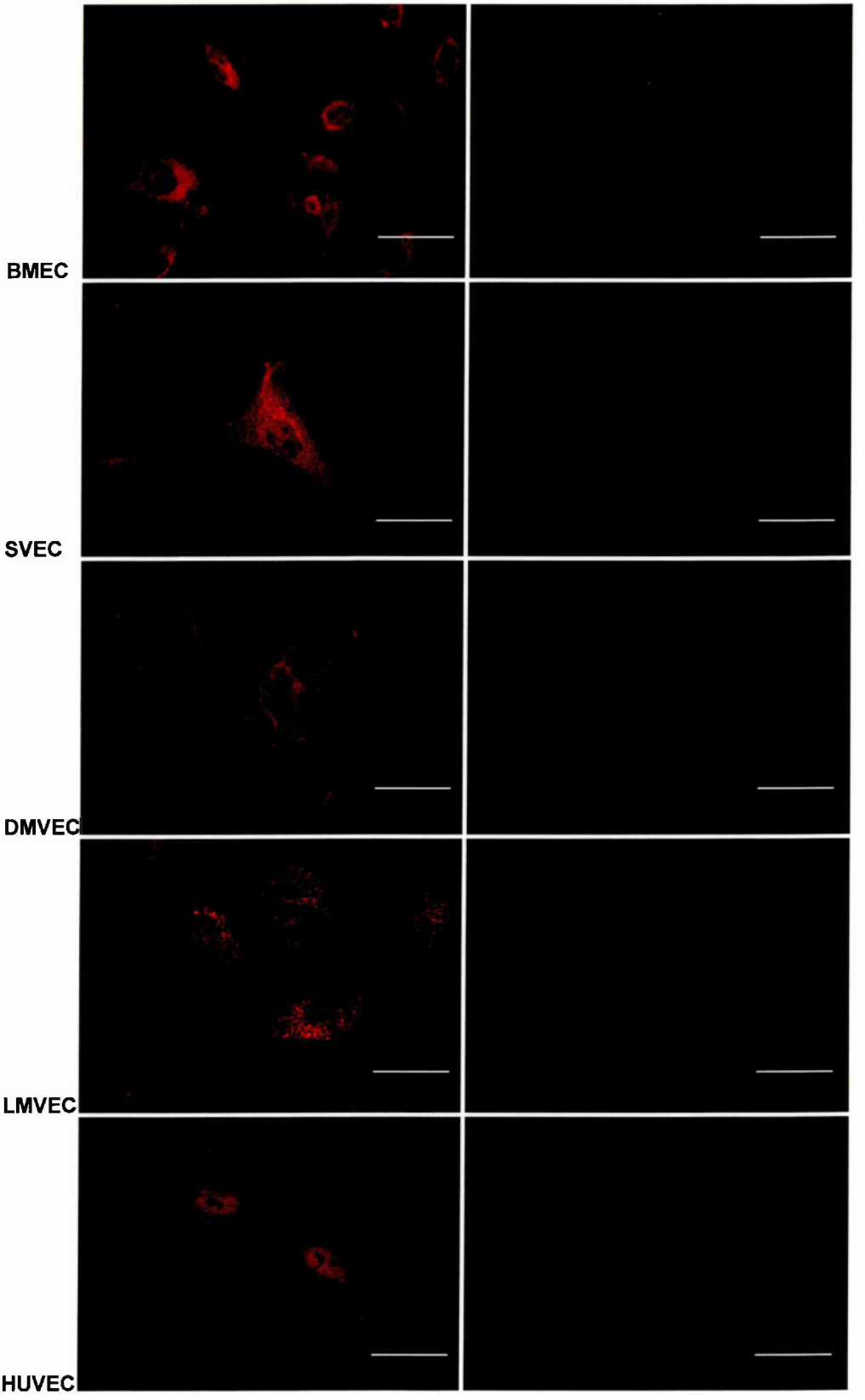


Figure 2.1 Immunocytochemistry staining of permeabilised endothelial cells showing localisation of vWF within different endothelial cell types
Left column stained with anti vWF followed by goat anti rabbit cy3 conjugated secondary. Right column stained with goat anti rabbit cy3 alone as a control. Scale bar = 40μm.

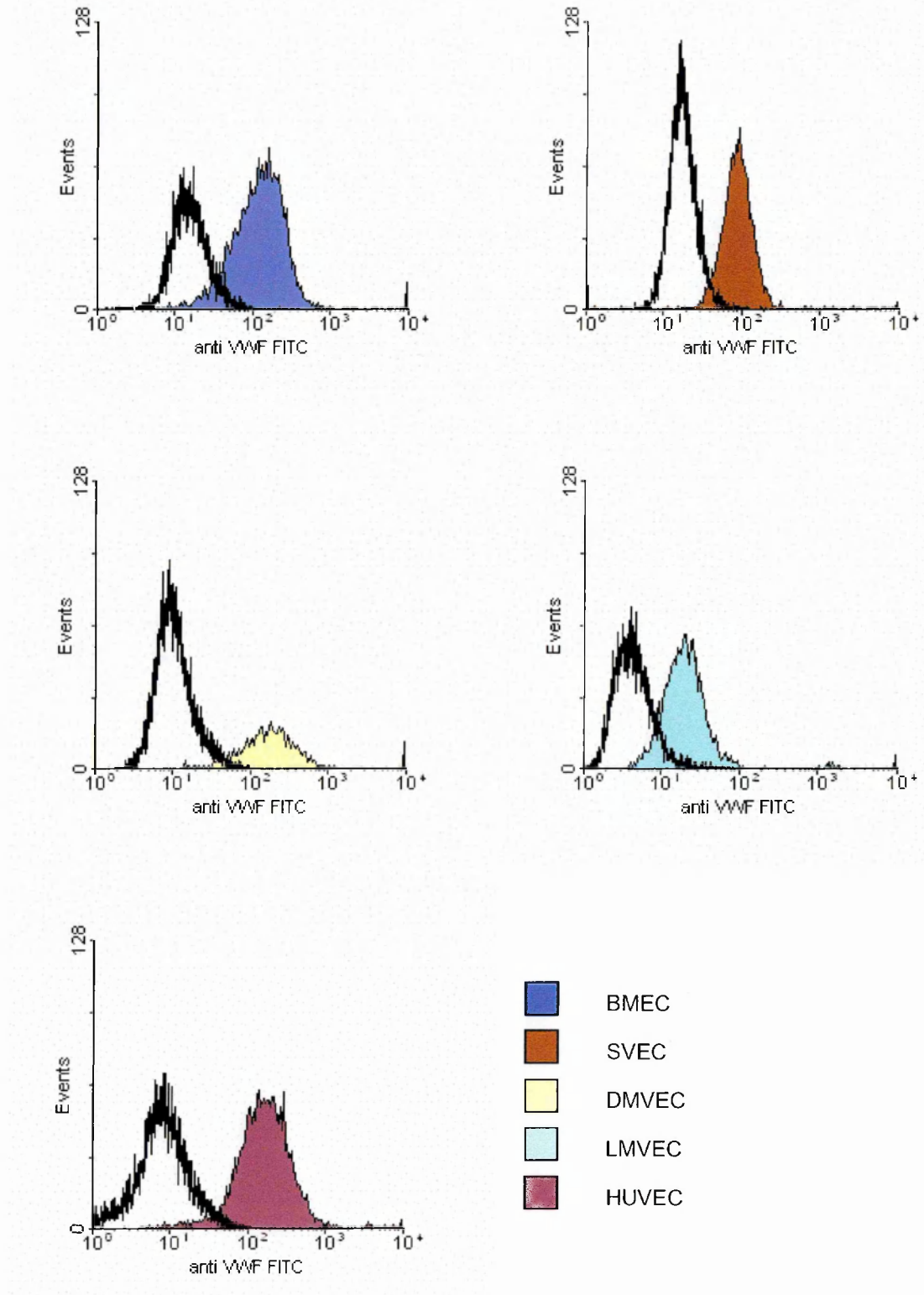


Figure 2.2 Flow cytometry histograms of vWF immunofluorescence on resting endothelial cells

Different endothelial cell types were harvested by trypsinisation, then the level of vWF detected using a human vWF specific antibody followed by goat anti rabbit Ig FITC conjugated secondary antibody. Filled peaks show vWF, unfilled show secondary alone.

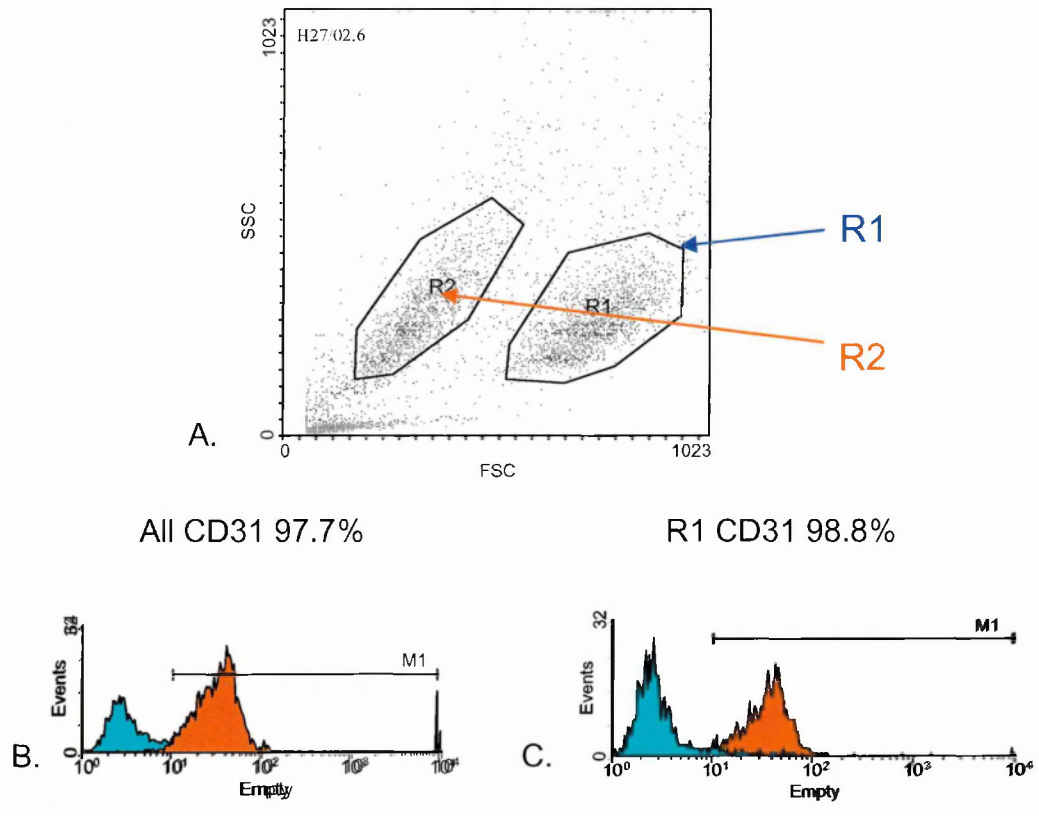


Figure 2.3 Flow cytometry data showing the phenotype of liver endothelial cells

There are two distinct populations of liver endothelial cells which can be seen in A. marked R1 and R2, they are showing differences in size, but less difference in surface granularity. The two populations both exhibit a similar overall level of CD31 (PECAM) shown in B and C. (data courtesy of Adam Linke).

Table 2.1 Markers detected on liver endothelium used in this study

Data courtesy of Adam Linke

MARKER	PERCENT POSITIVE
CD31	89.65%
CD14	2.85% (negative)
VCAM-1	4.15% (negative)
ICAM-1	16.5%
CD4	3.55% (negative)
HLA-DR	0.00% (negative)
CD80	0.20% (negative)
CD86	0.10% (negative)
HLA-ABC	100%

2.2 Cell based assays

A cell based binding assay system was used for several different experiments with various modifications, which wiil be described later in detail.

2.2.1 Solutions used

Assay medium: M199 with 2 mM glutamine, suppiemented with 10% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin.

ELISA diluent: HBSS (Gibco) with 4 mg/ml BSA (Sigma), 100 μg/ml rabbit IgG (Sigma) and 0.1% tween 20 (Sigma).

Wash buffer: PBS with 0.1% tween 20

2.2.2 Cell culture

Cells were plated onto the central 60 wells of a 0.2% gelatin coated 96 well plate at 3.5×10^4 cells per well in 200 μl of growth medium. Cells were grown overnight to confluence, then rested for 24 hours in assay medium (see 2.2.1).

2.2.3 ELISA assay

The monolayer was washed four times in HBSS, then fixed for 10 mins. at room temp. using 100 μl of 0.1% glutaraldehyde in PBS. The glutaraldehyde was removed, then 200 μl of 0.05 M tris/HCl (pH 7.5) was used to block the plate for 20 mins. at room temp. before washing three times in 0.1% tween 20 in PBS. 100 μl per well of appropriate anti human antibody or lectin was added for 1 hour at 37°C, in ELISA diluent (see 2.2.1 for details). 10 μg/ml of MHC class I antibody (Serotec, Oxford, UK) was used in each experiment on unstimulated cells to standardise between experiments as there have been shown to be approximately the same levels of MHC class I molecules across different cell types when unstimulated (Lidington *et al.* 1999). This has also been previously demonstrated on different strains of rats (personal communication D. Male). At the end of 1 hour, the plate was washed three times in 0.1% tween 20 in PBS and 100 μl of appropriate blotin labelled secondary antibody was added in ELISA diluent for those wells where it was necessary. Biotin labelled rabbit anti mouse immunoglobulins (Dako, Ely, Cambs. UK) added at 1:700 dilution in ELISA diluent was used for those wells containing MHC class I primary antibody. The cells were incubated at 37°C for 45 mins, then washed again in wash buffer. 100 μl of streptavidin

horseradish peroxidase complex (Amersham, Little Chalfont, Bucks.) was added at 1:700 in ELISA diluent for 45 mins. at 37°C. The reaction was visualised using 100 µl of 0.1 mg/ml of tetramethylbenzidine (Sigma) in 0.1 M citrate acetate buffer containing 0.03% hydrogen peroxide (Sigma). It was stopped with 50 µl of 10% sulphuric acid and then read at 450 nm.

2.3 Enzyme digestions

Enzyme digestions were performed in two different experiments to investigate the composition of the proteoglycan layer. In both cases, the confluent monolayer was washed three times in HBSS, then 65 µl of 10 U/ml heparinase III from *Flavobacterium heparinum*, 1 U/ml chondroitinase ABC *from Proteus vulgaris* or 1 U/ml neuraminidase from *Clostridium perfringens* (diluted in HBSS, all from Sigma) was added to triplicate wells for 1 hour then washed off prior to commencing the chemokine binding or lectin assays. Digestions using these enzymes at these concentrations have previously revealed that neuraminidase is selective only for sialic acid, chondroitinase ABC is selective for chondroitin 4 and 6 sulphate, dermatan sulphate and hyaluronic acid while heparinase III was only effective against heparan sulphate (Dos Santos 1995).

2.4 Statistical Analysis

In all experiments statistical significance was determined by the performance of a one or two way ANOVA followed by a post hoc t-test (Tukeys unless otherwise stated).

Chapter 3
Chemokine production by endothelial cells

3. Chemokine production by endothelial cells

3.1 Introduction

Activation of EC induces a multi-step cascade of events, which results in leukocyte recruitment. These events include upregulation of selectins and other adhesion molecules on the lumenal surface of the endothelium, thus allowing leukocytes to roll along the surface of the endothelia using the selectins. Then chemokines activate firm adhesion using the integrins and finally the leukocyte will transmigrate (Springer 1994). Chemokines are a key element in the multi-step process of leukocyte recruitment (Baggiolini 1998; Mackay 2001) in particular in the co-ordination and control of the process. They are thought to exert their effect while immobilised on endothelial cell surface proteoglycans. This immobilisation is necessary, as *in vivo* the forces exerted on free chemokines by the flow of blood would easily remove them from the site where they are required.

Chemokines are important not only for their chemotactic properties, but also for their activation of firm leukocyte binding by integrins (Shimaoka *et al.* 2002). However, their chemotactic properties ensure that specific subsets of leukocytes will be recruited. This effect is often described as combinatorial as more than one chemokine can be involved in the recruitment of each cell.

Chemokines are produced by endothelial cells in response to various molecules involved in inflammatory reactions, immunity and thrombosis. The chemokine repertoire of the endothelium includes members of both the CXC and CC families of chemokines. Most studies so far have focused on IL-8 and MCP-1.

Most of the data concerning endothelial cell functions has been obtained by studying HUVECs. These have great advantages in their ease of isolation and ready availability, however they are not necessarily the best representative of all endothelia. The primary site of leukocyte migration across the blood vessel walls is the post capillary venules, but in the dermis and lung, leukocytes migrate directly across the capillary wall. HUVEC are taken from large vessels of the umbilical vein and are foetal in origin and have hence undergone less cell division than EC found within the adult body. All endothelial cell types show induction of certain chemokines after cytokine stimulation and there is increasing evidence which shows structural and functional differences between endothelium obtained from different sites (Swerlick *et al.* 1992a; Swerlick *et al.* 1992b; Petzelbauer *et al.* 1993;

Swerlick and Lawley 1993; Groger *et al.* 1996). More recently, studies investigating microvascular endothelium show distinct differences in chemokine production profiles in response to cytokines.

Differences between endothelial cell types in cytokine regulation of adhesion molecules have been observed (Swerlick *et al.* 1992b). It is therefore important to compare the chemokine secretion patterns of different endothelial cells.

Several chemokines have been found to be produced by HUVECs, these include MCP-1, RANTES, Gro and IL-8 (Schroder and Christophers 1989; Rollins *et al.* 1990; Marfaing Koka *et al.* 1995).

In this study, BMEC, SVEC, DMVEC, LMVEC and Liver EC were examined and compared to HUVECs. We measured the secretion of five pro-inflammatory chemokines (IL-8, RANTES, MCP-1, IP-10 and MIP-1α) unstimulated, and in response to TNF-α, IFN-γ, TGF-β and combinations of these cytokines.

3.2 Methods

3.2.1 Collection of cell culture supernatants

The six different types of endothelial cells were grown in their normal culture medium to confluent monolayers on 0.2% gelatin in 24 well plates. They were then rested for 24 hours in assay medium (M199 with 10% FCS and penicillin streptomycin) before being stimulated. To do this, the medium was removed and replaced with fresh assay medium containing either TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatant was collected from unstimulated wells at 0, 5, 24 and 48 hours and from stimulated wells at 24 and 48 hours. The supernatants were stored at –20°C before measuring the amounts of chemokines present by ELISA.

3.2.2 Detection of chemokines by ELISA

Wash buffer: 0.05% tween 20 in PBS

ELISA diluent: 0.1% BSA, 0.05% tween 20 in trls buffered saline (pH 7.3)

A 96 well ELISA plate (Nunc, Maxisorb) was sensitised overnight at room temperature with 100μi per well of mouse anti human chemokine moncional capture antibody (R & D systems, Abingdon, England) diluted to the appropriate concentration in PBS (2-4 μg/ml, see table 3.1). After 3 washes with wash buffer, the non-specific reactions were blocked for 1 hour at room temperature using 200 μi per well of PBS containing 1% BSA and 5% sucrose. The wells were then washed and incubated for 2 hours at room temperature with 100 μl of samples in duplicate at an appropriate dilution (1:5, 1:25 or 1:100) in ELISA diiuent or standards at 0 – 8000 pg/ml in ELISA diluent. The plates were washed three times, then 100 μl of biotinylated goat anti human chemokine detection antibody (R & D systems) was added in ELISA diluent for 2 hours at room temperature (for concentrations used see table 3.1). After 3 more washes, 100 μl/well of streptavidin – biotin horseradish peroxidase (Amersham) diluted 1:700 in ELISA diluent was added for 1 hour at room temperature. Finally, the plate was washed 3 times, then developed, stopped and read as described previously (section 2.2.3).

Table 3.1 Optimal concentrations of antibodies for detection of chemokines in supernatants by sandwich ELISA

CHEMOKINE	CAPTURE ANTIBODY CONCENTRATION (μg/ml)	DETECTION ANTIBODY CONCENTRATION (ng/ml)
IL-8	4	20
MIP-1α	4	40
RANTES	4	5
MCP-1	2	50
IP-10	2	100

3.2.3 Standard curve properties

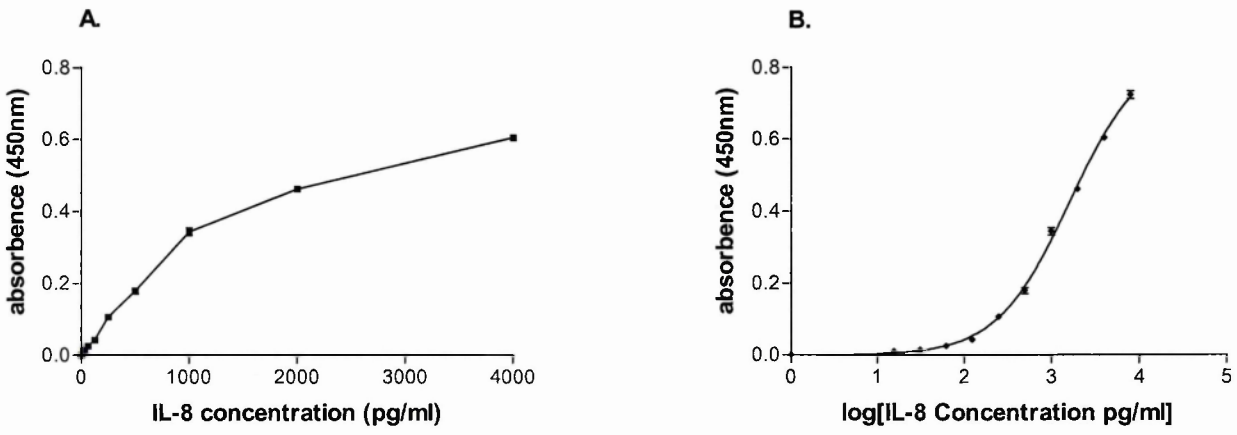


Figure 3.1 Standard curves for IL-8

This represents a typical standard curve produced using the protocol outlined in section 3.2.2 (A). B. Log standard curve for IL-8 showing fitted curve from which our data has been extracted for table 3.3.

Table 3.2 shows the range, sensitivity and variance of the assay, but the detection threshold is determined by the dilution factor used to detect the chemokine. This means that in the results table, where we have shown a result of 0, this data point is below the sensitivity of the assay, which is < 0.5 ng/ml. The maximum standard error shown here is that determined by reading three different values off the linear region of the standard curve (fig. 3.1a between 100 and 1000 pg/ml).

Table 3.2 Properties of the standard curves produced for each chemokine

CHEMOKINE	RANGE	SENSITIVITY	STANDARD ERROR
IL-8	30 - 4000 pg/ml	30 pg/ml	60 pg/ml
MIP-1α	30 - 4000 pg/ml	100 pg/ml	50 pg/ml
RANTES	30 - 4000 pg/ml	60 pg/ml	43 pg/ml
MCP-1	30 - 4000 pg/ml	100 pg/ml	120 pg/ml
IP-10	30 - 4000 pg/ml	100 pg/ml	26 pg/ml

3.3 Results

In order to determine how endothelia vary in their chemokine secretion profiles, the production of IL-8, RANTES, MCP-1, IP-10 and MIP-1α was measured by ELISA, on duplicate samples of supernatants from cells treated with different combinations of cytokines. The data for IL-8, RANTES, MCP-1 and IP-10, from one set of assays is given in table 3.3. Data showing the results of cytokines on chemokine production at 24 and 48 hours are shown in figures 3.3 and 3.4 respectiveiy. MIP-1α was not detectable in any of the endothelial culture supernatants in this assay.

3.3.1 Constitutive chemokine production

Production of the chemokines by resting endothelium was measuredby harvesting tissue culture supernatants at 0, 5, 24 and 48 hours (Table 3.3, rows 1-4 and fig. 3.2). MCP-1 & IL-8 were produced by all resting EC tested. HUVEC was the highest producer of both chemokines of all the different cell types, producing 24 ng/ml of MCP-1 and 30 ng/ml of IL-8 after 48 hours in culture. Unike the HUVECs, both the BMEC line and SVECs produced more MCP-1 than IL-8 however, only very low levels were detected (2.1 ng/ml or less) for both these cell types. In the primary microvascular cells and Liver EC more IL-8 was produced than MCP-1. RANTES was constitutively produced at a low level by DMVEC, but not by any other endothelial cell type. IP-10 was not produced by any of the unstimulated endotheiiai cells tested.

The rate of production of IL-8 by HUVEC suffered a slight lag effect at the beginning (until 5 hours), then was constant, while ali 5 other cell types showed a constant rate of IL-8 production (fig. 3.2a). A lag effect was also shown in the rate of MCP-1 production by HUVEC and microvascular endothelium up until 5 hours, with BMEC not beginning to produce MCP-1 until after 24 hours. SVEC and Liver EC produced MCP-1 at a constant rate (fig. 3.2b).

We have also shown that while the SVEC are relatively consistent in the lack of production of constitutive chemokines, the high levels of IL-8 and MCP-1 produced by HUVECs is variable between individuals.

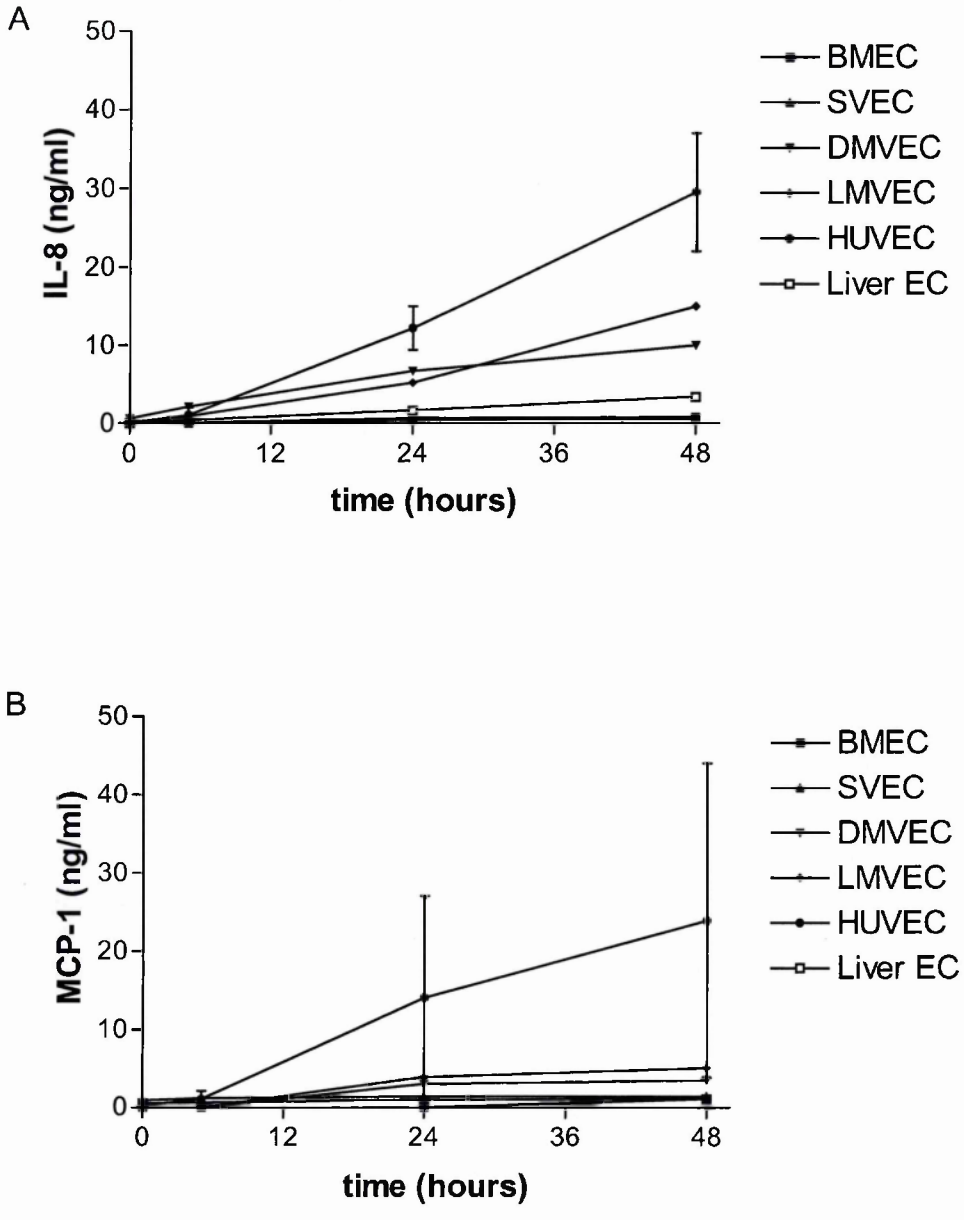


Figure 3.2 Constitutive production of MCP-1 and IL-8 by endothelial cells over time

Supernatants were collected from confluent resting endothelial cells at 0, 5, 24 and 48 hours, then assayed by sandwich ELISA for IL-8 and MCP-1. A. IL-8, B. MCP-1. SVEC n=4, HUVEC n=2, all other cell types n=1 Error bars show SEM.

Table 3.3 Chemokine production by endothelial cells

Confluent endothelial cells were treated with TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatants were collected and assayed by sandwich ELISA for IL-8, RANTES, MIP-1α, MCP-1 and IP-10. No MIP-1α was produced by any of the 6 cell types tested. B = Bone Marrow EC, S = Saphenous vein EC, H = HUVEC, D = Dermal microvascular EC, L = Lung microvascular EC, Liv = liver endothelial cells. SVEC n=4, HUVEC n=2, all others n=1.

		IL-8 production (ng/ml)						RANTES production (ng/ml)						MCP-1 production (ng/ml)						IP-10 production (ng/ml)					
Treatment	**hrs**	**B**	**S**	**D**	**L**	**H**	**Liv**	B	**S**	**D**	**L**	**H**	**Liv**	**B**	**S**	**D**	**L**	**H**	**Liv**	**B**	**S**	**D**	**L**	**H**	**Liv**
Control	**0**	0	0	0.6	0	0	0	0	0	0	0	0	0	0	0.8	0	0	0	0.5	0	0	0	0	0	0
	5	0	0	2.1	0.9	1	ND	0	0	0	0	0	ND	0	1.2	0	0	1.1	ND	0	0	0	0	0	ND
	24	0.6	0.4	6.7	5.2	12	1.6	0	0	0.6	0	0	0	0	1.4	3	3.9	14	1.0	0	0	0	0	0	0
	48	0.8	0.5	10	15	30	3.4	0	0	1.5	0	0	0	1.1	1.4	3.5	5.1	24	1.1	0	0	0	0	0	0
TNFα	**24**	14	37	48	52	141	106	10	0	26	6.4	3.0	1.6	47	9.2	19	40	18	15	17	0	3.8	2.1	0.6	0
	48	70	67	89	122	313	132	51	4.1	62	11	6.8	3.2	146	21	15	44	55	17	24	1.3	9.8	4.8	0.8	0.8
IFN-γ	**24**	0	0	2.4	2.9	5.0	1.6	0	0	0.9	0	0	0	3.9	5.9	4.0	4.9	16	1.7	3.7	1.0	11	17	3.5	3.3
	48	0.5	0	4.0	8.4	9.4	7.1	1.7	0	2.2	1.0	0.5	0	7.4	7.6	4.3	10	19	2.0	16	3.8	26	34	4.3	6.1
TGF-β	**24**	0	6.3	3.4	1.5	11	ND	0	0	0	0	0	ND	0	3.0	1.8	1.6	8.4	ND	0	0	0	0	0	ND
	48	0.8	4.9	5.9	3.2	31	ND	1.5	0	0	0	0	ND	1.7	3.7	2.0	2.3	17	ND	0	0	0	0	0.5	ND
TNFα + IFN-γ	**24**	7.5	16	24	28	94	59	18	11	32	9.2	17	17	49	19	14	13	35	10	171	39	44	50	77	39
	48	27	26	60	73	194	64	58	48	87	21	112	33	93	52	15	19	60	11	429	96	90	49	253	37
TNFα + TGF-β	**24**	5.2	53	21	20	112	31	11	0	13	4	4.4	1.0	32	12	7.0	12	18	11	3.0	0	0	1.2	1.0	0
	48	18	75	53	35	258	55	59	2.0	37	9.0	13	4.0	80	26	32	23	62	11	9.8	0	2.2	1.7	2.6	0
IFN-γ + TGF-β	**24**	0	3.3	1.8	1.2	9.5	1.7	0.5	0	0	0	0	0	5.6	22	3.5	4.1	22	1.4	3.1	3.0	17	14	8.1	2.7
	48	0	4.1	3.1	2.3	14	4.2	1.9	1.0	1.9	0	0.8	0.7	15	14	4.1	5.7	30	1.8	9.9	5.5	23	23	20	4.8

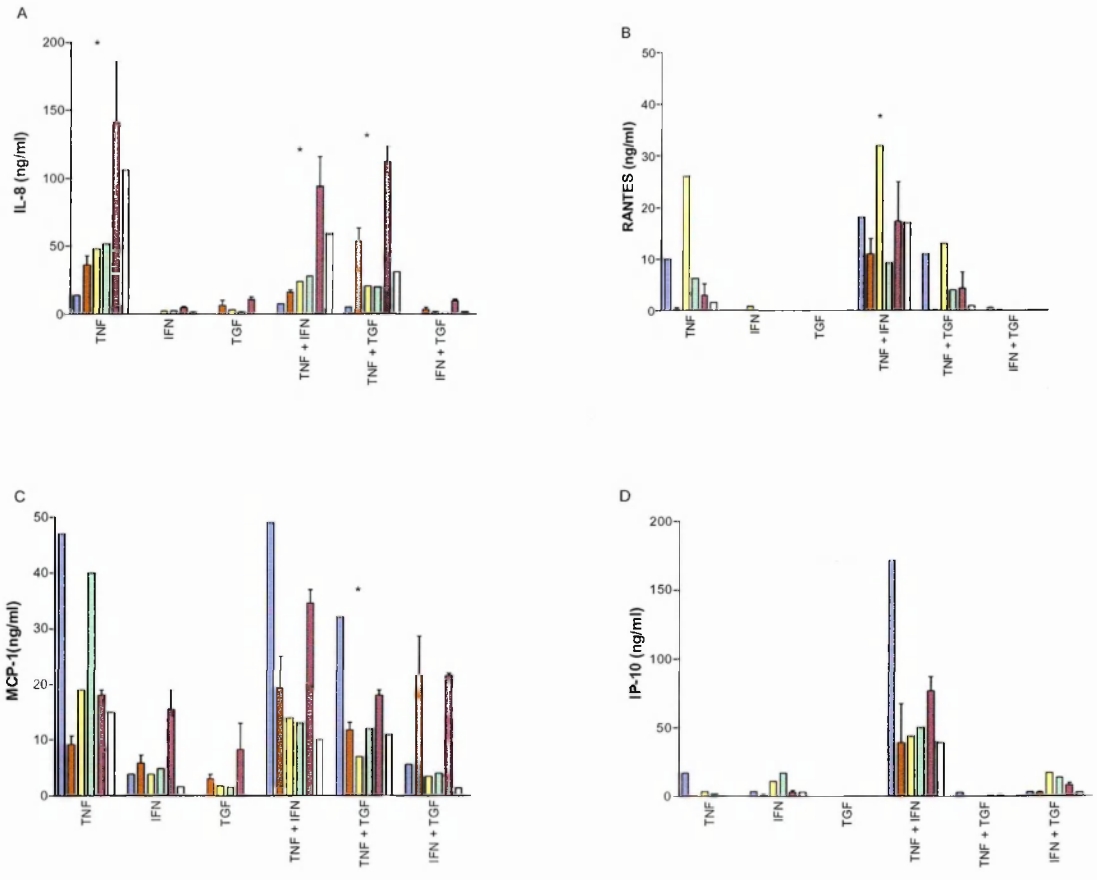


Figure 3.3 Chemokine production by endothelial cells after stimulation by cytokines for 24 hours

Supernatants were collected from confluent cytokine stimulated endothelial cells at 24 hours post stimulation, then assayed by sandwich ELISA for chemokine production. A. IL-8, B. RANTES, C. MCP-1, D. IP-10. Blue = BMEC, Red = SVEC, Yellow = DMVEC, Green = LMVEC, Purple = HUVEC, White = Liver EC. * denotes a statistically significant difference between SVEC and HUVEC ($p<0.05$). SVEC n=4, HUVEC n=2, other cell types n=1, therefore no statistical tests on these cells.

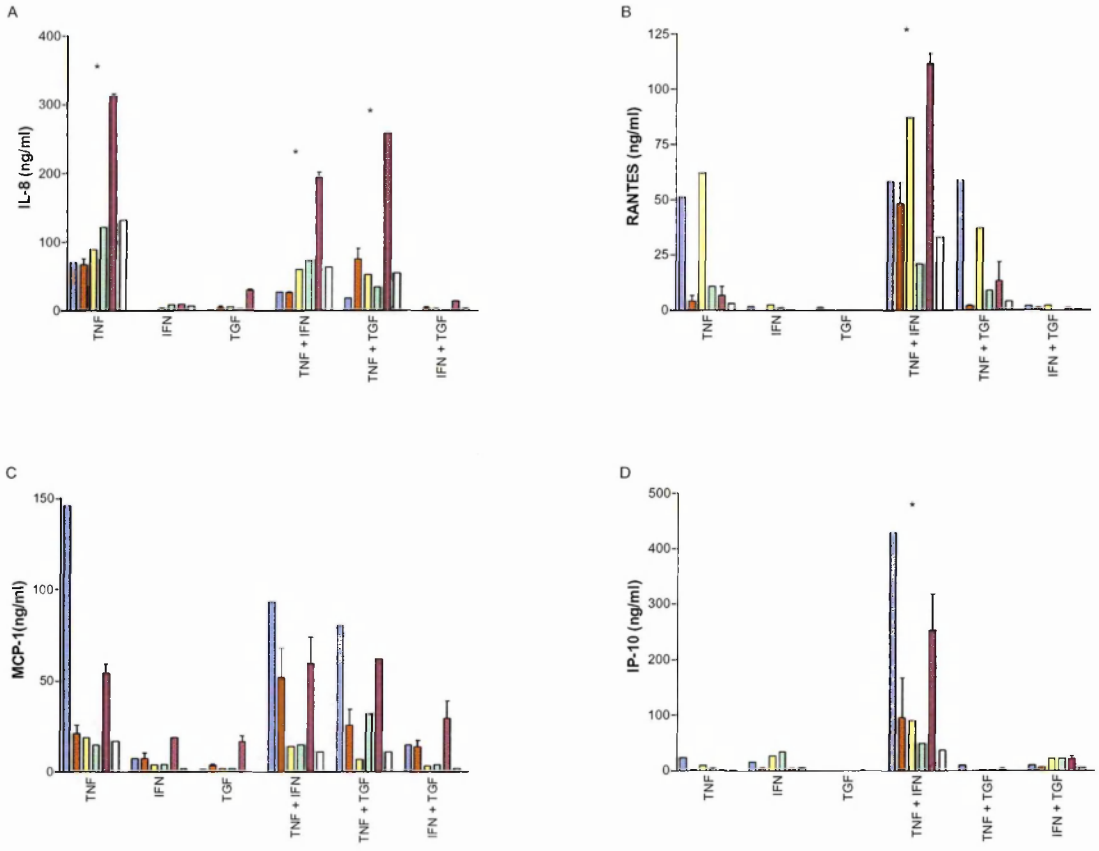


Figure 3.4 Chemokine production by endothelial cells after stimulation by cytokines for 48 hours

Supernatants were collected from confluent cytokine stimulated endothelial cells at 48 hours post stimulation, then assayed by sandwich ELISA for chemokine production. A. IL-8, B. RANTES, C. MCP-1, D. IP-10. Blue = BMEC, Red = SVEC, Yellow = DMVEC, Green = LMVEC, Purple = HUVEC, White = Liver EC. . * denotes a statistically significant difference between SVEC and HUVEC ($p<0.05$). SVEC n=4, HUVEC n=2, other cell types n=1, therefore no statistical tests on these cells.

3.3.2 TNF-α upregulates chemokine production

The addition of 25 ng/ml of TNF-α to the tissue culture medium for 24 or 48 hours increased production of IL-8, RANTES and MCP-1 by all 6 endotheiial cell types tested. It aiso upregulated IP-10 production but to a lesser extent. IL-8 showed the biggest increase in secretion levels upon addition of TNF-α across five out of the six celi types (BMEC being the exception) with HUVECs secreting as much as 313 ng/ml after 48 hours. RANTES production increased to only modest levels of 3 – 11 ng/ml after 48 hours by most cell types, however for DMVEC and BMEC, the increase was more pronounced – up to 62 ng/ml on DMVEC and 51 ng/ml on BMEC after 48 hours. MCP-1 was also produced at high levels (146 ng/ml) by BMEC; however, the DMVECs produced less than any other cell type (less than 20 ng/ml) at 48 hours. Upon stimulation with TNF-α, BMEC produced the most MCP-1, all other EC types tested produced IL-8 as their major chemokine after TNF-α stimulation. IP-10 production was increased slightly on addition of TNF-α in all cell types. Highest levels were demonstrated by BMEC as they secreted 24 ng/ml after 48 hours, while other cell types all produced less than 10 ng/ml with virtually none produced by HUVEC and Liver EC.

3.3.3 IFN-γ decreases production of IL-8 by endothelial cells

The addition of IFN-γ had very little effect on IL-8 production by BMEC and SVEC at 24 or 48 hours and no effect on Liver EC production at 24 hours, though an increase from 3.4 to 7.1 ng/ml was observed by 48 hours. On the other hand, in the two microvascular endothelia and in HUVEC, IFN-γ reduced the high constitutive levels by approximately half e.g. LMVEC showed constitutive levels of 15 ng/ml of IL-8 after 48 hours, but with IFN-γ added, they showed only 8.4 ng/ml, a decrease of 44%.

3.3.4 IFN-γ upregulates MCP-1 and IP-10 production

As previously reported (Luster and Ravetch 1987a) there was no constitutive expression of IP-10, but IFN-γ induced production of IP-10 by all 6 EC types. The highest increases were observed for the two types of microvascular endothelia (26 and 34 ng/ml at 48 hours for dermal and lung respectively). IFN-γ also increased production of MCP-1 significantly by BMEC, SVEC, LMVEC and to a much lesser extent by DMVEC, though not to the same degree as TNF-α. Liver EC showed only low levels – a maximum of 2.0 ng/ml was produced at 48 hours. HUVEC showed a slight

increase from constitutive ieveis at 24 hours, but by 48 hours the leveis were decreased from 24 ng/mi without IFN-γ to 19 ng/ml when IFN-γ was added to the maintenance medium. Interestingly, SVEC and HUVEC showed more MCP-1 than IP-10 production after IFN-γ stimulation, whereas BMEC, DMVEC LMVEC and Liver EC showed less.

3.3.5 Effects of TGF-β on MCP-1 and IL-8 production

Addition of TGF-β to the maintenance medium reduced the constitutive secretion of IL-8 by LMVEC and DMVEC. Unstimulated DMVEC secreted 10 ng/ml after 48 hours, after the same time with TGF-β added, only 5.9 ng/ml was secreted. The reduction was more pronounced for LMVEC as the constitutive levels of 15 ng/ml dropped to just 3.2 ng/ml after addition of TGF-β to the maintenance medium. TGF-β also reduced MCP-1 production by both microvascular EC and HUVECs, though to a lesser extent than seen for IL-8. This was particularly noticeable in the LMVEC and DMVEC as only low constitutive ievels were produced. We did not have enough Liver EC to investigate TGF-β stimulation (marked ND on table 3.3).

3.3.6 TNF-α and IFN-γ act in synergy to induce high IP-10 and RANTES production

RANTES production increased across all five ceils types upon stimulation with TNF-α, the highest level (62 ng/ml) being observed for DMVEC after 48 hours in culture. However all cell types showed some increased production in the presence of TNF-α or IFN-γ and showed high levels of secretion in the presence of these two cytokines in combination. The levels produced by a combination of the two cytokines were much higher than that of TNF-α alone. Although IFN-γ had little effect on RANTES production alone, when put into the maintenance medium in combination with TNF-α, there is a distinct synergistic effect. IP-10 was also produced by all EC upon stimulation with TNF-α or IFN-γ and extremely high ievels were produced with both together – again showing synergy. BMEC, SVEC and HUVEC produced over 200 ng/mi of IP-10 after 48 hours of stimulation with 25 ng/ml of TNF-α and 200 U/ml of IFN-γ together. In both the expression of IP-10 and RANTES, DMVEC show much higher secretion in comparison to LMVEC.

3.3.7 IFN-γ and TNF-α effects on MCP-1 and IL-8

For IL-8 production there was no synergy between TNF-α and IFN-γ. In fact, IFN-γ reduced the high chemokine produced by addition of TNF-α to endothelial cells. This suggests an antagonism between TNF-α and IFN-γ with regards to IL-8 production on all EC tested. However, the levels of IL-8 were not reduced to constitutive levels, which suggests that there is still an overall pro-inflammatory effect. MCP-1 levels were increased from those with TNF-α or IFN-γ alone showing a possible synergy between the two cytokines on MCP-1 production by SVEC and HUVEC only. An antagonistic effect was seen on LMVEC and Liver EC, with little change or inconsistent effects shown in DMVEC and BMEC.

3.3.8 Effects of TGF-β and TNF-α in combination

There was little significant difference between TNF-α acting on endothelial cells alone and TNF-α acting in combination with TGF-β for either SVEC or HUVEC. The other 4 cell types however appeared to show lower levels of IL-8 and MCP-1 produced after the addition of TGF-β as well as TNF-α to the maintenance medium as compared to TNF-α alone. For example, after 48 hours LMVEC constitutively produce 15 ng/ml of IL-8, they produced 122 ng/ml after stimulation with TNF-α alone and 3.2 ng/ml with TGF-β stimulation, but when TNF-α and TGF-β act together only 35 ng/ml of IL-8 was produced. This is not reduced to constitutive levels, but is a 65% reduction from those observed with TNF-α alone. TGF-β can reduce the effects of TNF-α on endothelial chemokine production by up to 75% depending on the cell type and chemokine investigated. Without more samples from different individuals though, it is difficult to tell whether this might be a consistent finding. There is little difference in the effects of TNF with or without TGF on RANTES or IP-10 production by any cell type tested..

3.3.9 Effects of TGF-β and IFN-γ in combination

No IP-10 was detectable after TGF-β stimulation of endothelial monolayers, but TGF-β was found to have a small downregulatory effect on IFN-γ induced IP-10 secretion by all endothelia except HUVEC. TGF-β again appears to stimulate HUVECs to produce high levels of chemokines rather than reduce the effects of other cytokines. This was noticeable when TGF-β was used in combination with IFN-γ as high secretion of MCP-1 and IP-10 was observed. HUVEC produced

low levels of IP-10 when stimulated with IFN-γ (4.3 ng/ml) and virtually none (0.5 ng/ml) when stimulated with TGF-β, however with both together 20 ng/ml of IP-10 was detected. SVEC from some individuals also show this trend, whereas others show the opposite. IFN-γ stimulates all other endothelia to produce 6.1 – 34 ng/ml of IP-10 after 48 hours. When TGF-β was added as well as IFN-γ, levels of production were reduced in each individual cell type producing 4.8 – 23 ng/ml IP-10.

It also appears that IFN-γ and TGF-β acting together suppress much of the production of IL-8 observed in LMVEC and DMVEC after stimulation with either cytokine alone.

3.3.10 Inter – individual variation in chemokine production

To address the question of whether there is any variation between individuals in their endothelial chemokine production and cytokine responsiveness, we repeated the assay using SVECs from a further three different donors (Tables 3.4 – 3.7). The tables show the mean results from duplicate wells for each individual donor, and the mean and standard error for the four donors pooled. From this data, we can show that there is variation between individuals with combinations of cytokines exhibiting greater differences between individuals than stimulation with a single cytokine. IP-10 also appears to have greater variation than others due to very high production by donor 4. Therefore we suggest that in general small differences between tissue from different donors could be due to inter-individual variation, where larger differences are exhibited we can conclude they are significant differences between cell types.

Treatment	IL-8 production (ng/ml)				Mean	Standard Error
	1	2	3	4		
Control 0 hr	0	0	0	0	0	0
Control 5 hr	0	-	0	0	0	0
Control 24 hr	0	0.8	0	0.7	0.38	0.22
Control 48 hr	0	-	0.8	0.7	0.50	0.25
TNFα 24 hr	56	30	27	33	36.5	6.6
TNFα 48 hr	72	-	49	79	66.7	9.1
IFN–γ 24 hr	0.6	0	0	0	0.15	0.15
IFN–γ 48 hr	0	-	0.7	0	0.23	0.23
TGF–β 24 hr	14	-	4.2	0.8	6.33	4.0
TGF–β 48 hr	10	-	3.9	0.8	4.90	2.7
TNFα & IFN–γ 24 hr	16	13	18	18	16.3	1.2
TNFα & IFN–γ 48 hr	25	-	25	29	26.3	1.3
TNFα & TGF–β 24 hr	73	-	46	41	53.3	9.9
TNFα & TGF–β 48 hr	105	-	67	53	75.0	15.5
IFN–γ & TGF–β 24 hr	4.1	-	5.8	0	3.30	1.7
IFN–γ & TGF–β 48 hr	6.2	-	6.1	0	4.10	2.0

Table 3.4 Variation between individuals in IL-8 production by SVECs

Confluent endothelial cells from four different donors were treated with TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatants were collected and assayed by sandwich ELISA for IL-8, RANTES, MCP-1 and IP-10. Means of duplicate wells for each individual donor are shown, with an overall mean and standard error of the mean for the four individuals pooled.

Treatment	RANTES production (ng/ml)				Mean	Standard Error
	1	2	3	4		
Control 0 hr	0	0.7	0	0	0.18	0.18
Control 5 hr	0	-	0	0	0	0
Control 24 hr	0	0	0	0	0	0
Control 48 hr	0	-	0	0	0	0
TNFα 24 hr	0	0	0	1.2	0.30	0.3
TNFα 48 hr	1.4	-	1.5	9.3	4.11	2.6
IFN–γ 24 hr	0	0	0	0	0	0
IFN–γ 48 hr	0	-	0	0	0	0
TGF–β 24 hr	0	-	0	0	0	0
TGF–β 48 hr	0	-	0	0	0	0
TNFα & IFN–γ 24 hr	9.8	3.4	17	14	11.1	2.9
TNFα & IFN–γ 48 hr	41	-	36	67	48.0	9.6
TNFα & TGF–β 24 hr	0	-	0.5	0	0.17	0.17
TNFα & TGF–β 48 hr	2.4	-	1.5	2.1	2.00	0.26
IFN–γ & TGF–β 24 hr	0	-	0.5	0	0.17	0.17
IFN–γ & TGF–β 48 hr	1.0	-	1.9	0	0.97	0.55

Table 3.5 Variation between individuals in RANTES production by SVECs

Confluent endothelial cells from four different donors were treated with TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatants were collected and assayed by sandwich ELISA for IL-8, RANTES, MCP-1 and IP-10. Means of duplicate wells for each individual donor are shown, with an overall mean and standard error of the mean for the four individuals pooled.

Treatment	MCP-1 production (ng/ml)				Mean	Standard Error
	1	2	3	4		
Control 0 hr	0.9	1.2	1.4	0	0.88	0.31
Control 5 hr	1.2	-	1.2	1.20	1.20	0
Control 24 hr	1.6	0.9	1.2	1.9	1.40	0.22
Control 48 hr	1.1	-	1.1	2.1	1.43	0.33
TNFα 24 hr	14	8.0	6.9	7.8	9.18	1.6
TNFα 48 hr	27	-	11	25	21.0	5.0
IFN–γ 24 hr	9.5	4.2	3.2	6.8	5.93	1.4
IFN–γ 48 hr	5.2	-	4.6	13	7.60	2.7
TGF–β 24 hr	4.9	-	2.1	2.1	3.03	0.9
TGF–β 48 hr	5.3	-	2.9	2.8	3.67	0.8
TNFα & IFN–γ 24 hr	32	16	5.7	24	19.4	5.6
TNFα & IFN–γ 48 hr	49	-	25	81	51.7	16.2
TNFα & TGF–β 24 hr	14	-	12	9.5	11.8	1.3
TNFα & TGF–β 48 hr	43	-	14	20	25.7	8.8
IFN–γ & TGF–β 24 hr	31	-	26	7.7	21.6	7.1
IFN–γ & TGF–β 48 hr	20	-	13	8.7	13.9	3.3

Table 3.6 Variation between individuals in MCP-1 production by SVECs

Confluent endothelial cells from four different donors were treated with TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatants were collected and assayed by sandwich ELISA for IL-8, RANTES, MCP-1 and IP-10. Means of duplicate wells for each individual donor are shown, with an overall mean and standard error of the mean for the four individuals pooled.

Treatment	IP-10 production (ng/ml)				Mean	Standard Error
	1	2	3	4		
Control 0 hr	0	0	0	0	0	0
Control 5 hr	0	-	0	0	0	0
Control 24 hr	0	0	0	0	0	0
Control 48 hr	0	-	0	0	0	0
TNFα 24 hr	0	0	0	0.5	0.13	0.13
TNFα 48 hr	0	-	0	4.0	1.33	1.3
IFN–γ 24 hr	1.4	0	0	2.7	1.03	0.65
IFN–γ 48 hr	1.3	-	1.1	8.9	3.77	2.6
TGF–β 24 hr	0	-	0	0	0	0
TGF–β 48 hr	0	-	0	0	0	0
TNFα & IFN–γ 24 hr	14	4.4	13	124	38.9	28.5
TNFα & IFN–γ 48 hr	31	-	19	237	95.7	70.8
TNFα & TGF–β 24 hr	0	-	0	0	0	0
TNFα & TGF–β 48 hr	0	-	0	0	0	0
IFN–γ & TGF–β 24 hr	3.8	-	3.6	1.5	2.97	0.74
IFN–γ & TGF–β 48 hr	5.0	-	5.6	5.9	5.50	0.26

Table 3.7 Variation between individuals in IP-10 production by SVECs

Confluent endothelial cells from four different donors were treated with TNF-α (25 ng/ml), IFN-γ (200 U/ml), TGF-β (25 ng/ml), combinations of these or left unstimulated. The supernatants were collected and assayed by sandwich ELISA for IL-8, RANTES, MCP-1 and IP-10. Means of duplicate wells for each individual donor are shown, with an overall mean and standard error of the mean for the four individuals pooled.

3.4 Discussion

There are now numerous studies demonstrating important structural, biochemical, antigenic and functional differences in endothelial cells from different morphological areas. Here we contribute a large comparative study of the effects of pro- and anti- inflammatory cytokines on chemokine production by endothelium from different areas of the body.

Our data showed great variability between endothelia in production of chemokines – with results from 0 ng/ml produced by unstimulated endothelia, up to 430 ng/ml from cytokine stimulated endothelia after 48 hours. Since we have shown there to be some variability between individual donors (tables 3.4 – 3.7) we cannot make inferences from small differences between endothelia. However, in the instances where the differences were great we can conclude that these were significant. There was variability between experiments of less than 5%, but this was far less than that between individuals.

In general, chemokines are physiologically active at levels of 2 – 200 ng/ml with maximum chemotactic activity at 10 – 50 ng/ml depending on the chemokine and the responding cell type. So for instance, our data suggests that only DMVEC, LMVEC, Liver EC and HUVECs produced physiological levels of IL-8 constitutively.

In this experiment, we particularly need to take into account that cells are grown under static conditions and the levels of chemokine build up, as they would not do *in vivo*. Under flow conditions, the chemokines would be much diluted, however since production is constant, a low concentration would be maintained at the cell surface.

The earliest study on the production of chemokines by EC was in 1987, when Luster and Ravetch showed the production of IP-10 by HUVEC cultured *in vitro*. Other early studies identified a soluble molecule, at first named leukocyte adhesion inhibitor, which was secreted by endothelial cells upon stimulation with IL-1, TNF-α or LPS and which was found to reduce the adherence of leukocytes to the endothelial cell surface. This molecule was deduced to be (Ala-IL-8)77 a form of IL-8 extended at the NH_2 terminus, which has similar effects to IL-8 derived from other cell types (Gimbrone *et al.* 1989). It has since been shown that the inhibitory effect could be due to the disruption of chemotactic gradients.

At a similar time, it was demonstrated by RNA blot analysis that TNF-α, LPS and IL-1 were each able to induce gene expression for IL-8 in HUVEC (Strieter *et al.* 1989a) and studies by several other labs confirmed these findings (Schroder and Christophers 1989; Sica *et al.* 1990a). It was also shown that cytokines such as IL-1 and TNF-α induce the production of MCP-1 by HUVEC (Rollins *et al.* 1990; Sica *et al.* 1990b; Rollins and Pober 1991). Since these early experiments, there have been many more investigations into the different chemotactic molecules produced by endothelium under different conditions. In a large proportion of these studies, HUVEC were used, as they are the easiest endothelial cell type to obtain, isolate and culture.

3.4.1 Constitutive chemokine production

Several groups have shown that IL-8 mRNA is present at very low levels in unstimulated EC, with slightly higher levels of MCP-1 mRNA. This has been demonstrated in HUVEC (Rollins *et al.* 1990; Sica *et al.* 1990b), DMVEC by *in situ* hybridisation (Goebeler *et al.* 1997), LMVEC (Brown *et al.* 1994) and Liver EC, but not sinusoidal cells (Afford *et al.* 1998) determined by *in situ* hybridisation on frozen sections. Basal levels of protein expression of these two chemokines have also been shown after 24 to 48 hours in culture (Rollins *et al.* 1990; Brown *et al.* 1994; Beck *et al.* 1999) In unstimulated frozen sections of liver EC, IL-8 mRNA was found in sinusoidal and vascular EC while MCP-1 mRNA was found only in vascular EC. Our results showed that unstimulated DMVEC, HUVEC, LMVEC and Liver EC produced physiologically relevant levels of IL-8. The two microvascular cell types and HUVECs also produced MCP-1 constitutively as did the SVEC, however, BMEC and Liver EC produced only 1 – 1.1 ng/ml which *in vivo*, would probably not be physiologically relevant. MCP-1 mRNA, but not the protein, has been shown to be present in transformed BMEC cell lines without stimulation (Vanderkerken *et al.* 2002). Particularly worth noting are the very high levels of MCP-1 and IL-8 (24 ng/ml and 30 ng/ml respectively at 48 hours) produced by the HUVECs in comparison to the other endothelial cell types. This begins to suggest that although HUVECs are often used as a model for endothelial cell systems, their chemokine production is atypical. It is not certain whether the high constitutive secretion of MCP-1 and IL-8 is related to its tissue of origin, or its age. SVEC, the other macrovascular endothelial cell type, differs from HUVECs in both the location and age of the tissue and produced very little in the way of inflammatory chemokines. Also notable, were the high levels of constitutive production of IL-8 by DMVEC and LMVEC. IL-8 is particularly important in controlling migration of neutrophils into tissues (Larsen *et al.* 1990; Leonard *et al.* 1990).

We demonstrated slight constitutive production of RANTES by DMVEC, though at only 1.5 ng/ml after 48 hours in a static culture, this is would probably not be relevant *in vivo*. In agreement with our study Goebeler *et al.* (1997) found no RANTES mRNA in cultured DMVEC without stimulation, this has also been shown in HUVECs - no RANTES was present in supernatants, nor was any mRNA found under unstimulated conditions (Marfaing Koka *et al.* 1995).

In all of our experiments, we did not find any detectable levels of MIP-1α under any stimulatory conditions. This is in agreement with previous results (Goebeler *et al.* 1997; Sundstrom *et al.* 2001). When endothelial cells are stimulated with different inflammatory cytokines, it becomes clear that TNF-α has an important role in chemokine production during an inflammatory response. We have shown that this cytokine induces production of IL-8, RANTES, MCP-1 and to a lesser extent IP-10.

3.4.2 Chemokine production by endothellal cells after cytokine stimulation

The increases in levels of MCP-1 and IL-8 in response to the inflammatory cytokines TNF-α and IL-1 have been well documented, both on HUVECs (Gimbrone *et al.* 1989; Rollins *et al.* 1990; Sica *et al.* 1990a; Sica *et al.* 1990b; Randolph and Furie 1995; Parks *et al.* 1998) and on other endothelial cell types (Brown *et al.* 1994; Goebeler *et al.* 1997; Beck *et al.* 1999; Anderegg *et al.* 2000; Briones *et al.* 2001). However, the effects of TNF-α on RANTES and IP-10 production are less clear.

When investigating IL-8, very few mRNA transcripts were detectable in unstimulated DMVEC, however upon stimulation with TNF-α, the number of positive cells and the signal density increased (Goebeler *et al.* 1997). At 1 – 10 ng/ml of TNF-α, maximal IL-8 protein secretion was detectable after anything more than 8 hours in culture.

BMEC expressed MCP-1 as their major pro-inflammatory chemokine after TNF-α stimulation with IL-8 of secondary importance, while all other endothelia tested expressed IL-8 the highest. The reduction in MCP-1 levels between 24 and 48 hours detected for DMVEC could be due to internalisation by specific receptors on the surface (e.g. CCR5 see chapter 5). It is suggested that in fact, CCR2 expression is reduced on the cell surface of some cell types after stimulation with TNF-α or LPS. This has not yet been shown on endothelium.

We showed that DMVEC and BMEC both secreted high levels of RANTES after 48 hours stimulation by TNF-α (62 and 51 ng/ml respectively), all other cell types secreted 11 ng/ml or less of RANTES after this stimulation. RANTES is specifically chemotactic for memory T cells, monocytes and eosinophils (Schall *et al.* 1988; Alam *et al.* 1993). RANTES mRNA and protein have both been shown by *in situ* hybridisation to be increased in DMVEC at 8 hours after 1 ng/ml TNF-α stimulation with a further increase demonstrated up to 24, then 48 hours (Goebeler *et al.* 1997). The maximum amount of RANTES demonstrated was approximately 100 pg/ml at 48 hours, which was much less than we have found, however, this is likely to be due to the lower amount of TNF-α used in the stimulation protocol (we used 25 ng/ml compared to their 1 ng/ml). Marfaing Koka *et al.* (1995) showed very little induction of RANTES protein (<0.5 ng/ml) after TNF-α (100 U/ml) stimulation of HUVEC for 20 hours but low levels of RANTES mRNA were found by *in situ*. In our experiment, we have detected a low, but probably physiologically relevant level of only 3 ng/ml after 24 hours. In a comparison of HUVEC with coronary artery endothelial cells (CAEC) (Briones *et al.* 2001), HUVEC did not produce a significant amount of RANTES protein upon stimulation, whereas CAEC produced significant amounts (approx. 5 ng/ml after 48 hours TNF-α stimulation and 25 ng/ml after IL-1 stimulation). Similar results were also shown using an RNA protection assay, demonstrating that the RANTES production is due to protein synthesis, and is not released from intracellular stores. The major discernible difference between our experimental protocol and that of Briones *et al.* is that their cells were continually grown with growth supplements. They used a medium similar to that used for growth of our lung and dermal endothelia, whereas, we put our cells into a maintenance medium containing only 10% FCS and antibiotics. We have detected more RANTES than MCP-1 secreted by DMVEC, which has also been shown at the transcription level when higher levels of RANTES mRNA than MCP-1 mRNA were found to be present in TNF–α stimulated DMVEC (Anderegg *et al.* 2000).

We showed some IP-10 production was induced in all cell types upon stimulation with TNF-α, however, levels were only physiologically significant for BMEC, SVEC, DMVEC and LMVEC. It was shown by Rollins *et al.* (1990) that there was no induction of IP-10 mRNA after TNF-α stimulation of HUVECs for either 3 or 24 hours. Briones *et al.* (2001) showed induction of IP-10 protein and mRNA in HUVECs, but in CAEC although the mRNA levels were increased after TNF–α stimulation for 24 hours, the levels were reduced by 48 hours, which suggests some sort of feedback mechanism.

In contrast, IFN-γ stimulation appears to block the production of IL-8, had little effect on RANTES production, but increased the production of MCP-1 from constitutive levels in some cell types and induced all endothelial cell types to produce IP-10 as expected from an IFN-γ – inducible protein (Luster and Ravetch 1987a). This induction of IP-10 protein and mRNA has also been shown in studies of various other endothelial cell types including HUVEC (Rollins *et al.* 1990), DMVEC (Goebeler *et al.* 1997), LMVEC (Sundstrom *et al.* 2001) and sinusoidal Liver EC, but not in Liver portal vein EC (Shields *et al.* 1999).

Other groups have also shown no increase of IL-8 after stimulation with IFN-γ, but as many of them have shown lower constitutive levels of production than we have seen, they would not have observed any noticeable reduction in expression. For example: Rollins *et al.* (1990) showed induction of MCP-1 but not IL-8 mRNA after IFN-γ treatment of HUVECs. Similar results have been shown in DMVEC (Goebeler *et al.* 1997) and LMVEC (Brown *et al.* 1994). We have shown that SVEC, BMEC and LMVEC show significant increases in MCP-1 production after 48 hours. DMVEC and Liver EC show little change, while HUVEC show a slight increase at 24 hours, which is lost by 48 hours. Goebeler *et al.* showed no change in MCP-1 protein secretion or mRNA expression by *in situ* hybridisation after IFN-γ stimulation of DMVECs, but a slight induction of mRNA and protein - up to 1.5 ng/ml above control levels at 24 hours in HUVECs. In our experimental system this would not be a physiologically significant induction, but they used a different system and have stimulated with lower amounts of TNF-α. Two other groups have also shown an induction of MCP-1 after LMVEC stimulation, but the investigation of BMEC and SVEC in this situation is novel. Beck *et al.* (1999) showed an increase in MCP-1 protein in LMVEC with an approximate doubling after 48 hours, (but not significant) while HUVEC did not respond. MCP-1 mRNA and protein have also been shown to increase slightly on LMVEC upon IFN-γ stimulation (Brown *et al.* 1994). There was no expression of IL-8 mRNA in unstimulated LMVEC, and also no increase of IL-8 after stimulation with up to 500 U/ml of IFN-γ, however there was about a six fold increase in the levels of MCP-1 found after IFN-γ stimulation. We have shown an approximate doubling in MCP-1 production at both 24 and 48 hours compared to unstimulated LMVECat the same time point..

IFN-γ and TNF-α act in synergy to produce high levels of MCP-1 from SVEC and high levels of IP-10 and RANTES from all endothelial cell types that we have examined. This synergy between TNF-α and IFN-γ has been observed in several different cellular events including adhesion molecule

expression (Detmar *et al.* 1990; Doukas and Pober 1990) and production of oxygen radicals as well as in chemokine expression (Piali *et al.* 1998).

Goebeler *et al.* (1997) found that TNF-α, but not IFN-γ, induced DMVECs to express MCP-1 mRNA after 8 hours of incubation and that at this time, protein was also detectable. RANTES was also produced; however, protein was not detectable until 24 hours after stimulation with TNF-α. Treatment of DMVECs with both TNF-α and IFN-γ together revealed no effect on MCP-1 levels, but a synergistic effect resulting in increased levels of RANTES was demonstrated.

Brown *et al.* (1994) also showed synergy in the production of MCP-1, but not IL-8 by LMVEC, however, they were using 20 U/ml of TNF-α and 50 U/ml of IFN-γ, whereas we have been using higher levels 25 ng/ml of TNF-α and 200 U/ml of IFN-γ.

Sundstrom *et al.* (2001) showed synergy only in the production of RANTES – not in the case of either IL-8 or MCP-1 on LMVEC. Terada *et al.* (1996), looking at cultured endothelial and epithelial cells from the nasal mucosa found that RANTES was again enhanced from the level of either TNF-α or IFN-γ to a much increased level when both were used together. Marfaing Koka *et al.* (1995) showed that production of RANTES by HUVEC was optimally induced by a combination of IFN-γ and TNF-α. Approx. 5 ng/ml of RANTES was observed after 20 hours in culture in 48 well plates with 100 U/ml of TNF-α and 100 U/ml of IFN-γ. IL-1β was shown to have little additive effect when TNF-α and IFN-γ were used simultaneously. They showed this by both ELISA of supernatants and by *in situ* hybridisation of mRNA. The mechanism of such a synergy appears to involve post receptor events perhaps such as activation of activator protein 1 or NF-KB acting on the 5' promotor region of the gene

On certain endothelia TGF-β significantly reduced high constitutive levels of IL-8 and MCP-1, but had no effect on levels of IP-10 or RANTES. It was found to have very little effect on IL-8 production by HUVEC, but reduced MCP-1 levels. We have shown that it reduced the TNF-α stimulated secretion of IL-8 and MCP-1 when both cytokines were applied together across several EC types. Similar results have been shown in other studies, but all on HUVEC (Chen and Manning 1996; Weiss *et al.* 1999). Weiss *et al.* also show that the p75 TNFR is downregulated by TGF-β, although this helps explain reductions in chemokine levels from those shown after the addition of TNF-α alone, this mechanism cannot explain the lower production in TGF-β stimulated cells

compared to unstimulated. We have shown that adding TGF-β to TNF-α has little effect on RANTES production an effect also shown by Crane *et al.* (2000)

3.4.3 Summary and future work

We have tested the production of chemokines by six different endothelial cell types; BMEC, SVEC, DMVEC, LMVEC, HUVEC and Liver EC. These cell types do not produce MIP-1α under any of the stimulation protocols tested. We have shown constitutive production of physiologically relevant levels of IL-8 by HUVEC, Liver EC and microvascular EC and MCP-1 by SVEC, HUVEC and the microvascular EC.

When cytokines were applied, most cell types reacted in a similar way, but to differing degrees. We have shown that TNF-α increases production of IL-8, RANTES and MCP-1 in all six cell types while IFN-γ increases production of IP-10 in all cell types except HUVEC and Liver EC. The addition of TGF-β reduces production of IL-8 and MCP-1 from constitutive levels and TNF-α and IFN-γ used in combination induce synergistic production of IP-10 and RANTES by all cell types tested.

HUVEC were found to produce high levels of IL-8 under all stimulation protocols, Liver EC also produced high levels of IL-8 in response to TNF-α.

To improve this study, it would have been ideal to have more donors of microvascular endothelium and Liver EC. Due to monetary and time constraints this was not possible. We have however, been able to give a good overview of chemokine production by endothelial cells from different tissues after stimulation by different cytokines.

Chapter 4
Binding of chemokines to endothelial monolayers

4. Binding of chemokines to endothelial monolayers

4.1 Introduction

In the previous chapter, we have shown that chemokines are produced by endothelium *in vitro*, we also know that many other cell types produce them both constitutively and during inflammation. To enable firm adhesion of leukocytes to take place, the leukocytes must sense chemokine signals during the rolling stage of recruitment. As soluble gradients cannot persist at the endothelial cell surface due to their removal by the force of the blood flow, chemokines are presented on the glycocalyx. Endothelial cells contain a thick glycocalyx rich in proteoglycans, which serve as the anchoring structures for chemokines. By this mechanism, chemokines are immobilised and presentation to leukocytes can be achieved (Rot 1992b; Tanaka *et al.* 1993b)

Proteoglycans are proteins, which have been modified post-translationally by the addition of glycosaminoglycan (GAG) side chains at serine residues. The most abundant GAG species (50 – 90% of total endothelial proteoglycans) is the polyanionic species heparan sulphate, which is structurally and biosynthetically related to heparin. Chemokines contain a heparin binding site and have been shown to bind with high affinity to heparan sulphate *in vitro* (Huber *et al.* 1991).

It has been demonstrated that IL-8 binds to the lumenal surface of post-capillary venules and small vein endothelium in the dermis *in vivo* (Rot 1992a). This is the normal site of leukocyte transmigration in most tissues. Studies of cross sections of veins have shown that binding was to the EC on the lumen of the veins. There was no accumulation of IL-8 within the endothelial cells – suggesting that it was not taken up by the cells and remained bound to the surface. Whether this binding was specifically via GAGs was not studied, although the kinetics and affinities suggested retention on the glycocalyx. Binding sites for IL-8 have more recently been viewed using electron microscopy and it was also found that heparinase digestion reduced IL-8 binding, which suggests HSPG to be a ligand for this chemokine (Middleton *et al.* 1997). *In vitro* studies have shown that MIP-1β or RANTES immobilised on various different proteoglycans are as effective as in their soluble form at activating integrin dependent adhesion of T cells (Tanaka *et al.* 1993a; Tanaka *et al.* 1993b; Gilat *et al.* 1994). MIP-1β has also been localised to the high endothelial venules (HEV) of lymph nodes where lymphocyte migration occurs (Tanaka *et al.* 1993a). Immobilisation of MIP-1α and β on rheumatoid arthritis endothelial cells via HSPG actually enabled more efficient integrin

mediated adhesion of T cells as compared to the chemokines in their soluble form (Tanaka *et al.* 1998).

GAG structures can vary considerably between and even within tissues (Gallagher *et al.* 1992; Gallagher 1997), so it is thought that differences in the binding of chemokines to GAGs may act as a further molecular method of recruiting leukocytes to specific anatomical sites. For example, the heparin binding site has on several chemokines been found to be in proximity to the carboxyl terminus and consists of several residues of lysine, surrounded by other basic residues. IL-8 and Gro-α exhibit single acidic residues within this area, which are thought to allow them to bind to specific types of heparin, specifically those with low sulphation levels. IL-8 binds heparan sulphate better than heparin, perhaps because HS is less highly modified than heparin and shows a domain type structure with regions of high sulphation, alternating with regions of low sulphation (Witt and Lander 1994).

It has been suggested that the binding of chemokines to HSPG has several advantages – high chemokine concentrations can be maintained in the correct location, HSPG can activate some chemokines, aid presentation to specific receptors and the soluble molecule is afforded some protection from damage by enzymes because of its changed conformation. By binding the GAGs on the cell surface, a chemokine can polymerise them and is thought by this method to enhance cellular responses to chemokines (Tanaka *et al.* 1993a; Hoogewerf *et al.* 1997).

Each tissue of the body has a different function, so the needs of a particular tissue will determine what is required of the endothelial cells in its blood vessels. Because chemokines are fundamental to leukocyte recruitment, their varied presentation on different endothelial cells helps determine which particular cell types will be recruited into which tissues. Different chemokines are produced at different sites and under different conditions, but it appears that their presentation to circulating leukocytes could also depend on which proteoglycans are present on the surface of the endothelial cell.

Chemokine binding to extracellular matrix components is also important during the later stages of leukocyte recruitment once the leukocyte is in the interstitium and is migrating to the specific site of damage or injury. At this stage, chemokines form a gradient from high (near the injury) to low along which the leukocyte can migrate towards the higher concentrations.

The aim of this study is to determine the levels of binding of our five inflammatory chemokines to different endothelia *in vitro* and to then further investigate using enzyme digestions whether binding is via particular components of the proteoglycan layer or by a different mechanism.

4.2 Methods

4.2.1 Chemokine binding assay

The cells were set up and rested as described in section 2.2 then washed four times in HBSS. 65 µl of recombinant human chemokine (IL-8, MIP-1α, RANTES, MCP-1 or IP-10, all R & D systems) was added in triplicate at 0, 250 and 500 ng/ml diluted in HBSS and incubated at 37°C for 1 hour. The plate was washed, fixed and chemokines remaining bound to the monolayer were detected using the cell based ELISA assay as described in section 2.2.3.

100 µl per well of blotin conjugated anti human chemokine antibody (IL-8, MIP-1α, RANTES, MCP-1 or IP-10, all R & D systems) at a 1:500 dilution in ELISA diluent (see section 2.2.1) was used to detect bound chemokine. The chemokine antibodies were directly conjugated antibodies to biotin, so no second layer was necessary and the wells were filled with ELISA diluent alone while the secondary antibody layer required to detect MHC class I was incubated.

4.2.2 Chemokine binding assay on extracellular matrix components

This experiment was also performed on extracellular matrix components to investigate which ones can hold chemokines. 10 µg/ml of laminin (Sigma), type I collagen (Sigma), heparan sulphate proteoglycan (HSPG, Sigma) or superfibronectin (Sigma) was used in place of the confluent monolayer, incubated overnight at 4°C, then blocked using 100 µl/well of 5 mg/ml BSA for 30 mins. The layer was washed four times in HBSS and then chemokines added for 1 hour, fixed and assayed as described previously (section 4.2.1).

4.2.3 Enzyme digestion

Enzyme digestions of the proteoglycan layer were performed to investigate its composition. In this experiment, a confluent monolayer of BMEC was washed three times in HBSS. Then enzymes were added as described in section 2.3 prior to commencing the chemokine binding assay. Briefly 65 µl of 10 U/ml heparinase III, 1 U/ml chondroitinase ABC or 1 U/ml neuraminidase (diluted in HBSS,) was added to triplicate wells for 1 hour at 37°C before being washed off for the chemokine binding assay as described in section 4.2.1.

4.2.4 Competition binding of RANTES and MIP-α to endothelium

This was performed as described in 4.2.1, except that mixtures of different concentrations of chemokines were used in triplicate wells. Mixtures of chemokines were made up which had increasing amounts of RANTES from 0 to 1000 ng/ml mixed with 500 ng/ml of MIP-1α and added to BMEC for 1 hour as in the previous binding assays. This assay was also repeated with increasing concentrations of MIP-1α competing against 500 ng/ml RANTES.

4.2.5 Chemokine binding at different temperatures

To check whether any of the chemokines were being internalised during the time of the experiment, we tested chemokine binding at different temperatures. The assay was performed exactly as described in section 4.2.1 except each plate was incubated at 4°C, 25°C or 37°C after the addition of chemokine until fixation of the cells.

4.2.6 Statistics

To analyse assays in this chapter a one way or two way ANOVA was performed followed by tukeys post hoc t-test comparing all pairs of values where necessary.

4.3 Results

4.3.1 Chemokine binding to different endothelial monolayers

We determined the levels of chemokine binding to different endothelia by means of a chemokine binding assay (described in 4.2.1). Figure 4.1 shows the binding of five different chemokines (IL-8, MIP-1α, RANTES, MCP-1 and IP-10) to our five different endothelial cell types. For each cell type, we compared chemokine binding to the amount of MHC class I expressed. This was to help standardise between cell types. We have shown that MIP-1α and RANTES bound at significant levels to all five different types of endothelium, whereas IL-8, and MCP-1 did not bind under static conditions. Each of the five endothelial cell types bound MIP-1α and RANTES at differing levels while microvascular endothelia also bound a significant amount of IP-10 above controls when 500 ng/ml of chemokine was added.

When 500 ng/ml of chemokine was added to the BMEC monolayer (fig. 4.1a), the endothelium bound slightly more RANTES than MIP-1α after a 1 hour incubation with means of 63% and 54% of the MHC class I levels respectively. However when 250 ng/ml of chemokine was added, there was a marked difference in binding as MIP-1α bound 3% (approx. one fifteenth of that bound at 500 ng/ml) while RANTES bound 31% of MHC class I levels (approximately half of the amount bound at 500 ng/ml).

SVEC bound much higher amounts of RANTES than MIP-1α at both concentrations (fig. 4.1b), while DMVEC, LMVEC and HUVEC all bind much more MIP-1α than RANTES with HUVEC showing the highest levels of chemokine binding and LMVEC the lowest (figs 4.1c-e).

We have shown two distinct profiles of binding of chemokine to endothelium. The first type of binding is represented by RANTES binding to BMEC and SVEC and showed a proportional increase in binding in relation to increased concentration. The second binding pattern, shown by the binding of MIP-1α to all five different endothelial cell types, is where the bound chemokine increases out of proportion with concentration increases. The binding at 500 ng/ml was typically 5 to 20 times that at 250 ng/ml. This suggests that MIP-1α uses a co-operative binding mechanism to bind to endothelium.

RANTES binding to LMVEC, DMVEC and HUVEC appeared to be part way between the two with approximately 4 to 7 times increase in binding from 250 ng/ml to 500 ng/ml.

This suggests that MIP-1α and RANTES could bind to endothelium by two different mechanisms. It is possible that the higher binding, could be mediated by a specific receptor for chemokines as opposed to binding to various residues of the proteoglycan layer, or it could indicate different affinities of binding to different components of the proteoglycan layer.

MIP-1α and RANTES both bind to similar specific receptors, both bind to CCR1 and CCR5, and RANTES also binds to CCR3. So both chemokines will have effects on similar subsets of leukocytes.

Having determined that all five cell types bound both MIP-1α and RANTES and that the means by which these two chemokines bind could be different, we then wanted to investigate further the methods by which these two chemokines were binding.

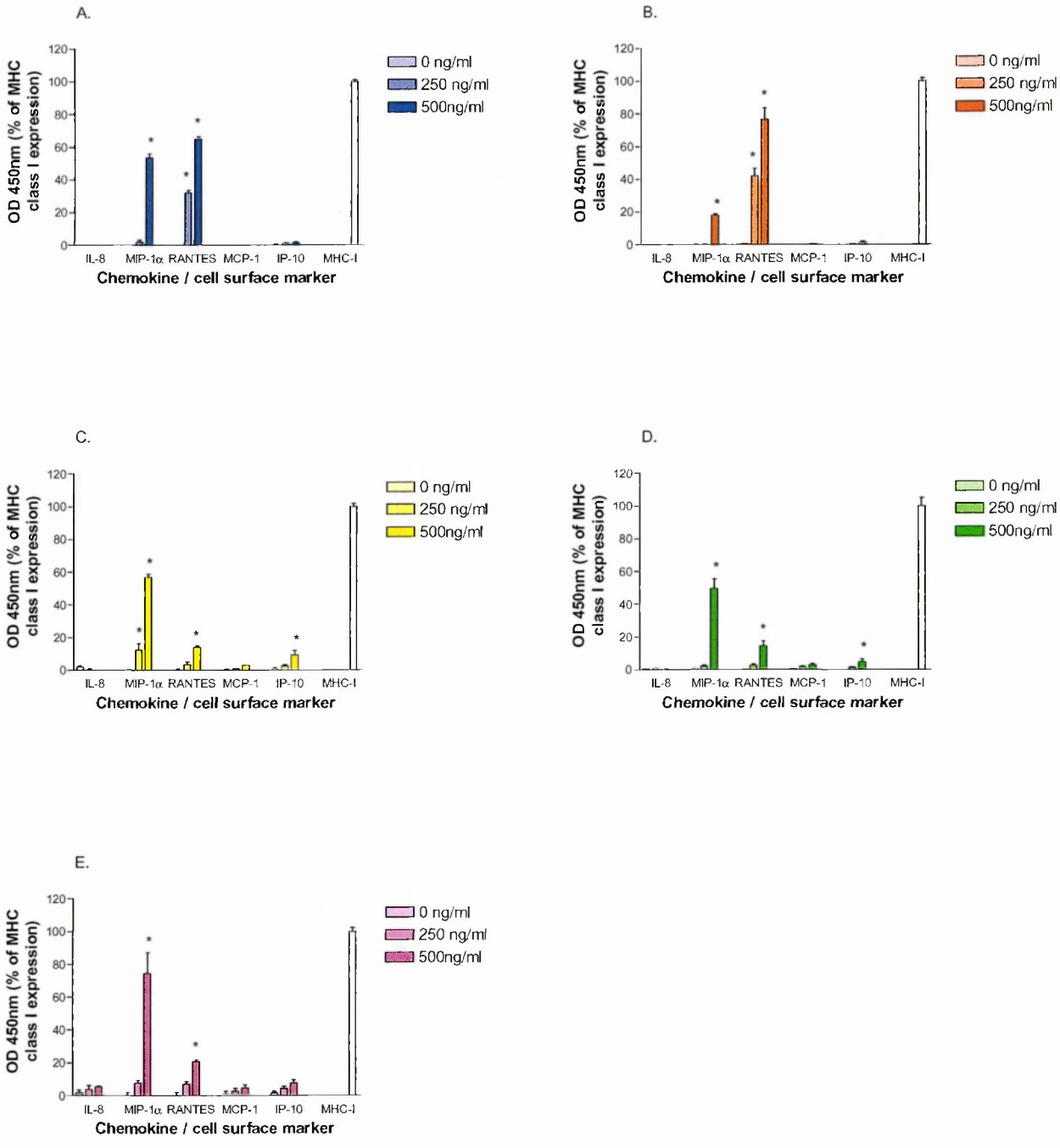


Figure 4.1 Chemokine binding to endothelial monolayers

Endothelial cells were grown to confluence in 96 well plates, rested for 24 hours. Then different concentrations of chemokines were added (0, 250 or 500 ng/ml of IL-8, MIP-1α, RANTES, MCP-1 or IP-10) for 1 hour at 37°C. The cells were then washed, fixed and bound chemokine was detected using biotin labelled specific anti human chemokine antibodies. A. BMEC, B. SVEC, C. DMVEC, D. LMVEC, E. HUVEC. The results are of triplicate wells from at least two experiments. Significance was tested by ANOVA with a post hoc t-test (*=P<0.05)

4.3.2 Chemokine binding to endothelial monolayers after enzyme digestion

To determine how chemokines might be binding to the endothelial cell, we chose to investigate BMEC further as these cells had shown similar binding levels of the two chemokines and clearly demonstrated the different types of binding as well as being particularly simple to culture.

We investigated whether the chemokines were binding to the proteoglycan layer by digestion of various components of the layer (figs. 4.2 and 4.3). We used the enzymes heparinase III which cleaves heparan sulphate proteoglycan, chondroitinase ABC lyase, which cleaves chondroitin sulphate proteoglycan and dermatan sulphate and neuraminidase which splits off sialic acid from other glycoproteins (for further details see section 2.3)

We found that the digestion of HSPG by heparinase significantly reduced the binding of RANTES at 500 ng/ml, but not at 250 ng/ml (fig 4.2). Heparinase had no effect on MIP-1α binding (fig 4.3), while chondroitinase and neuraminidase had no effect on the binding of either chemokine. Although Heparinase significantly reduced the binding of RANTES, it did not abolish binding completely, so this suggests that there is more than one method by which RANTES binds.

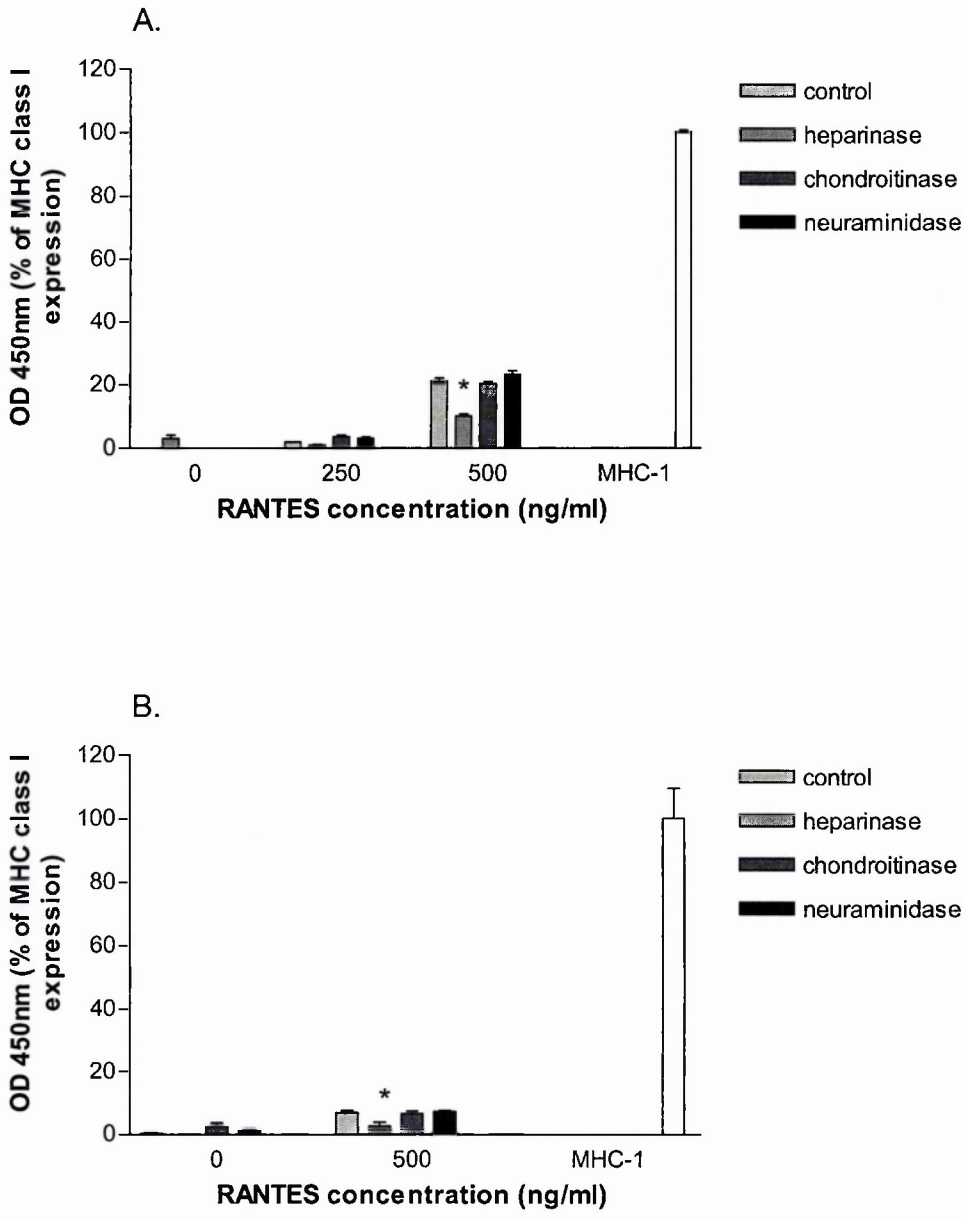


Figure 4.2 RANTES binding to BMEC monolayers after enzyme digestion of the proteoglycan layer

Endothelial monolayers were digested with 10 U/ml heparinase, 1 U/ml chondroitinase, 1 U/ml neuraminidase or no enzyme for exactly 1 hour at 37°C. Different concentrations of RANTES were added (0, 250 or 500 ng/ml) for 1 hour at 37°C. The cells were then fixed and bound chemokine was detected using biotin labelled specific anti human chemokine antibodies. A. and B. show duplicate experiments. Significance was tested by two way ANOVA with a post hoc t-test (*=P<0.05).

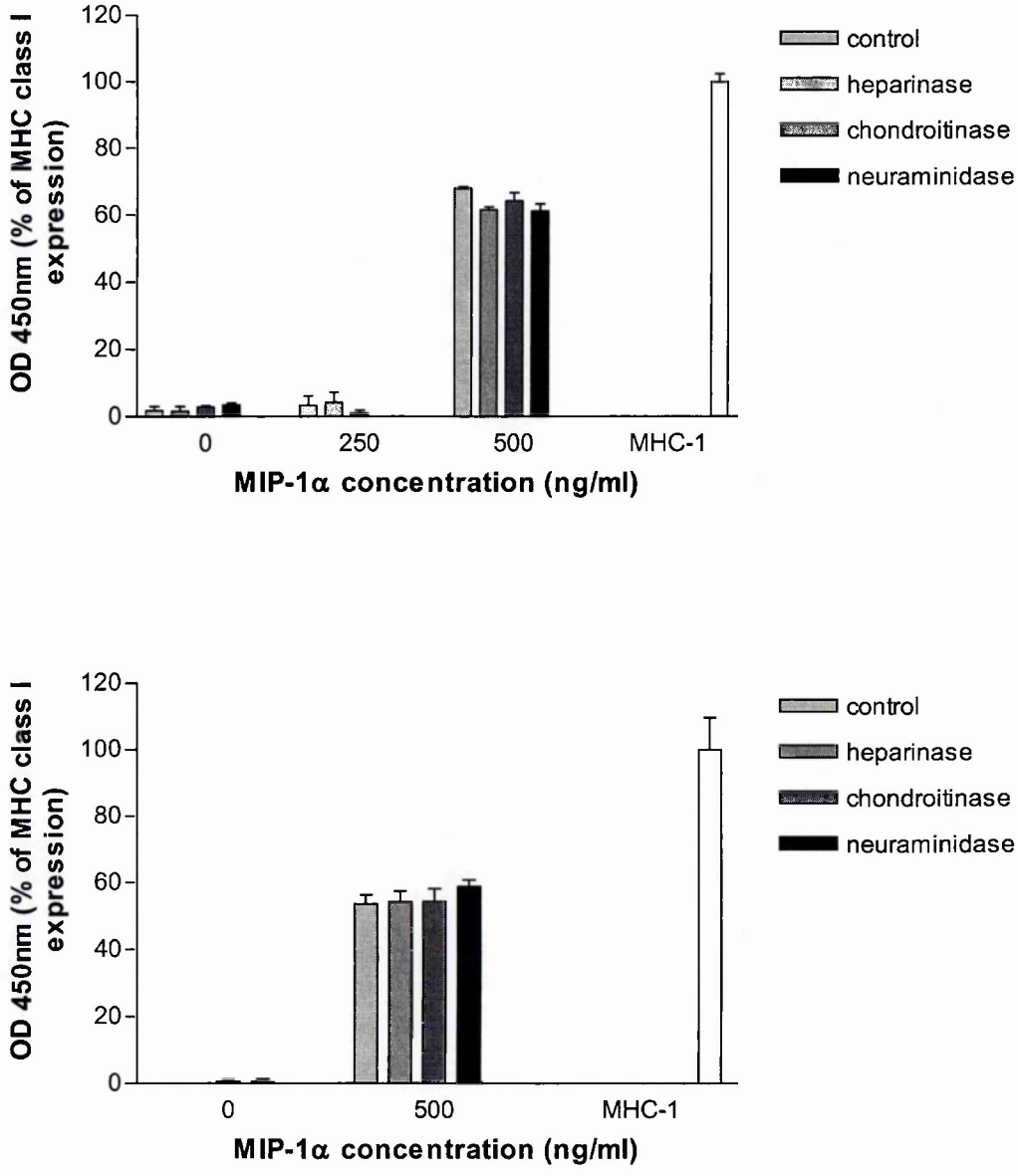


Figure 4.3 MIP-1α binding to BMEC monolayers after enzyme digestion of the proteoglycan layer

Endothelial monolayers were digested with 10 U/ml heparinase, 1 U/ml chondroitinase, 1 U/ml neuraminidase or no enzyme for exactly 1 hour at 37°C. Different concentrations of MIP-1α were added (0, 250 or 500 ng/ml) for 1 hour at 37°C. The cells were then fixed and bound chemokine was detected using biotin labelled specific anti human chemokine antibodies. A. and B. show duplicate experiments. No significant differences were detected using ANOVA

4.3.3 Chemokine binding to extracellular matrix components

During leukocyte migration through the tissues, chemokines are bound to ECM components allowing leukocytes to migrate via haptotaxis and become activated. To further investigate what substrates different chemokines could bind to, we coated 96 well tissue culture plates with different ECM components (10 μg/ml of HSPG, laminin, fibronectin or collagen) and then performed the binding assay directly onto these components (table 4.1). We found that IL-8 and MCP-1 did not bind to any of the ECM components tested, while RANTES bound all those tested to varying degrees. MIP-1α showed binding only to collagen, while IP-10 bound to all substrates tested.

Table 4.1 Chemokine binding to extracellular matrix components

Assayed by ELISA of bound chemokine to 10 μg/ml of HSPG, laminin, fibronectin or collagen. No significant binding (-), Low binding (+) approx. equivalent to 10 – 30% binding to MHC I on BMEC, High binding (++) approx. equivalent to >30% binding to MHC I on BMEC.

CHEMOKINE	EXTRACELULLAR MATRIX COMPONENT			
	HSPG	Laminin	Fibronectin	Collagen
IL-8	-	-	-	-
MIP-1α	-	-	-	++
RANTES	++	+	+	++
MCP-1	-	-	-	-
IP-10	++	++	++	++

4.3.4 Effects of temperature on chemokine binding

Our binding experiments were carried out at 37°C during the one hour incubation of chemokines with cells before fixation. We lowered the temperature at which this was carried out to slow the cells metabolism and turnover of molecules. We tested the binding of MIP-1α, RANTES and IP-10 to BMEC and SVEC at different temperatures. Using a two way ANOVA with post hoc t-test, we tested whether there were significant differences between our binding assays that were normally performed at 37°C and those where the cell metabolism was slowed by reducing the temperature to 25 or 4°C. The results have some implications for the kinetics of chemokine binding to endothelial cells. We have shown that at 500 ng/ml RANTES, the binding was reduced at 25 and 37°C as compared to 4°C (fig. 4.3a). When 250 ng/ml of MIP-1α was added to BMEC, the binding was reduced at 37°C compared to that at 4°C (fig. 4.3b). No significant differences were observed in IP-10 binding to BMEC at different temperatures (fig. 4.3c). On SVEC, RANTES binding was increased at 500 ng/ml, but reduced at 250 ng/ml when 37°C was compared to 4 or 37°C and no significant differences were detected in MIP-1α binding to SVEC (figs. 4.3 d, e). However the binding of IP-10 to SVEC was much increased at 37°C as compared to the lower temperatures (fig. 4.3f).

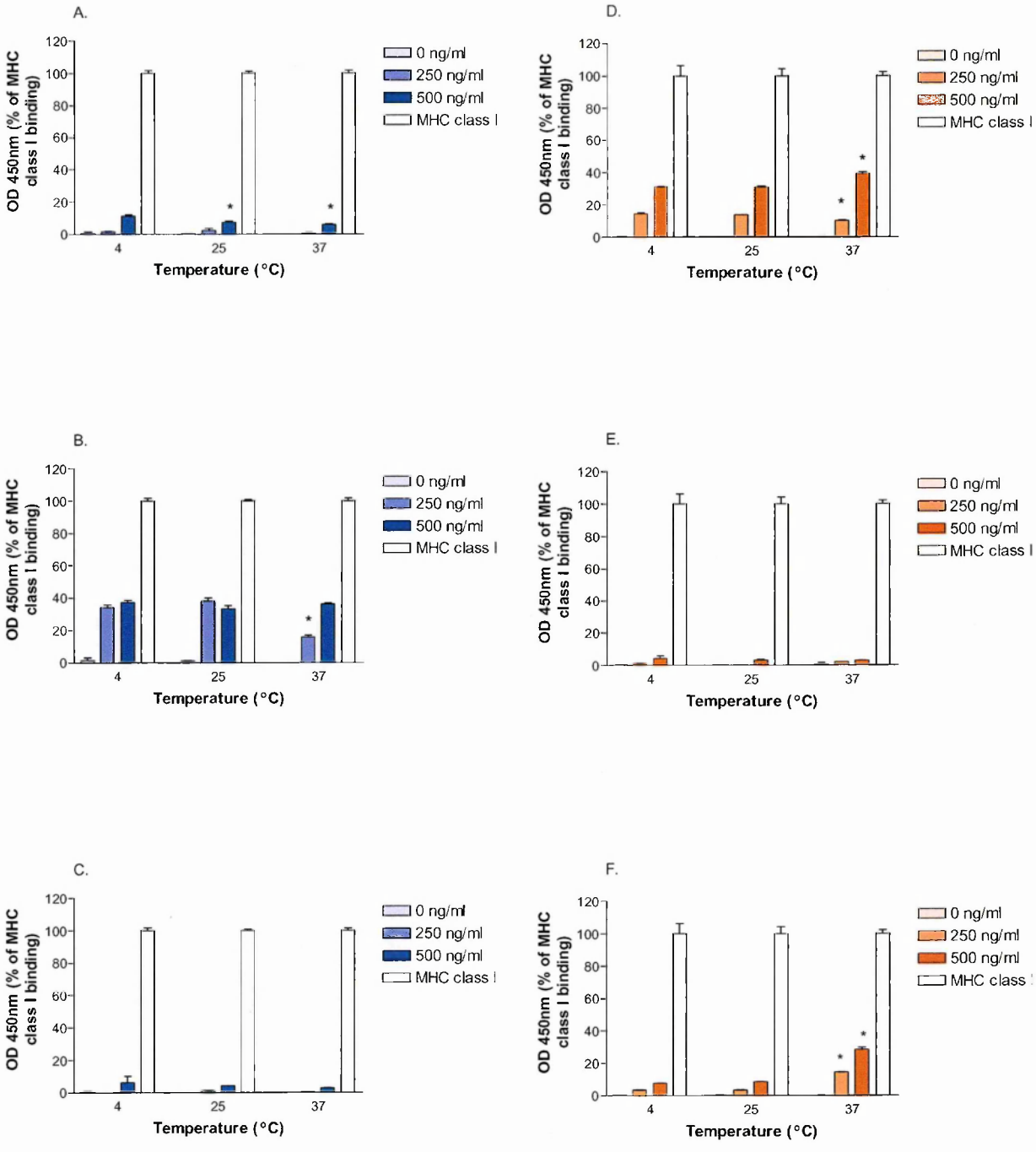


Figure 4.4 Binding of RANTES and MIP-1α to BMEC and SVEC at different temperatures

There is little difference observed in the binding of RANTES or MIP-1α to SVEC at different temperatures which indicates that the receptors that these chemokines bind to are not being internalised. The chemokine binding step and the subsequent reaction was done at 4, 25 and 37 ° C. A. RANTES binding to BMEC, B. MIP-1α binding to BMEC, C. IP-10 binding to BMEC, D. RANTES binding to SVEC, E. MIP-1α binding to SVEC, F. IP-10 binding to SVEC. The experiments shown are one representative of at least 3 performed. Significance was tested by ANOVA with a post hoc t-test (*=P<0.05).

4.3.5 Competition binding of RANTES and MIP-α to endothelium

Both MIP-1α and RANTES bind to BMEC at similar levels at 500 ng/ml and both are known to bind similar chemokine receptors. To test whether these two chemokines were competing for binding sites we used a competition-type binding assay. We have shown that there is no change in the binding of RANTES when different concentrations of MIP-1α are added, however, when increasing concentrations of RANTES are added, there is a slight but significant reduction in MIP-1α binding (fig 4.5a). When 1μg/ml of RANTES is added, the reduction in MIP-1α binding is approximately 10%. This shows that while MIP-1α cannot compete with RANTES binding to BMEC, RANTES is capable of displacing MIP-1α, suggesting that these two chemokines share a binding site.

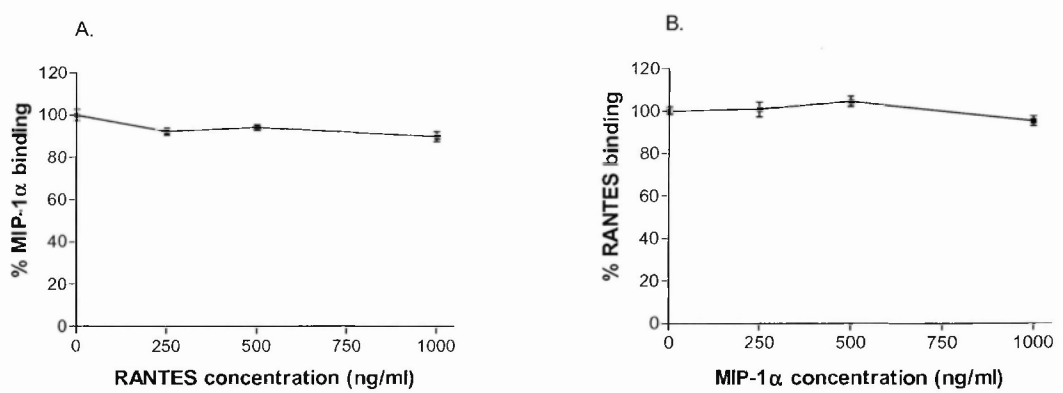


Figure 4.5 Competition assays between MIP-1α and RANTES on BMEC

We have found that there is no competition between RANTES and MIP-1α for binding sites in the assay system we are using. A. Increasing concentrations of RANTES competing with 500 ng/ml of MIP-1α. Results expressed as a percentage of MIP-1α bound as compared to no addition of RANTES. B. Increasing concentrations of MIP-1α competing with 500 ng/ml of RANTES. Results expressed as a percentage of RANTES bound as compared to no addition of MIP-1α. The results show the mean and standard error of three experiments. Significance was tested by ANOVA, no significant differences were detected.

4.4 Discussion

To investigate whether there are differences in the chemokine binding properties of different endothelia, we have measured the levels of chemokine binding to different endothelial monolayers using a cell based ELISA method. We have shown that MIP-1α and RANTES both bound to all five endothelia tested and that IP-10 bound at low, but significant levels to LMVEC and DMVEC. The binding profile of RANTES is also quite different to that of MIP-1α on all cell types. Since the system used is static we do have to bear in mind that *in vivo*, the chemokine would be subjected to removal by the flow of the blood around the body.

It has now been clearly shown that chemokines can bind to endothelium via GAGs present in the proteoglycan layer and it is also known that endothelial cells from different tissues / compartments have differences in proteoglycans present and the levels of sulphation of those proteoglycans. However, this is the first time that a comprehensive study has directly compared several different endothelial cell types for their chemokine binding properties. Several groups have investigated chemokine binding under both static and shear flow conditions, though once again mostly using HUVECs.

We chose to investigate BMEC further as they demonstrate high levels of binding of both RANTES and MIP-1α and clearly demonstrate the 2 distinct binding profiles. We have shown that some of the binding to RANTES is via HSPG, but no significant binding of MIP-1α to heparan sulphate was shown. However when we test the chemokine binding at different temperatures, there is reduced binding at certain concentrations. It appears that MIP-1α and RANTES may share a binding site, and that RANTES has a stronger affinity for this particular ligand. Possible candidates molecules include CCR1 or CCR5.

4.4.1 MIP-1α

We have shown significant binding of MIP-1α at 500 ng/ml to all endothelia tested, but have shown no binding to purified HSPG and no differences in chemokine binding after digestion of heparan sulphate, chondroitin sulphate and dermatan sulphate or sialic acid residues from the proteoglycan layer. At a concentration of 250 ng/ml there is higher binding at lower temperatures but this is not shown significantly at 500 ng/ml. In our original chemokine binding assay, the pattern of binding

suggests co-operation, which could mean that I MIP-1α is binding via its specific chemokine receptors, CCR1 and CCR5 as it is one of the few chemokines that does not bind to DARC (Szabo *et al.* 1995). We showed that MIP-1α did bind to collagen, though not to either fibronectin or laminin. *In vivo* studies have also shown no MIP-1α binding to human dermal EC (Middleton *et al.* 1997). However, this does not exclude MIP-1α from a role in leukocyte migration at other sites and they suggest that it may be more useful at anatomical sites where immobilisation is not necessarily required – for instance in the capillaries of the lung and kidney. It has also been suggested that perhaps MIP-1α has more important effects as a stimulatory chemokine acting on endothelial cells and leukocytes to produce other inflammatory mediators or other effects of the leukocytes. We have also demonstrated that at the concentrations used in these assays, there is no competition between MIP-1α and RANTES for binding sites.

Ali *et al.* (2000) showed on CHO cells that MIP-1α binding to CCR1 & 5 is independent of the presence or absence of GAGs, but at low concentrations (pM level), the presence of GAGs increased the affinity of MIP-1α for the specific receptors, possibly by some sort of conformational change brought on by binding to the GAGs.

IL-8 and RANTES were shown to bind at higher levels to dermal EC than MCP-1, 3 or MIP-1α did (Hub and Rot 1998). These results are contradictory to what I have observed - I have shown strong binding of MIP-1α to dermal endothelia and lower binding of RANTES. It must be noted though, that my experiments were performed *in vitro* on microvascular EC and used much higher concentrations of chemokines than these which were actually performed on pieces of skin via an *in situ* immunoassay. *In vivo*, the conditions will be different and there will be many additional components present (e.g. other cells or ECM components) that could change the affinity of a chemokine for a particular cell type. In particular it was noted that MIP-1α binds strongly to infiltrating macrophages. It is also likely that because I am using a higher concentration, that the MIP-1α is more likely to be oligomerised.

4.4.2 RANTES

We have shown that SVEC and BMEC bind significant amounts of RANTES when either 250 or 500 ng/ml is added to the culture. All other endothelia tested only show significant levels of binding at 500 ng/ml and show less overall binding than to MIP-1α. After the proteoglycans present on the

endothelial cell surface are digested with heparinase, there is decreased binding of RANTES at 500 ng/ml and although there is a reduction at 250 ng/ml, this result is not statistically significant. This shows that heparan sulphate is important in RANTES binding to endothelium, however, there could be more than one method by which RANTES binds, as heparinase does not totally abolish all RANTES binding to BMEC, it reduces it by approximately 50%. We also showed that RANTES could compete with MIP-1α for binding sites, which suggested that HSPG was not its only binding site as MIP-1α was not found to bind HSPG (the other RANTES ligand) significantly.

We have shown a slight but significant reduction in RANTES binding at 37°C as compared to 4°C on BMEC when 500 ng/ml of the chemokine is added. The results on SVEC are somewhat different, with increased binding noticeable at 500 ng/ml at 37°C, while slightly decreased binding is seen at 250 ng/ml at 37°C as compared to 4°C. Looking at these results overall suggest that RANTES may be binding by more than one method perhaps both to CCR1, 3 or 5 and to the proteoglycan layer and it could be dependent on the concentration of chemokine present.

RANTES has been shown to bind selectively to dermal venular ECs (not capillaries or arteries) *in vivo* and the binding sites were specific and saturable (Hub and Rot 1998). Since we are using cultured microvascular EC, this could explain the difference. Also, the dermal endothelial cells that we have used were at passage 5 – 7 and could possibly have lost some of their specific phenotype by this stage. Hub and Rot showed that unlabelled IL-8 competing with labelled RANTES was able to entirely block RANTES binding, while IL-8 binding was strongly reduced by competition with unlabelled RANTES but not abolished completely. Our data shows a slight reduction in MIP-1α binding when in the presence of high concentrations of RANTES.

RANTES binding on cultured microvascular EC has been shown to be in similar patterns to those on HUVEC, this concurs with my results, which show little difference between HUVEC and microvascular EC (Hoogewerf *et al.* 1997).

Using heparinase on HUVEC, the binding of RANTES was reduced by 10 – 40% (Kuschert *et al.* 1999). Although we have not tested this on HUVEC in this study, this is similar to what we have found for BMEC (approximately 50% reduction).

4.4.3 IP-10

We have shown that IP-10 bound significantly only to lung and dermal EC and then at a very low level. In some experiments, we showed IP-10 binding to SVEC, however this was inconsistent. In investigating the effects of different temperatures on SVEC, we found that IP-10 binding was significantly higher at 37°C than at 25 or 4°C.

Using a fusion protein of IP-10 and alkaline phosphatase, Luster *et al.* (1995) have demonstrated that the binding site for IP-10 is the same as that for PF-4 and that both of them are able to inhibit proliferation/angiogenesis. The binding of IP-10 to EC is specific, saturable, and PF-4, heparin and heparan sulphate all reduce its binding. They found that IL-8, MIP-1α, MIP-1β, MCP-1 and RANTES had no effect on IP-10 binding suggesting differences in the binding sites for GAGs.

4.4.4 IL-8

In these studies, we have used high (higher than physiological levels) of chemokines. The system we have used for detection requires many washing stages, which tend to loosen the endothelial cell from the gelatin and from the monolayer. If during the experiment, the endothelial cells become activated, then the cells lift off yhe plate. It appears that this might be happening on addition of IL-8. This is interesting to note as it is possible, that although in this assay, we cannot definitively demonstrate binding to the endothelium, activation by IL-8 could be important in the migration of endothelial cells. To improve the assay, it would perhaps be better to use a radiolabelled chemokine at a lower concentration as this would be more sensitive.

In the results presented for IL-8, some binding appears to be occurring, particularly to HUVEC, but due to the loss of up to 70% of the cells, we cannot draw any conclusions. Previous publications have found no binding of IL-8 to cultured HUVECs (Hechtman *et al.* 1991; Rot 1992a),

In other studies by Middleton *et al.* (1997) on the dermis *in vivo*, IL-8 was observed under the electron microscope on the lumenal EC surface particularly concentrated on the microvillus processes in close proximity to adhesion molecules. When radiolabelled IL-8 was injected intradermally, they showed that it rapidly crossed to the lumen via a form of transcytotic vesicular transport and was presented on the cell surface. The digestion of HSPG using heparinase reduced the IL-8 found in relation to EC and also if the C-terminus (GAG binding domain) is truncated they

found that the IL-8 did not trancytose so well. Hence the C-terminus GAG binding domain is thought to be required for transcytosis.

4.4.5 MCP-1

In these experiments, we have shown no binding of MCP-1 to any of the five endothelial cells tested. It has previously been described that MCP-1 did not associate with the HUVEC cell surface, whereas Gro-α did via HSPG – to detect this, FACS, cell surface ELISA and immunofluorescent staining were used (Weber *et al.* 1999b). This differs from published results *in vivo*, where MCP-1 and MCP-3 have been shown to bind selectively to venular ECs of the dermis (not capillaries or arteries) and it was found that the binding sites were specific and saturable (Hub and Rot 1998). These variations in results could be due to the heterogeneity of endothelium or due to the different methods employed.

4.4.6 General discussion

We have shown that heparinase significantly reduced RANTES binding to BMEC, suggesting that HSPG is important in RANTES binding. However it appears that MIP-1α binds by a different method. Figure 4.1 shows the binding of MIP-1α to the different endothelia. It is possible that it could be more specific than the RANTES binding as doubling the concentration more than doubles the amount of MIP-1α detected. It is possible that at 500 ng/ml, MIP-1α was in the form of a dimer, hence high levels of binding were detected. The binding could be via a chemokine receptor or via another molecule on the cell surface (another proteoglycan or a different type of receptor). MIP-1α has been previously shown to bind HSPG as a dimer, but heparin as a monomer (Hoogewerf *et al.* 1997; Koopmann and Krangel 1997; Stringer *et al.* 2002), but as digestion with heparinase had no effect in this model, we must discount this possibility.

We have shown that some of the binding of RANTES is via HSPG, but not all of it, and we have shown that there is potentially a different specific receptor method of binding of MIP-1α to endothelium. DARC has been found on post-capillary venules (Hadley *et al.* 1994; Peiper *et al.* 1995) and is known to bind several CC and CXC chemokines with the notable exception of MIP-1α (Neote *et al.* 1993a; Szabo *et al.* 1995). The other potential method for chemokine binding is via specific receptors – which we will investigate in more detail in the next chapter. There is the

potential for the involvement of DARC in RANTES binding – either alone or in co-operation with HSPG or CCR1,3 or 5.

Hoogewerf *et al.* (1997) found that RANTES, MCP-1, MIP-1α and IL-8 bound to HUVECs and that IL-8, MCP-1 and RANTES form oligomers when binding to the cell surface or to immobilised heparin, but that MIP-1α remains as a monomer under the same conditions. However later studies (Stringer *et al.* 2002) show that the dimer of MIP-1α binds strongly to HSPG, but not heparin. Mutation of the heparin binding site did not affect the binding or signalling by MIP-1α (Koopmann and Krangel 1997), therefore HS does not appear to be required for binding of MIP-1α. When the proteoglycan layer was digested using a combination of heparinases and chondroitinases (Hoogewerf *et al.* 1997) the binding of RANTES, MCP-1, MIP-1α and IL-8 was reduced but by different amounts (40 – 80%). In their study a 4 hour digest at 37°C was used, but with much lower enzyme concentrations. Since the primary binding site of MIP-1α to HUVEC is not via GAG binding, it might be via a specific high affinity chemokine receptor, but by RT PCR they could not detect any CXCR1-2, CCR1-5 or DARC mRNA.

RANTES, MIP-1α, MCP-1 and IL-8 bound to HUVEC could be selectively competed for by soluble GAGs (Kuschert *et al.* 1999). RANTES was the most selective – remaining bound to EC under very high concentrations of competing GAGs, while MIP-1α was the least selective – easily being competed for by heparin while IL-8 and MCP-1 were intermediate. Similar results were obtained when the chemokines were immobilised on heparin. By using a species of heparin with reduced sulphation levels, they found that binding required the N and O sulphated groups, however despite chondroitin and dermatan sulphate having similar sulphation levels they showed very different binding patterns.

Witt and Lander (1994) have demonstrated that IL-8 and Gro bind to the same fractions of HSPG, whereas PF-4 and NAP-2 bind to different specific moieties. They also showed that the specificity of binding is determined by the glutamate residues present in the GAG binding portion of the chemokine. This means that the sulphation patterns on the GAGs in the proteoglycan layer determine which particular chemokines will bind at which particular sites.

BMEC express more HSPG per cell than HUVEC and the sulphation patterns are different – BMEC show higher levels of sulphation as well as more N-sulphated domains although the proportions of heparan sulphate, chondroitin sulphate and dermatan sulphate did not change (Netelenbos *et al.*

2001). This could begin to explain some of the differences observed in chemokine binding between cell types.

Chemokine interactions with the glycocalyx of endothelium have an important role in modulating the presentation of chemokines to circulating leukocytes. Chemokines bound to the proteoglycan layer are not susceptible to being washed away under flow conditions and more efficiently recruit leukocytes. The binding of chemokines to HSPG promotes the accumulation at appropriate sites, and since GAGs have been found to display differential specificity for structural motifs, they have the potential to provide specificity to the process of presentation of chemokines at the vessel wall. GAG expression has been observed to be altered in certain disease states such as atherosclerosis and rheumatoid arthritis, so presentation of different chemokines may explain why certain cell types are recruited more during these disease states. So chemokine presentation on the vessel wall provides a mechanism for site and stimulus specific selective location of chemokines and hence leukocytes.

In this study we have shown MIP-1α and RANTES binding to all five endothelial cells and IP-10 binding to LMVEC and DMVEC at a low level and inconsistently on SVEC. We have shown by digesting HSPG that this is the mechanism by which RANTES binds, however, there is a possibility that there is another mechanism too as heparinase digestion did not reduce RANTES binding completely to zero. MIP-1α does not bind HSPG under these conditions, however it must bind via a different mechanism, possibly through specific CC receptors.

Chapter 5
Chemokine receptor expression

5. Chemokine receptor expression

5.1 Introduction

In 1991 the first specific chemokine receptors were discovered, they were two receptors for IL-8, now named CXCR1 and CXCR2, (Holmes *et al.* 1991; Moser *et al.* 1991; Murphy and Tiffany 1991; Schumacher *et al.* 1992). However until relatively recently, the actions of chemokines and the expression of their specific receptors had only been described for leukocytes. Now, there are several studies, which show that other cell types including endothelium are able to bind to and respond to chemokines (Rot 1992a; Rot 1992b; Horuk *et al.* 1997; Tanabe *et al.* 1997 and see table 5.1).

Chemokine receptors comprise a family of seven – transmembrane-spanning G protein receptors that signal mainly through G_i - type proteins. The receptors have a single polypeptide chain approximately 350 amino acids in length and consist of a short extracellular N-terminus and an intracellular C-terminus, with three intracellular and three extracellular loops in between. A disulphide bond links highly conserved cysteines in extracellular loops 1 and 2. The N-terminus and the third intracellular loop are thought to be essential for the specific binding of chemokines. These chemokine receptors exhibit 25 – 80 % sequence homology and are part of a much larger family that include receptors for neurotransmitters, inflammatory mediators, hormones, paracrine substances and many other molecules.

DARC was the first chemokine receptor to be identified on endothelial cells (Hadley *et al.* 1994). Since then, chemokine receptors have been found to be present on vascular endothelium by several different groups (reviewed in Murdoch and Finn 2000). Unfortunately most of these studies concentrate on one or maybe two different types of endothelium, almost always HUVEC. In this study, we examine how chemokine-receptor expression varies between endothelia from several different vascular beds. Evidence concerning the presence of endothelial expressed chemokine receptors has been conflicting (see table 5.1). Published results so far vary widely from no chemokine receptors present (Petzelbauer *et al.* 1995) to several receptors present (Gupta *et al.* 1998; Murdoch *et al.* 1999). Many of the studies have focused on HUVECs, however, brain microvascular, dermal microvascular and human coronary artery endothelial cells are also used as

comparisons in some studies. There do not seem to have been any studies done specifically on SVECs. In this study, we have examined five different endothelial cell types.

Table 5.1 Summary of chemokine receptors detected on endothelium

REFERENCE	ENDOTHELIUM	RECEPTORS EXAMINED	RECEPTORS FOUND
Schonbeck *et al.* (1995)	SVEC & HUVEC (no distinction)	CXCR1 & 2	CXCR1
Petzelbauer *et al.* (1995)	HUVEC DMVEC	CXCR1 & 2	none
Murdoch *et al.* (1997) (poster abstract)	HUVEC	CXCR1 & 2 CCR1 - 5	CXCR1 & 2 CCR1, 2B, 3, 4, 5
Feil and Augustin (1998)	HUVEC DMVEC	CXCR1 – 4 CCR1 – 6 DARC CXCR1 – 4 CCR1 – 6 DARC	CXCR3, 4 > CXCR2 > CXCR1 Weak CCR5 DARC CXCR3, 4 > CXCR1, 2 none DARC
Gupta *et al.* (1998)	HUVEC	CXCR1 – 4 CCR1 - 3	CXCR4 > CCR3 > CXCR1, CCR1, 2
Volin *et al.* (1998)	HUVEC & bovine	CXCR4	CXCR4 on all cell types
Berger *et al.* (1999)	CAEC Brain EC	CXCR1 – 4 CCR1 - 5	CXCR4, CCR3, CCR5 on all. CCR2A on CAEC only. CXCR1 & 3 on Brain EC only.
Murdoch *et al.* (1999)	HUVEC	CXCR1 - 4	CXCR4 > CXCR1 > CXCR2
Weber *et al.* (1999a)	HUVEC	CCR1 & 2	Low CCR2 > CCR1
Molino *et al.* (2000)	CAEC IAEC HUVEC	CXCR4	CXCR4
Salcedo *et al.* (2000a)	HUVEC DMVEC	CCR2	CCR2 more abundant on DMVEC than HUVEC
Salcedo *et al.* (2000b)	DMVEC HUVEC	CXCR1 – 4 CXCR1 - 4	CXCR4 > CXCR1 > CXCR3 > CXCR2 CXCR4 > CXCR3 > CXCR1 > CXCR2
Garcia Lopez *et al.* (2001)	Many tissues HUVEC	CXCR3 CXCR3	Found CXCR3 on larger vessels in many tissues including lung. Not found on skin EC. Not detected on HUVEC
Romagnani *et al.* (2001)	HMVEC from different sources	CXCR3	CXCR3 is cell cycle dependent
Salcedo *et al.* (2001)	DMVEC	CCR1 - 4	Low CCR1 - 4

The specific role of chemokine receptors on endothelial cells has yet to be fully determined, however it is already known that endothelial cells respond to chemokines via the chemokine receptors; for example during chemotaxis or proliferation. In this study, we have compared the expression of chemokine receptors CCR1-6 and CXCR1-5 by the same five endothelial cell types used in the previous chapter.

5.2 Methods

5.2.1 Detection of chemokine receptors by Flow cytometry

Endothelial cells were detached from 150 cm^2 culture flasks by enzyme digestion with trypsin 0.025% / EDTA 0.01%. After neutralisation of the trypsin using culture medium, the cells were spun at 1500 rpm for 5 mins. The pellet was resuspended in 1 ml of 4% formaldehyde diluted in PBS and incubated for 10 mins on ice. After another centrifugation, the pellet was resuspended in 1 ml of 0.1% Triton X-100 in PBS for 1 min. At the end of 1 min, 5 ml of PBS was added to the suspension and the cells were centrifuged again. The cells were blocked by resuspending the pellet in 0.1 mg/ml of rabbit IgG and 10% normal goat serum, in PBS for 30 mins on ice. The cells were transferred into microfuge tubes, allowing 1 x 10^5 cells per tube (25 µl of 4 x 10^6 cells/ml). An anti-chemokine receptor antibody directly conjugated to either PE or FITC was added to each tube and incubated at 4°C for 1 hour. Antibodies to CXCR1-5, CCR1-3, 5 & 6 and matching isotype control antibodies were purchased from R & D systems, 10 µl of each of these were used without dilution. The antibody to CCR4 from Santa Cruz Biotechnologies (Santa Cruz, CA. USA) was diluted 1:100 as was the anti-vWF from Sigma These two antibodies were not conjugated to fluorescent markers, hence required a secondary antibody to be added.. 500 µl of PBS was added to each tube and then the tubes were centrifuged at 2000 rpm in a microfuge for 1 min. For detection of CCR4 and vWF, a secondary FITC conjugated goat anti rabbit antibody (used at 1:400, Chemicon, Harrow, UK) was added to the washed cells and incubated at 4°C for a further 45 mins. 500 µl of PBS was also added to this, then these tubes were centrifuged for one final time before being resuspended in 300 µl of PBS in FACS tubes. The cells were analysed using a Becton Dickinson FACSCalibur. 10,000 events were analysed per sample tube, the medians were determined and the medians of the isotype controls or secondary antibody alone were subtracted. The results shown are one representative of at least three experiments.

5.2.2 Detection of chemokine receptors by Immunocytochemistry

EC were grown to 70 – 80% confluence on 0.2% gelatin coated glass coverslips in 12 well tissue culture plates. They were fixed for 10 mins at room temperature using 4% formaldehyde in PBS, then permeabilised for 5 mins at room temperature using 0.1 % Triton X-100 diluted in PBS and

blocked with 10% normal goat serum for 30 mins at room temperature. The cells were stained for one hour at 4°C using chemokine-receptor specific fluorescent antibodies (same as described for FACS experiments except the anti CCR4 was used at a 1:60 dilution in this experiment). The coverslips were washed three times in PBS. For CCR4 and vWF staining, goat anti rabbit cy3 conjugated antibody (1:400, Chemicon) was added for 1 hour at 4°C. The coverslips were then mounted onto slides using fluoromount, left overnight to dry and viewed using a confocal microscope. Images were taken in the horizontal plane at 1 μm intervals through the specimen. The images were then overlaid to produce a composite.

5.3 Results

We determined the expression of chemokine (CXC and CC) receptors by permeabilised resting endothelial cells both by flow cytometry and by immunocytochemistry using receptor specific antibodies. Figure 5.1 summarises the expression of chemokine receptors CCR1-6 and CXCR1-5. It shows one representative experiment with duplicate samples. We showed similar patterns across several experiments, although the actual values of mean fluorescence varied considerably from experiment to experiment. Figures 5.2 - 5.11 show FACS histograms from which the data in Figure 5.1 is derived, while Figures 5.12 - 5.21 show immunocytochemistry staining of these same receptors on the different endothelial cell types. In all five endothelial cell types examined, we have shown high levels of CXCR1 and CXCR3. CCR3 was also present at lower levels in endothelia from all tissues except the lung (which showed virtually no expression), CCR4 expression varied widely between cell types and from experiment to experiment. CXCR4 and 5 were expressed by SVEC and HUVEC only.

5.3.1 Flow cytometry

HUVECs 'the standard EC' expressed CXCR1 highly with similar amounts of CXCR3 and low levels of CCR3 and CCR4. CXCR4 was also shown to be present in small amounts on resting HUVECs.

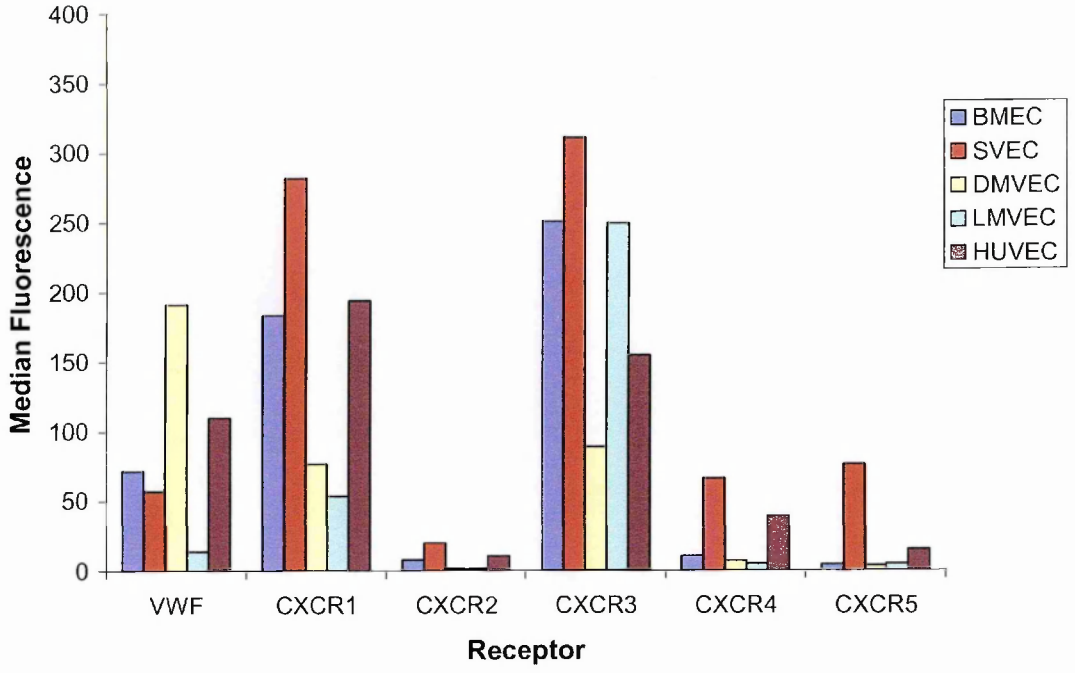
The level of CXCR1 (Fig. 5.1a) a receptor for IL-8, on the transformed bone marrow EC line is comparable to that on HUVEC, whereas SVEC showed slightly more CXCR1 than HUVEC and microvascular endothelia showed less than half of the amount found on HUVECs. CXCR2, the second receptor for IL-8, was found only at extremely low levels and not at all on microvascular endothelial cells.

CXCR3, the receptor for IP-10, I-TAC and Mig, was present on HUVECs at levels very slightly below that for CXCR1. SVEC, BMEC and LMVEC showed a higher level of expression of CXCR3 compared to that on HUVEC, with BMEC and LMVEC also showing CXCR3 at a higher level than they expressed CXCR1. However DMVEC again showed a lower level of this receptor than all other cell types.

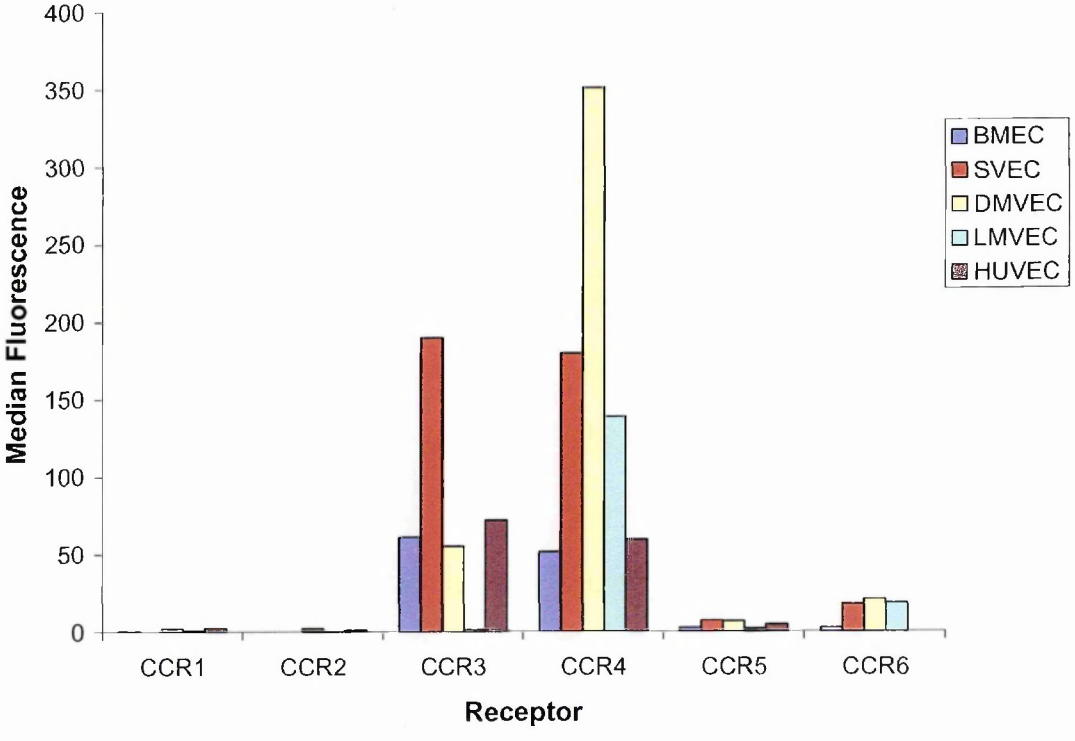
CXCR4 and CXCR5 were expressed only on SVEC and HUVEC, with higher levels seen on the SVEC. This suggests a difference between macrovascular and microvascular endothelium.

CCR3 is a receptor for many different chemokines including some of the monocyte chemoattractant proteins (MCPs) and eotaxin. It was found to be highly expressed (Fig. 5.2b) on SVEC, with lower expression on BMEC, DMVEC and HUVEC, but virtually no expression on LMVEC. CCR4 was expressed on all 5 cell types – low, but consistent between experiments on BMEC and HUVEC, but with very variable expression on SVEC, DMVEC and LMVEC. The fact that the expression is consistent on BMEC is likely to be due to the fact that it is a cell line, hence there is no heterogeneity between different cultures. The variation observed in primary cultures of SVEC, DMVEC and LMVEC could be due in part to differences between individual donors. The values shown in fig. 5.1b are the highest observed values of CCR4 (sometimes no expression was detected).

There was virtually no CCR1, CCR2 or CCR5 expression detected, and only low amounts of CCR6 were detected on SVEC and both types of microvascular endothelium.



A. vWF and CXC receptor expression



B. CC receptor expression

Figure 5.1 Summary of chemokine receptor expression on different resting endothelia

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using receptor specific antibodies and analysed by flow cytometry. A. expression of CXCR1-5, B. expression of CCR1-6. Results are expressed as the median fluorescence for each receptor of a representative experiment of three performed.

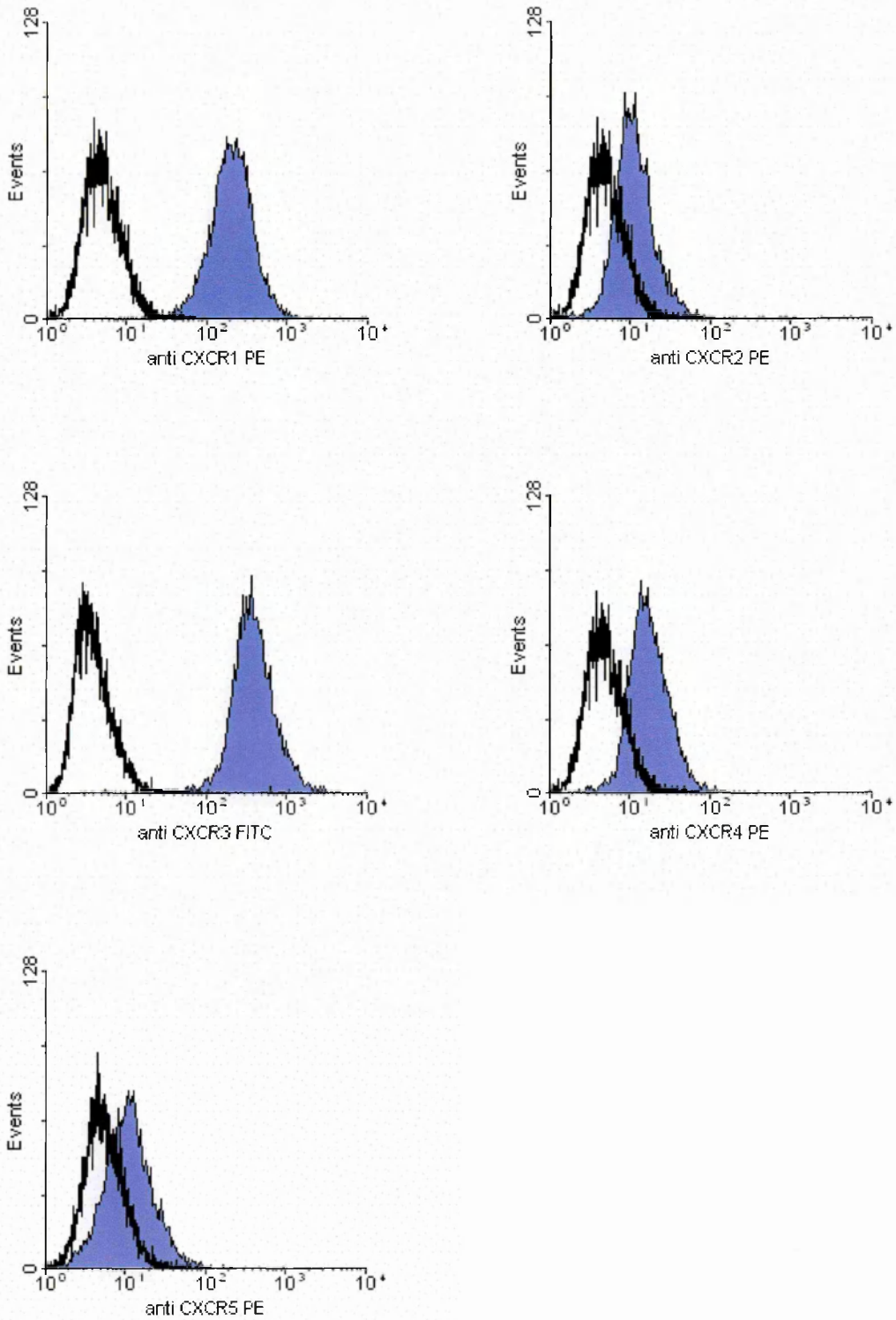


Figure 5.2 Flow cytometry histograms of CXCR1-5 immunofluorescence on resting BMEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (unfilled peaks) were used and 10,000 events analysed by flow cytometry.

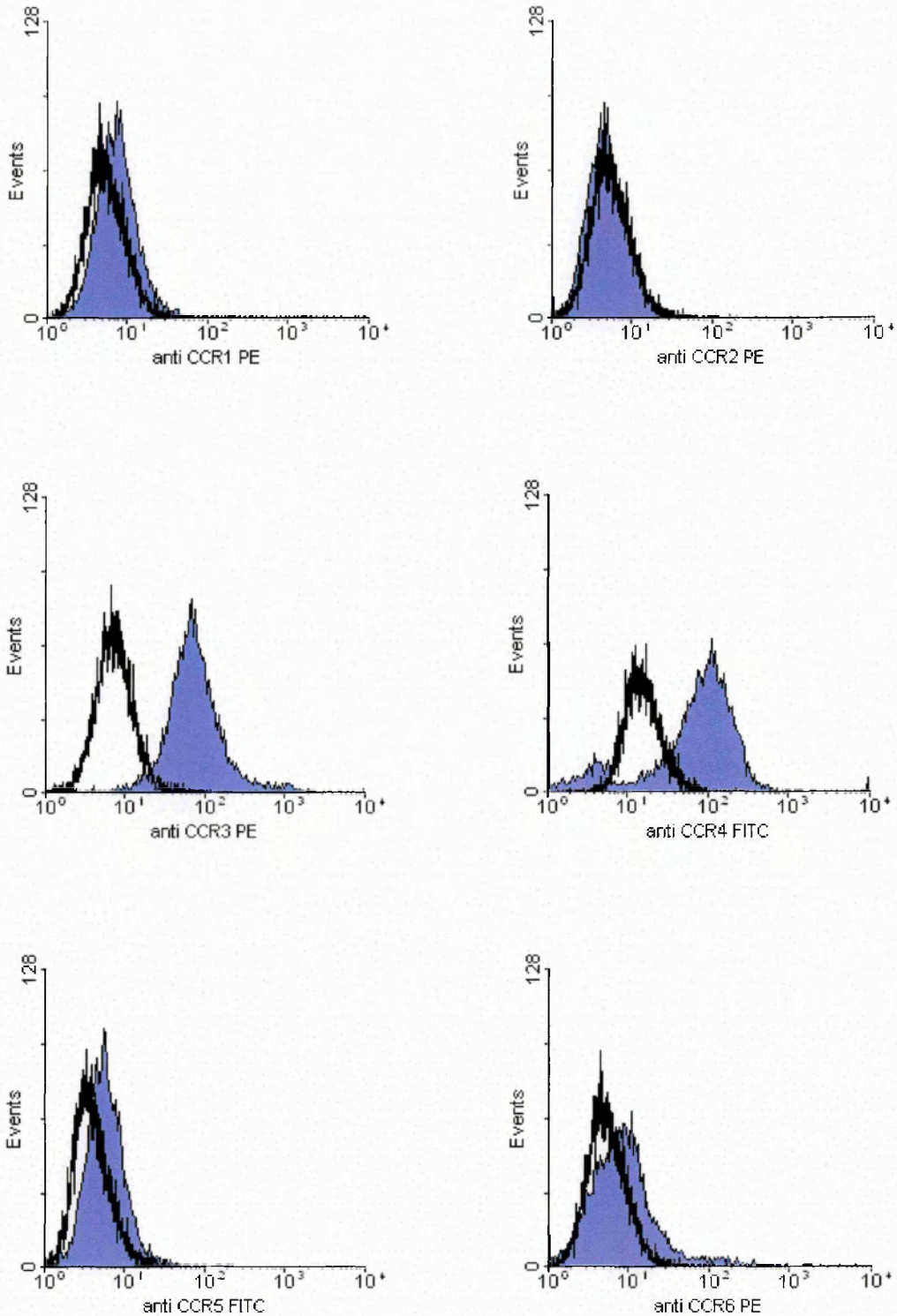


Figure 5.3 Flow cytometry histograms of CCR1-6 immunofluorescence on resting BMEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (CCR1-3, 5 and 6) or secondary antibody alone (CCR4) were used as controls (unfilled peaks) and 10,000 events analysed by flow cytometry.

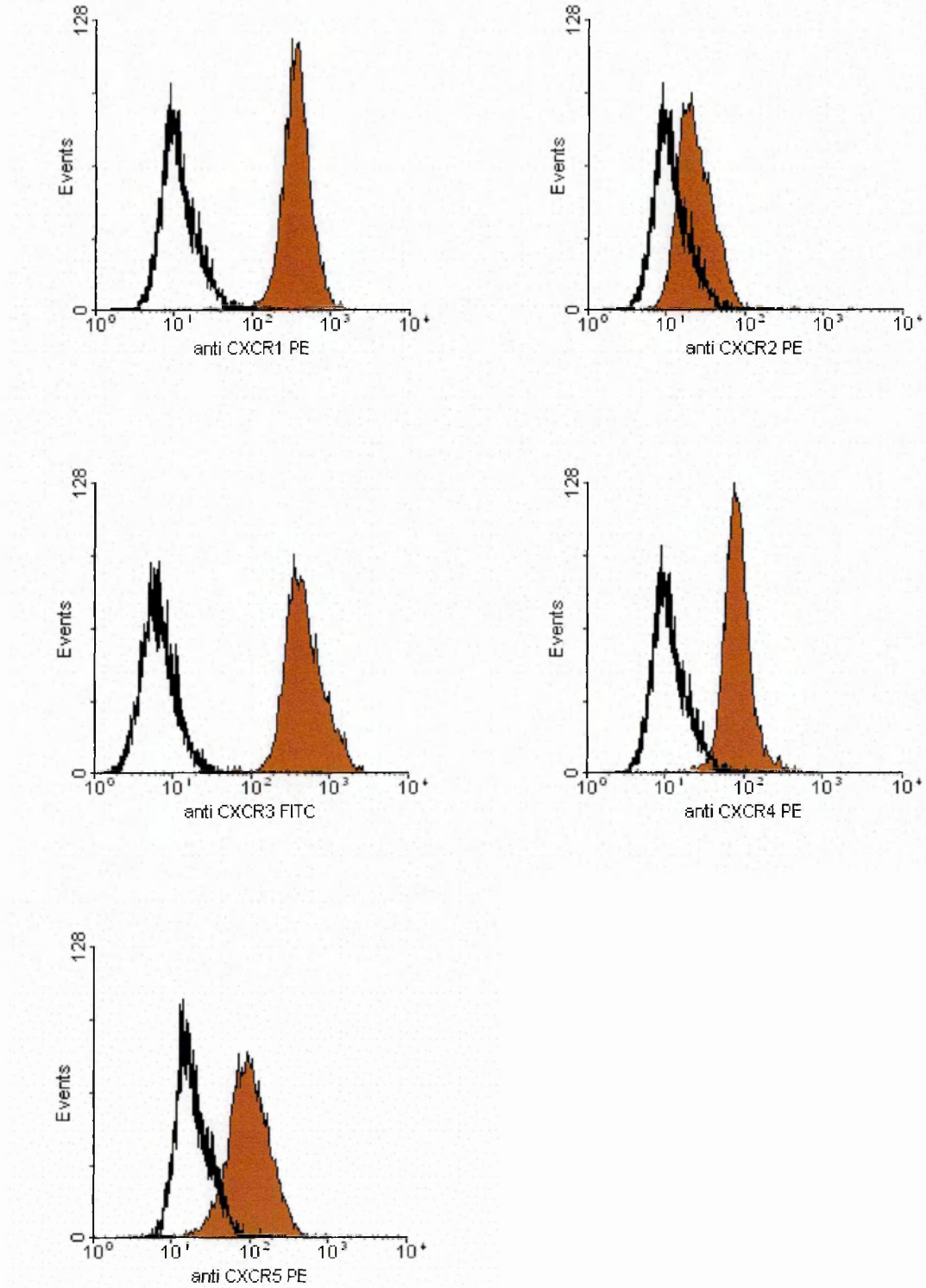


Figure 5.4 Flow cytometry histograms of CXCR1-5 immunofluorescence on resting SVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (unfilled peaks) were used and 10,000 events analysed by flow cytometry.

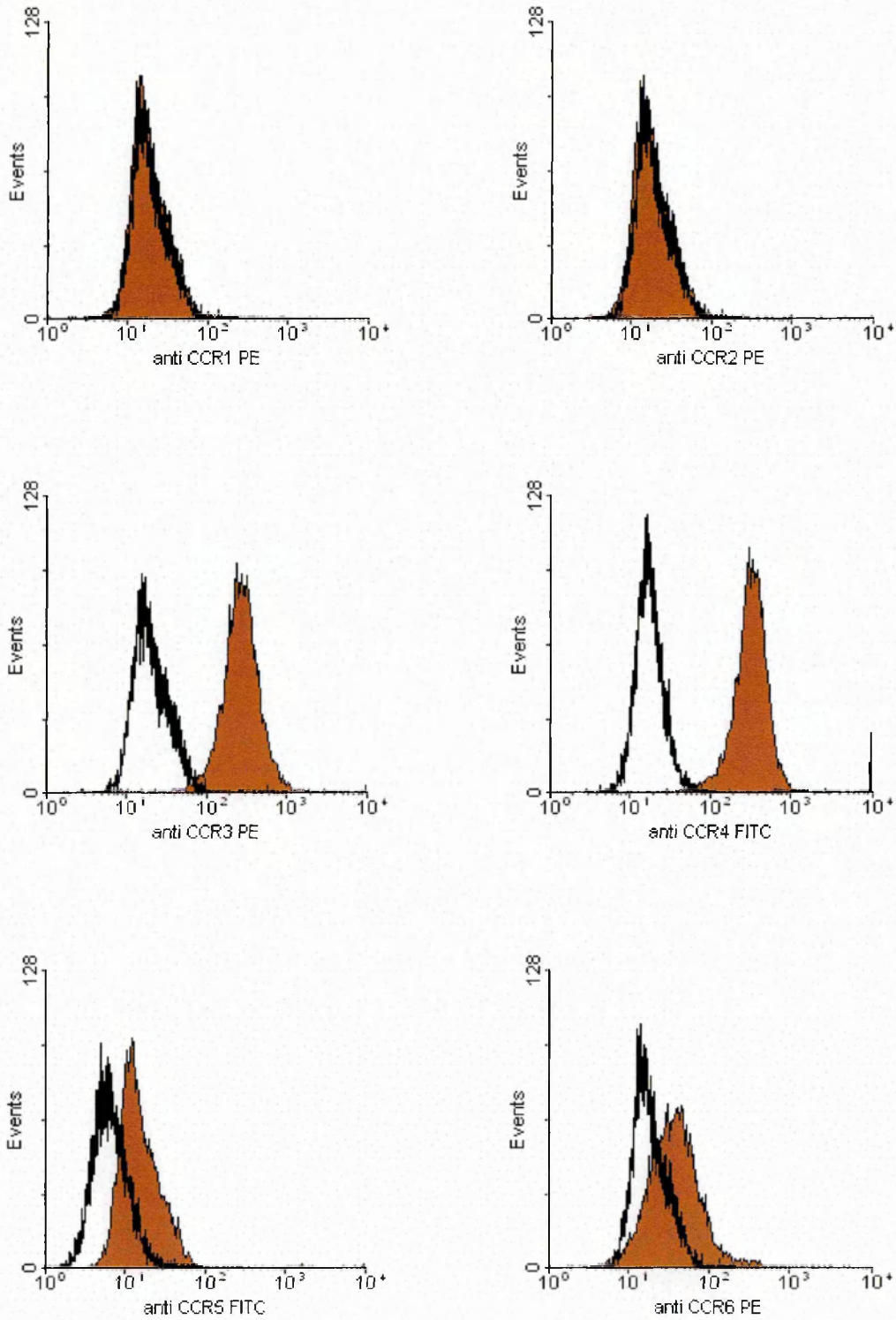


Figure 5.5 Flow cytometry histograms of CCR1-6 immunofluorescence on resting SVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (CCR1-3, 5 and 6) or secondary antibody alone (CCR4) were used as controls (unfilled peaks) and 10,000 events analysed by flow cytometry.

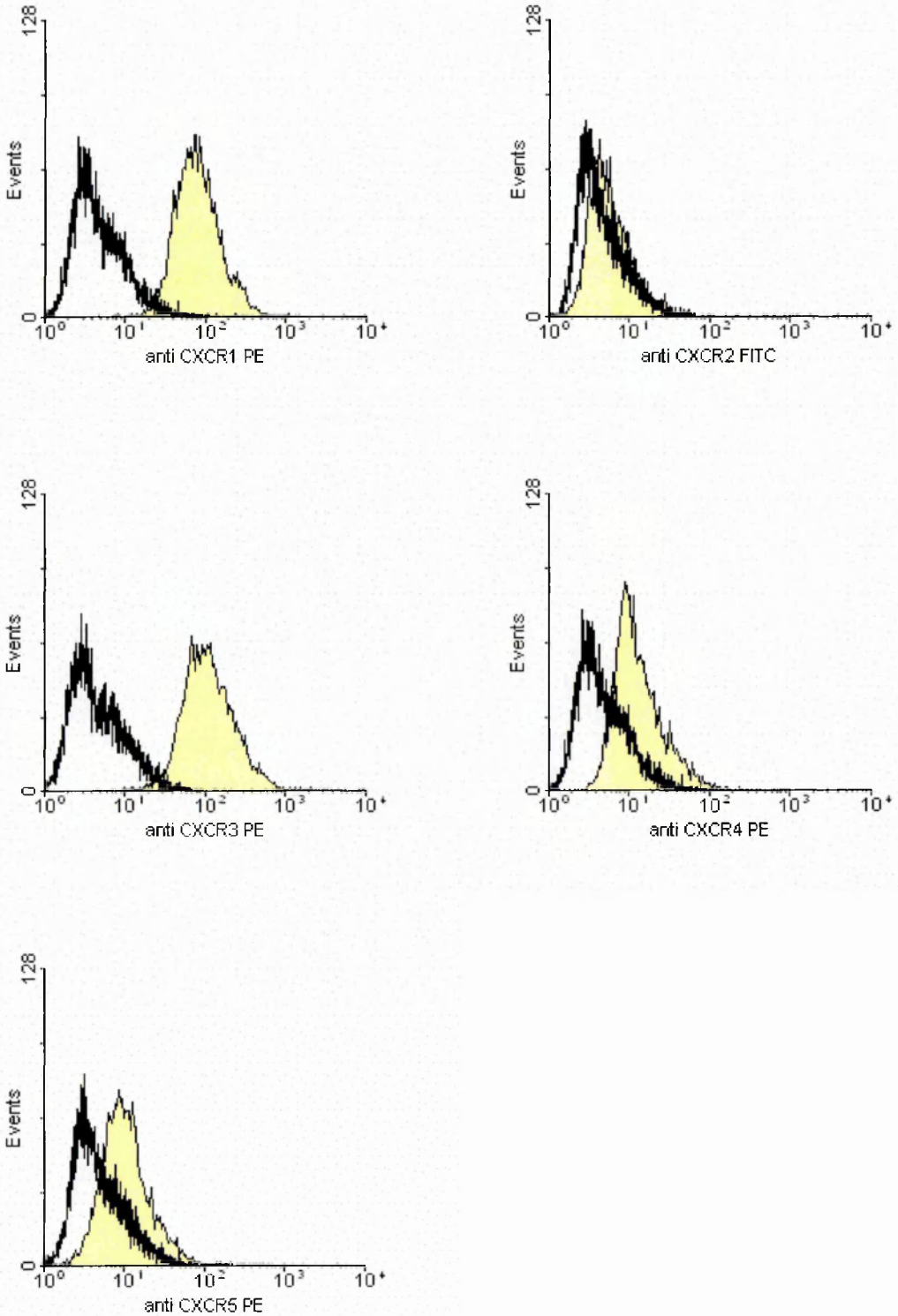


Figure 5.6 Flow cytometry histograms of CXCR1-5 immunofluorescence on resting DMVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (unfilled peaks) were used and 10,000 events analysed by flow cytometry.

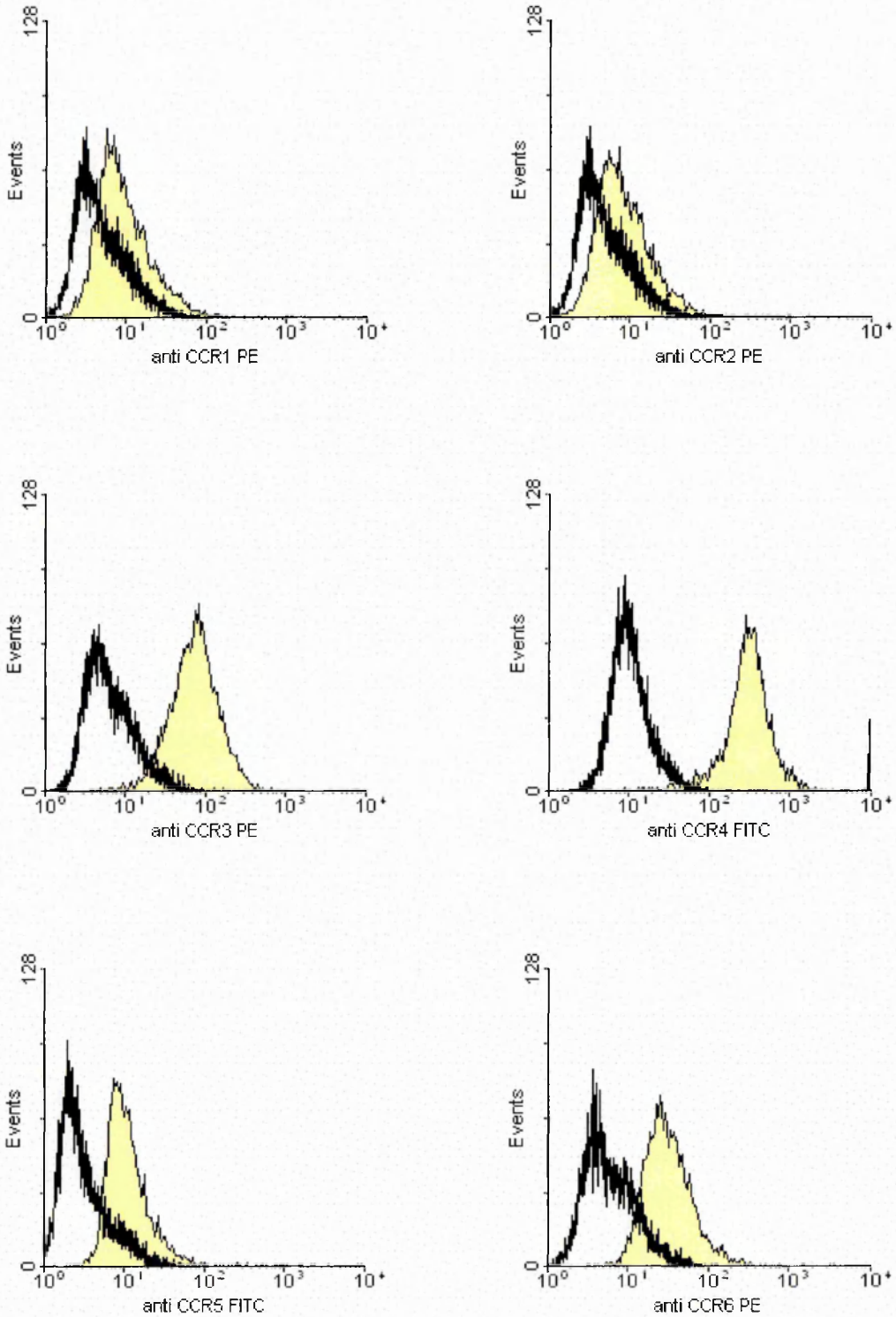


Figure 5.7 Flow cytometry histograms of CCR1-6 immunofluorescence on resting DMVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (CCR1-3, 5 and 6) or secondary antibody alone (CCR4) were used as controls (unfilled peaks) and 10,000 events analysed by flow cytometry.

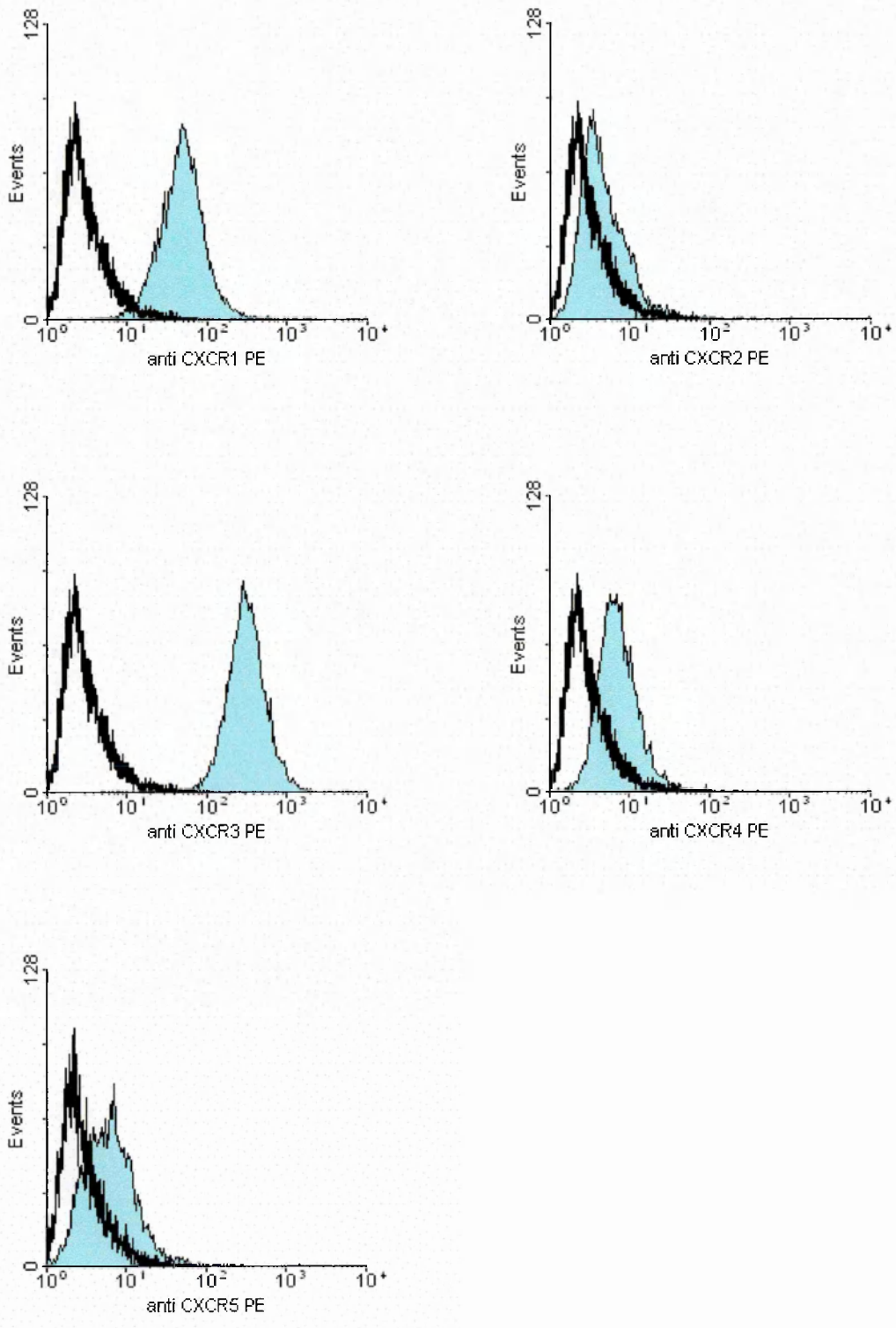


Figure 5.8 Flow cytometry histograms of CXCR1-5 immunofluorescence on resting LMVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (unfilled peaks) were used and 10,000 events analysed by flow cytometry.

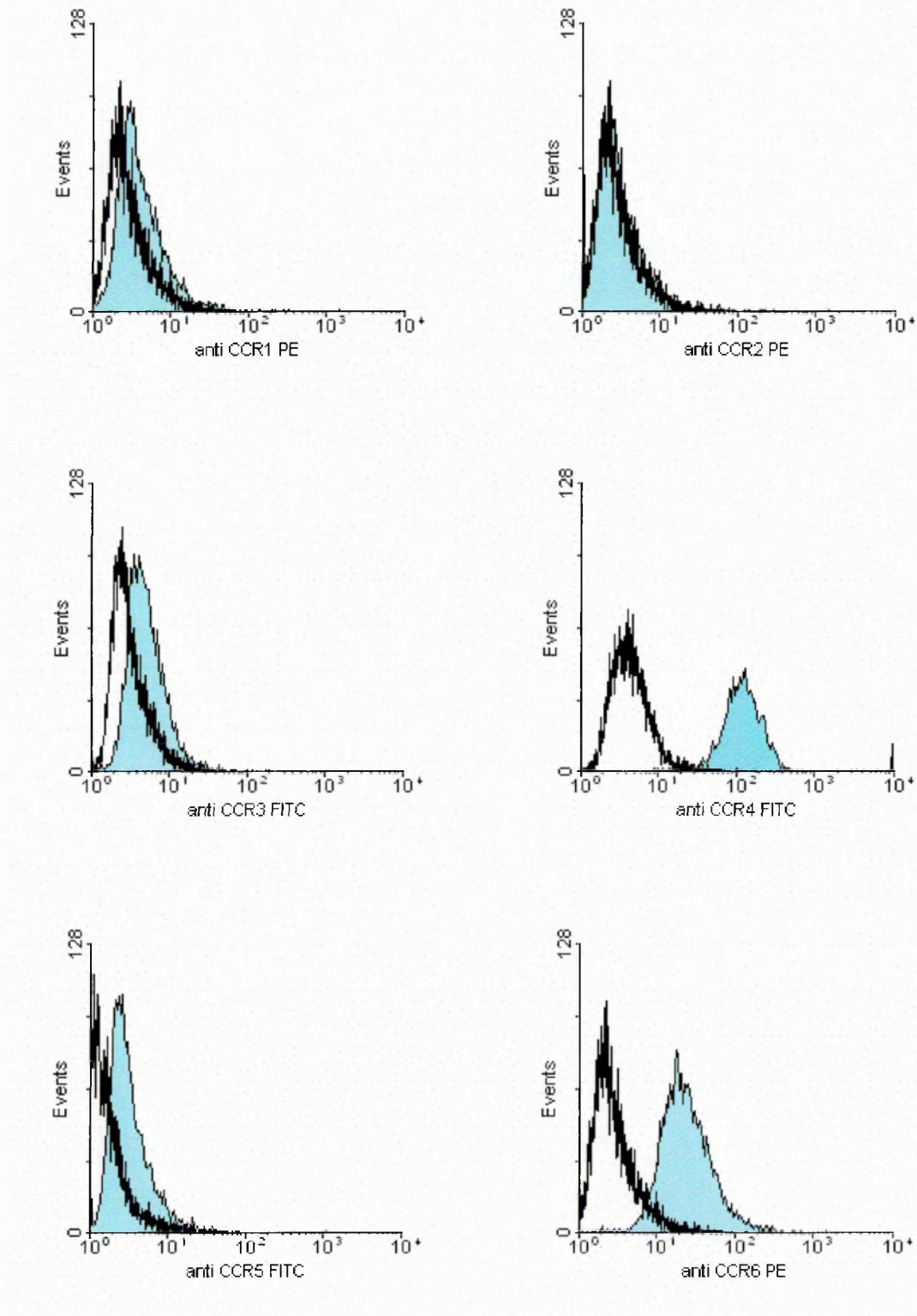


Figure 5.9 Flow cytometry histograms of CCR1-6 immunofluorescence on resting LMVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (CCR1-3, 5 and 6) or secondary antibody alone (CCR4) were used as controls (unfilled peaks) and 10,000 events analysed by flow cytometry.

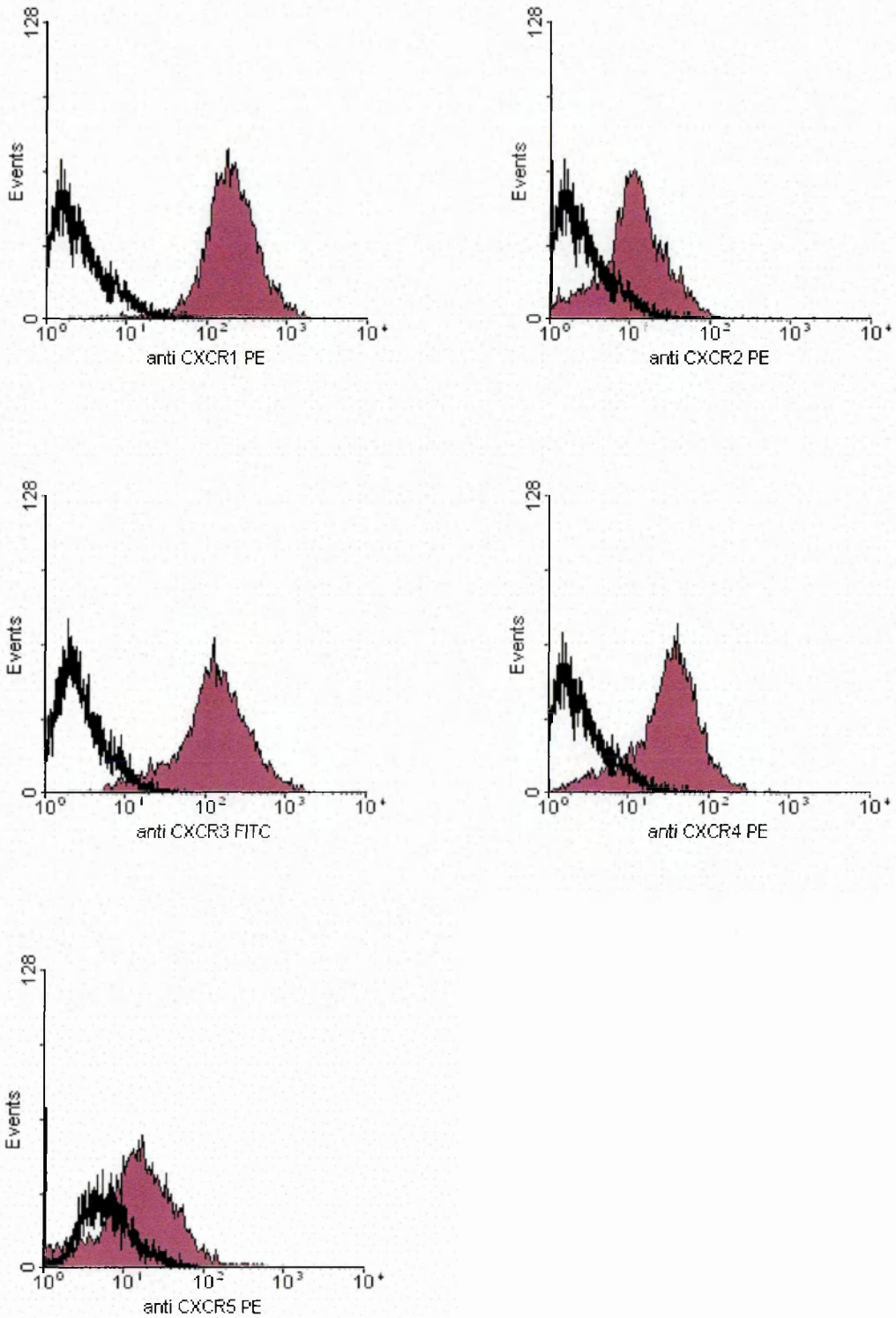


Figure 5.10 Flow cytometry histograms of CXCR1-5 immunofluorescence on resting HUVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (unfilled peaks) were used and 10,000 events analysed by flow cytometry.

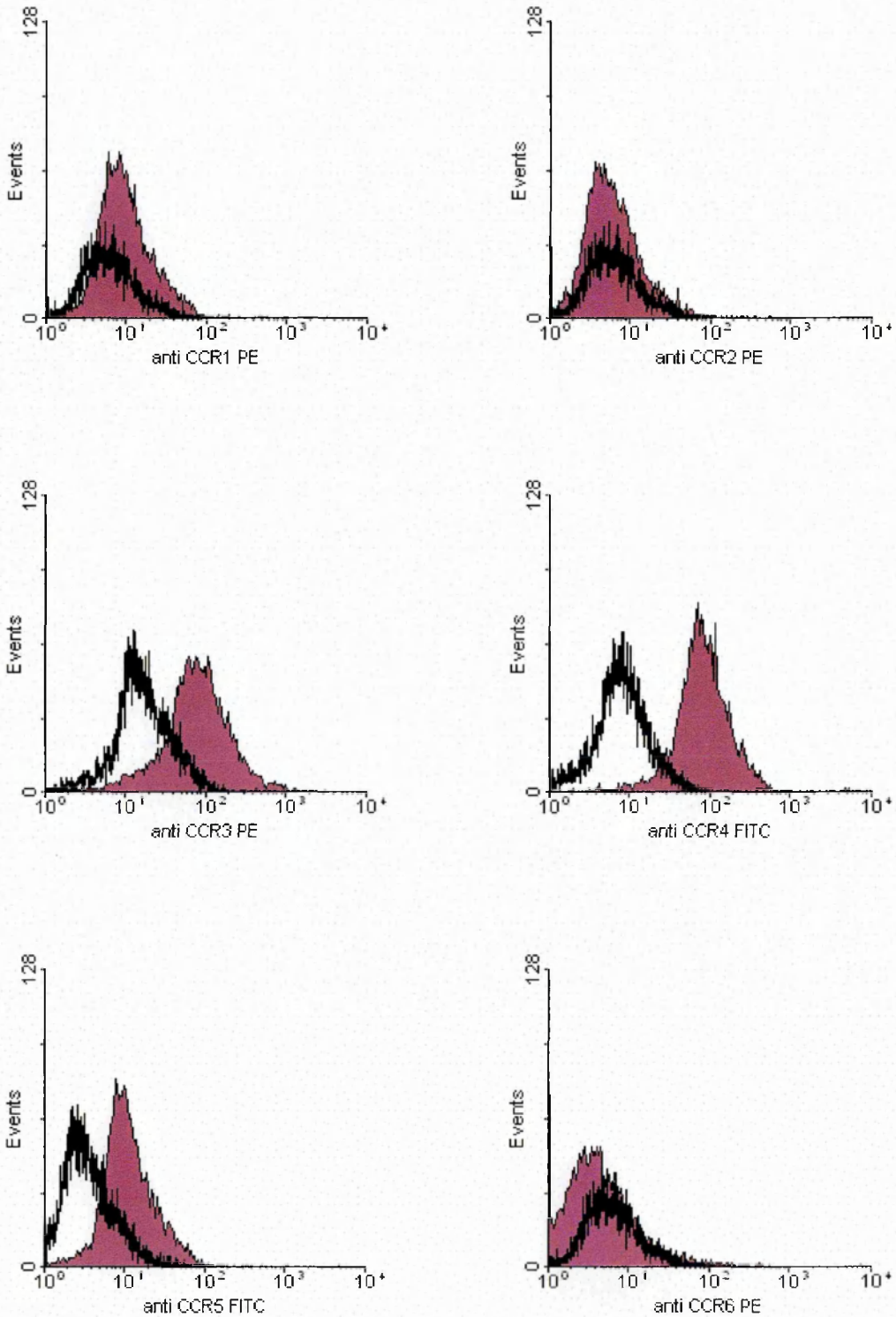


Figure 5.11 Flow cytometry histograms of CCR1-6 immunofluorescence on resting HUVEC

Resting endothelial cells were harvested by trypsinisation, then the levels of chemokine receptors were detected using anti human chemokine receptor specific antibodies (filled peak). Isotype matched controls (CCR1-3, 5 and 6) or secondary antibody alone (CCR4) were used as controls (unfilled peaks) and 10,000 events analysed by flow cytometry.

5.3.2 Immunocytochemistry

The confocal images from immunocytochemistry staining of subconfluent cells enabled us to localise chemokine receptors within the cell. Similar expression profiles to those found with flow cytometry were found (Figs 5.12 – 5.21). All receptors were localised throughout the cytoplasm, particularly in the area close to the nucleus. Staining was also localised to the cell surface. With the exception of CCR4, the chemokine receptors tested stained all cells uniformly. The CCR4 expression was very variable, appearing to be localised in vesicles of some cell types. It is uncertain whether this distribution could affect the flow cytometry data. With immunocytochemistry, it is possible to visualise the low levels of chemokine binding seen with several chemokines (e.g. CCR2). The receptors that bind at a low level appear to be sparsely distributed across the cell surface, with less obvious binding around the nucleus.

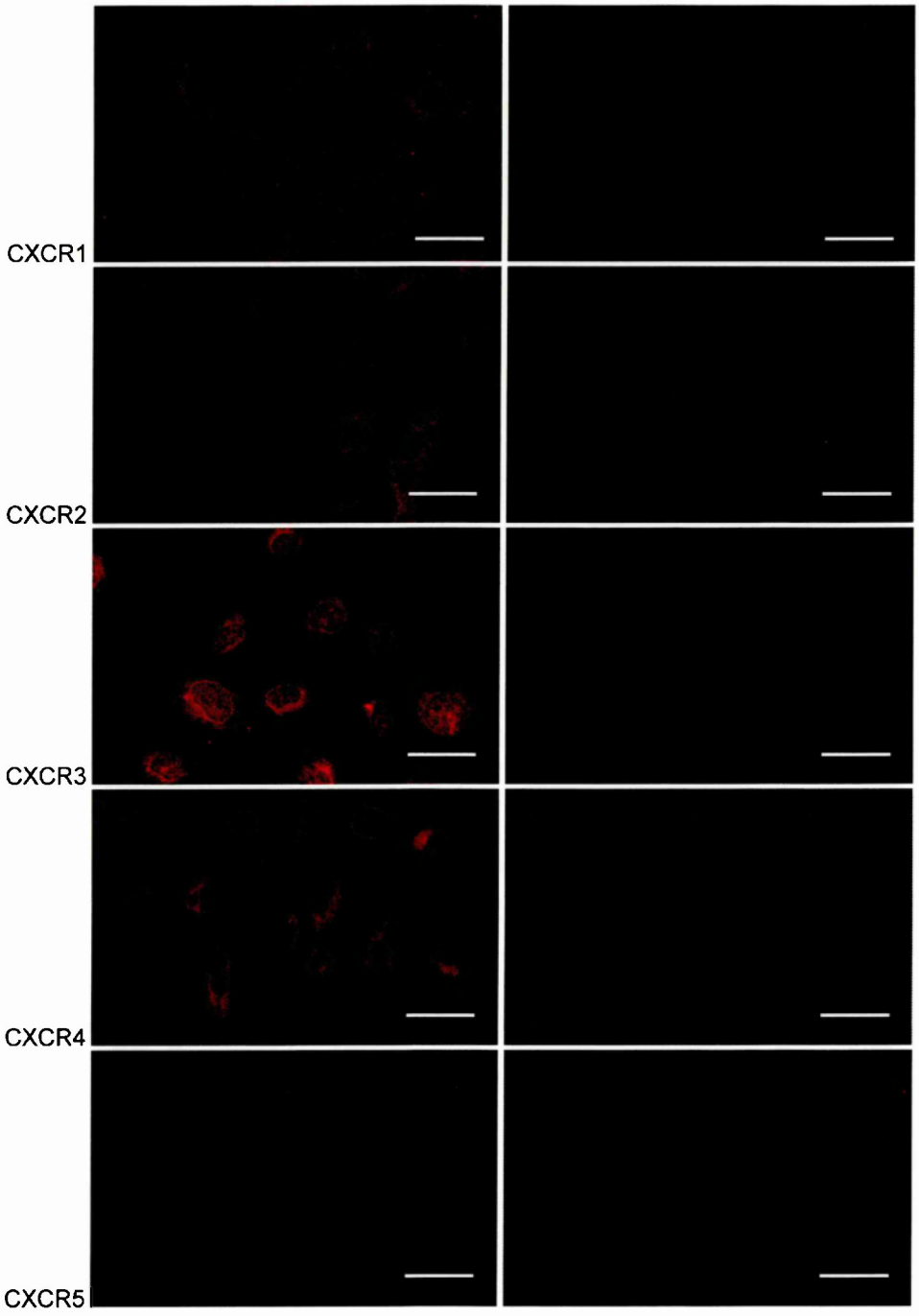


Figure 5.12 CXC chemokine receptor staining of permeabilised BMEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls were used as negative controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

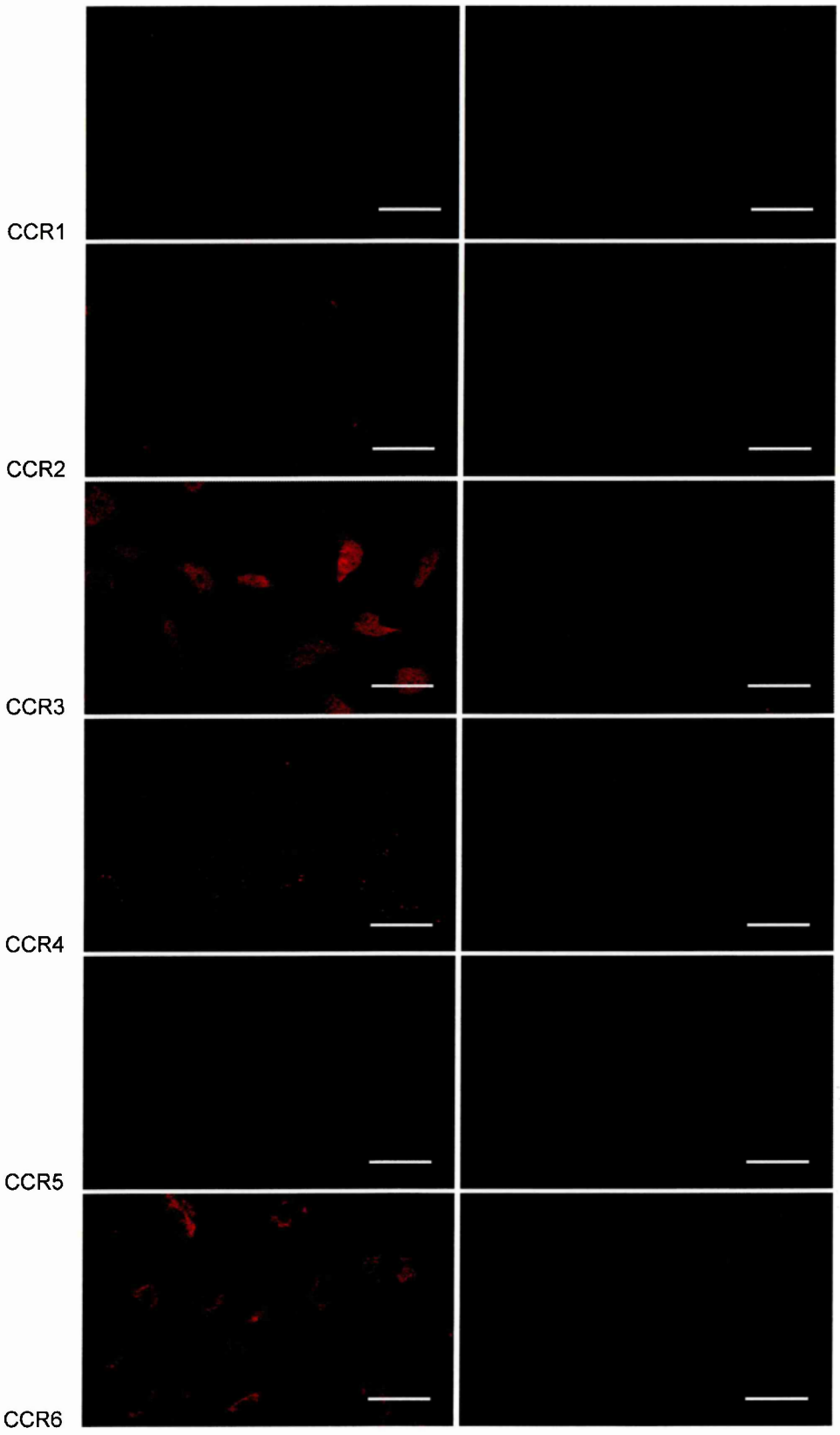


Figure 5.13 CC chemokine receptor staining of permeabilised BMEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls (CCR1-3,5 and 6) or secondary antibody alone (CCR4) were used as controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

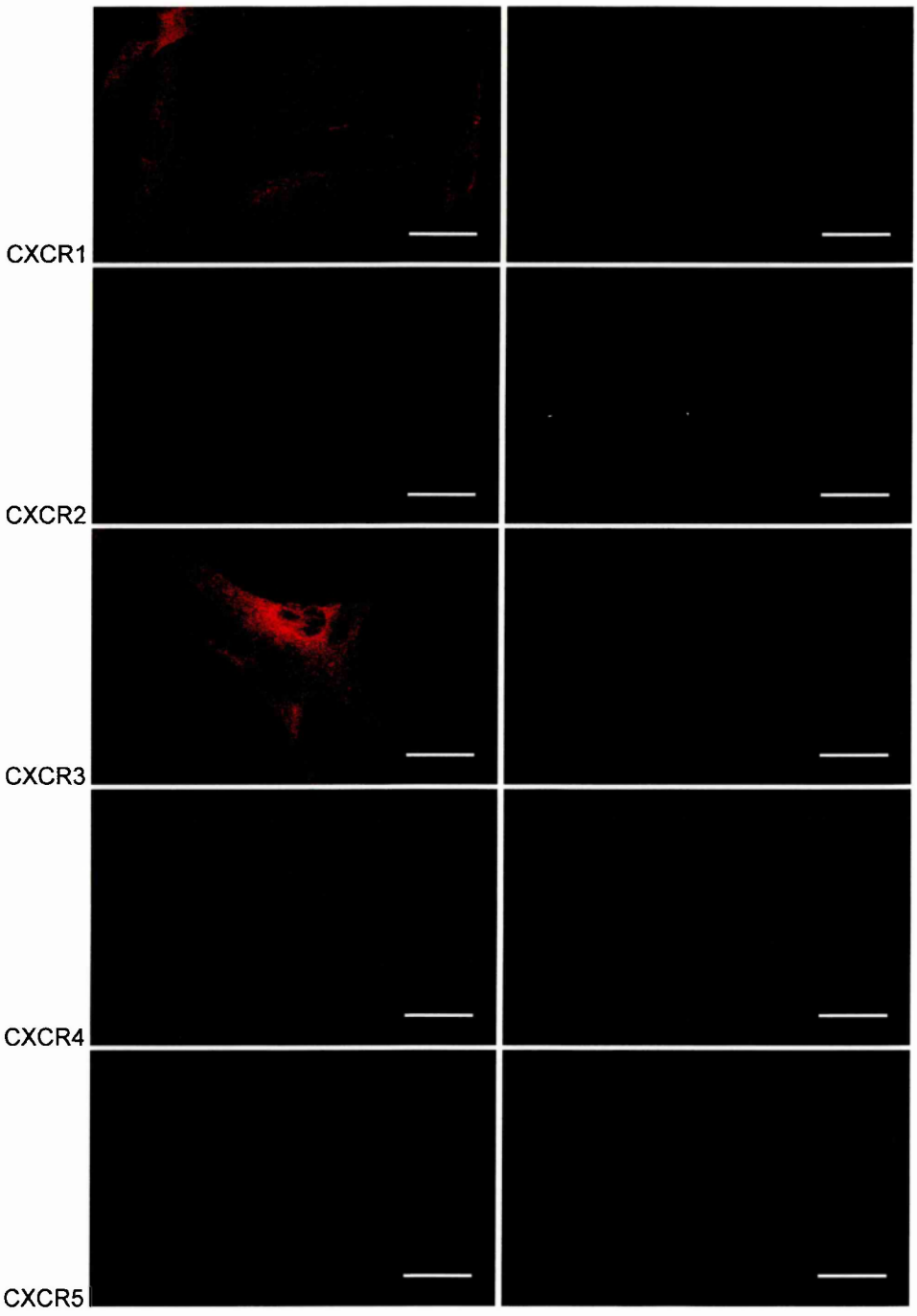


Figure 5.14 CXC chemokine receptor staining of permeabilised SVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls were used as negative controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

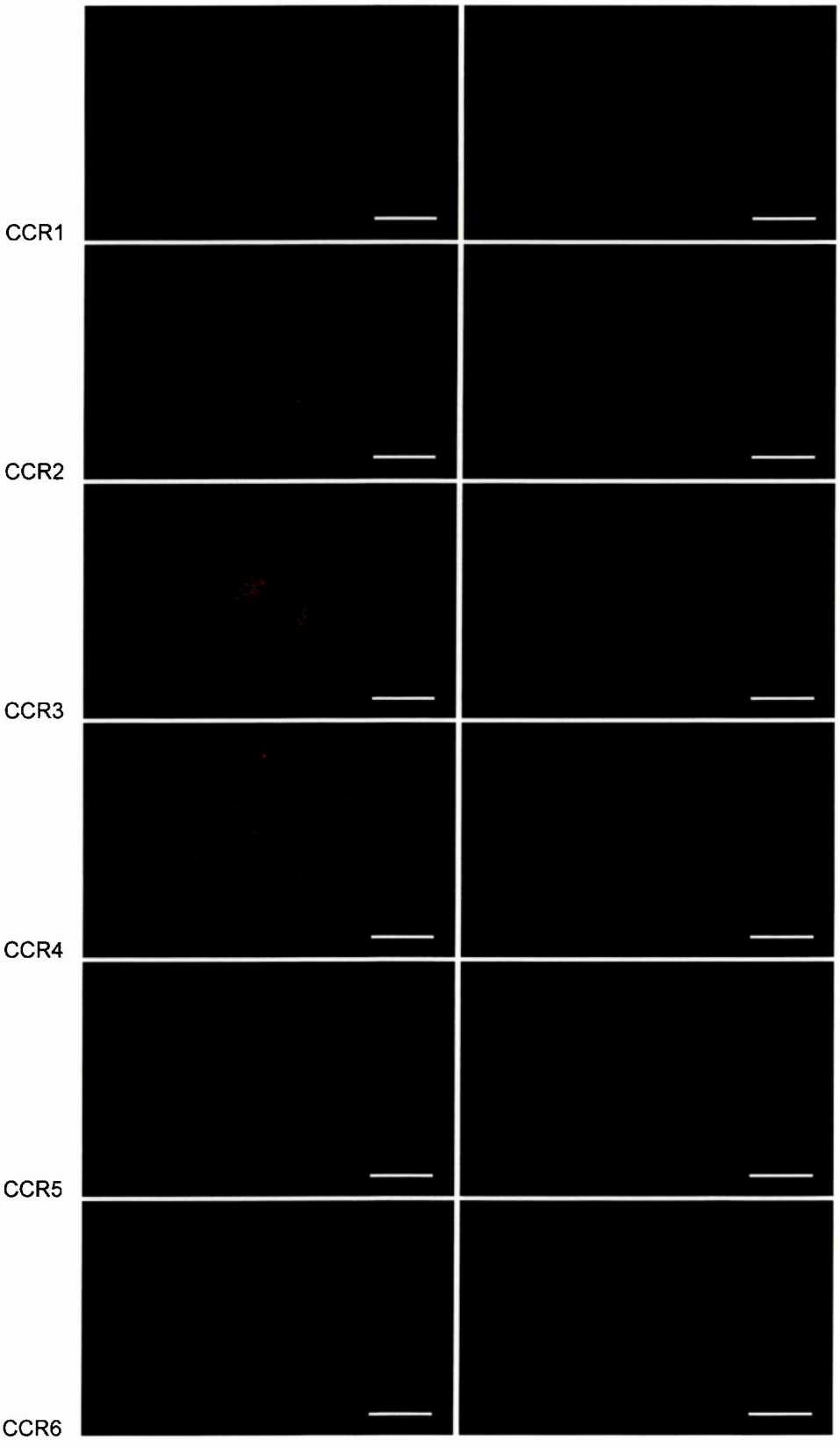


Figure 5.15 CC chemokine receptor staining of permeabilised SVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls (CCR1-3,5 and 6) or secondary antibody alone (CCR4) were used as controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

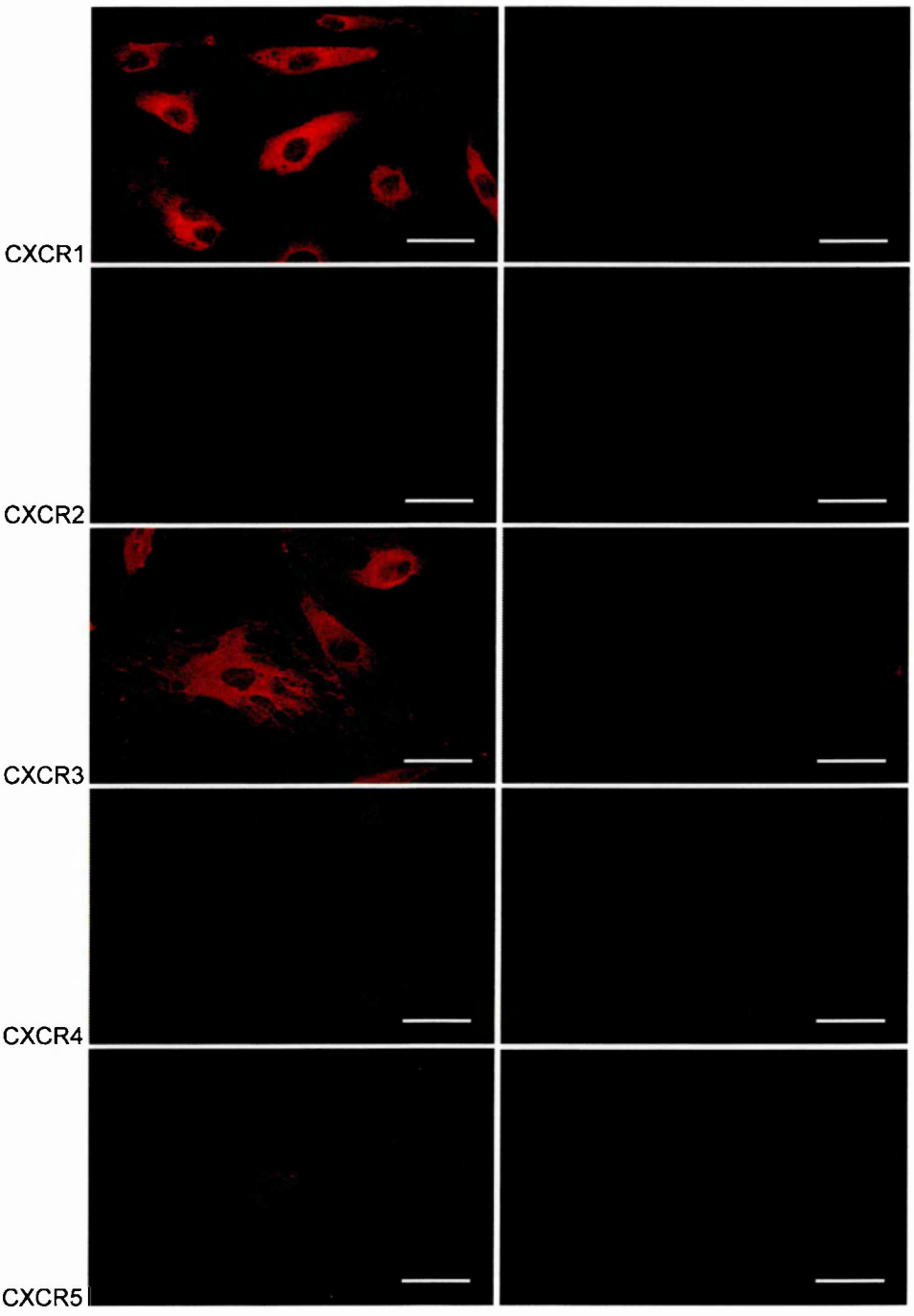


Figure 5.16 CXC chemokine receptor staining of permeabilised DMVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls were used as negative controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

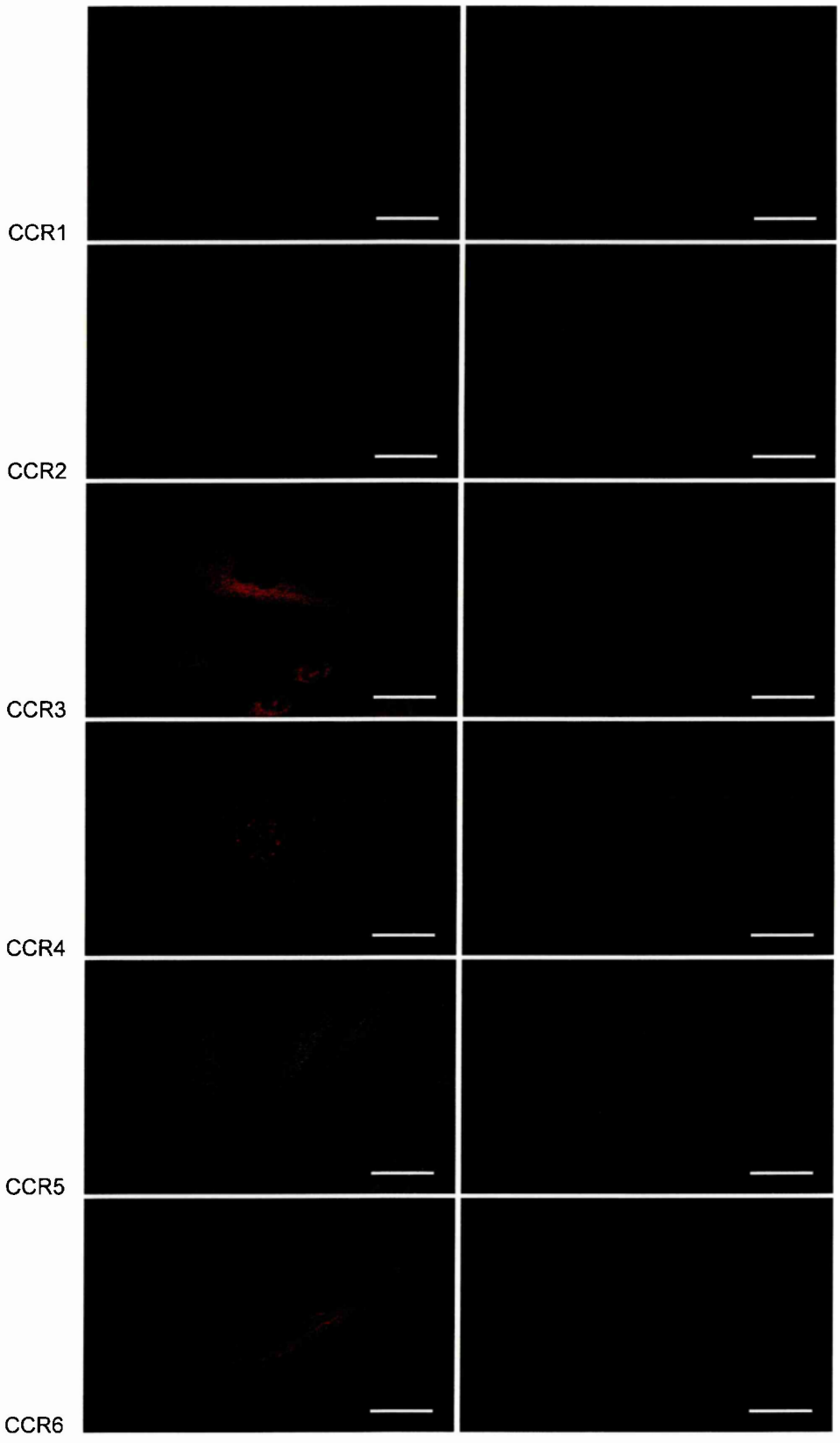


Figure 5.17 CC chemokine receptor staining of permeabilised DMVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls (CCR1-3,5 and 6) or secondary antibody alone (CCR4) were used as controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

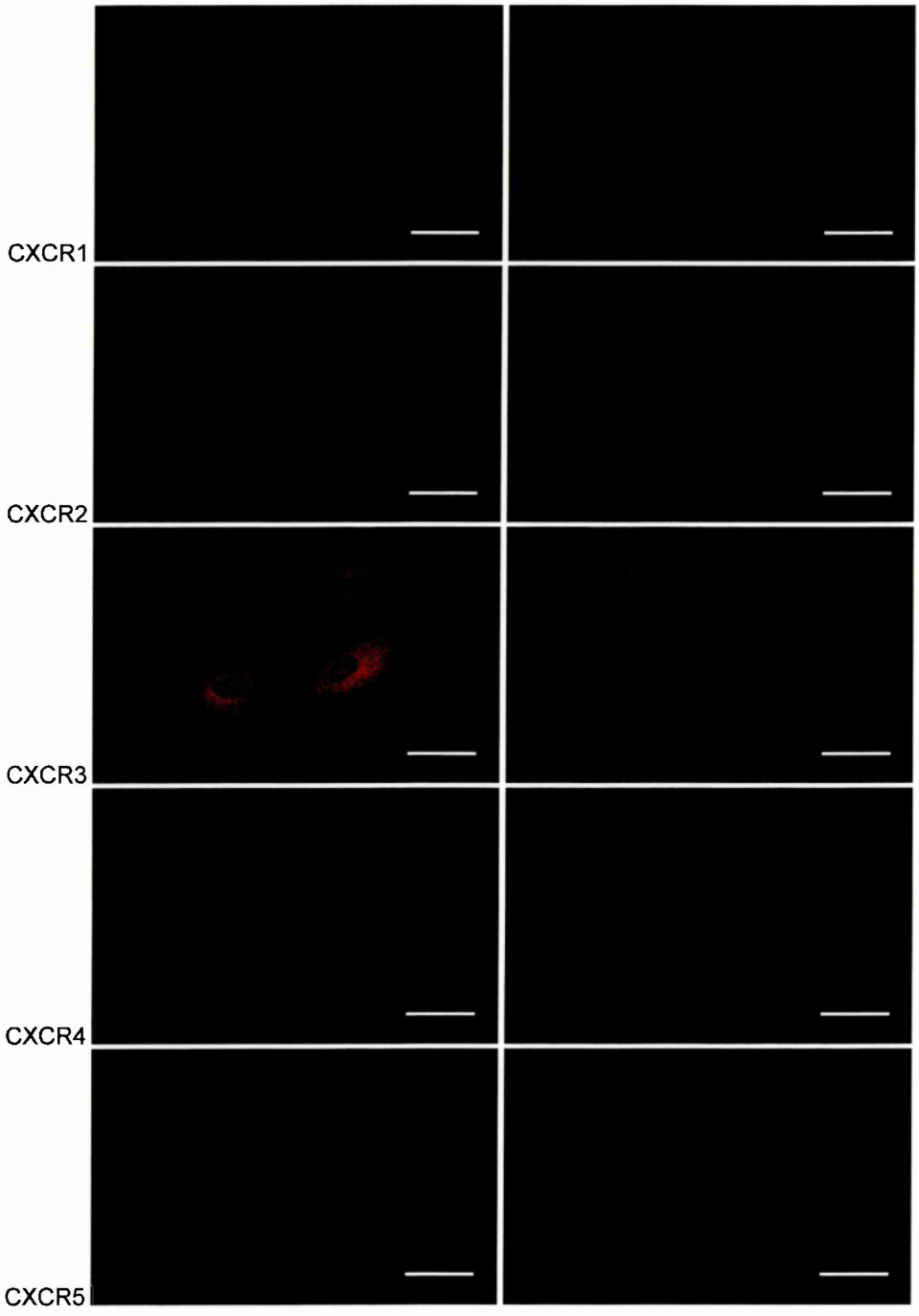


Figure 5.18 CXC chemokine receptor staining of permeabilised LMVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls were used as negative controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

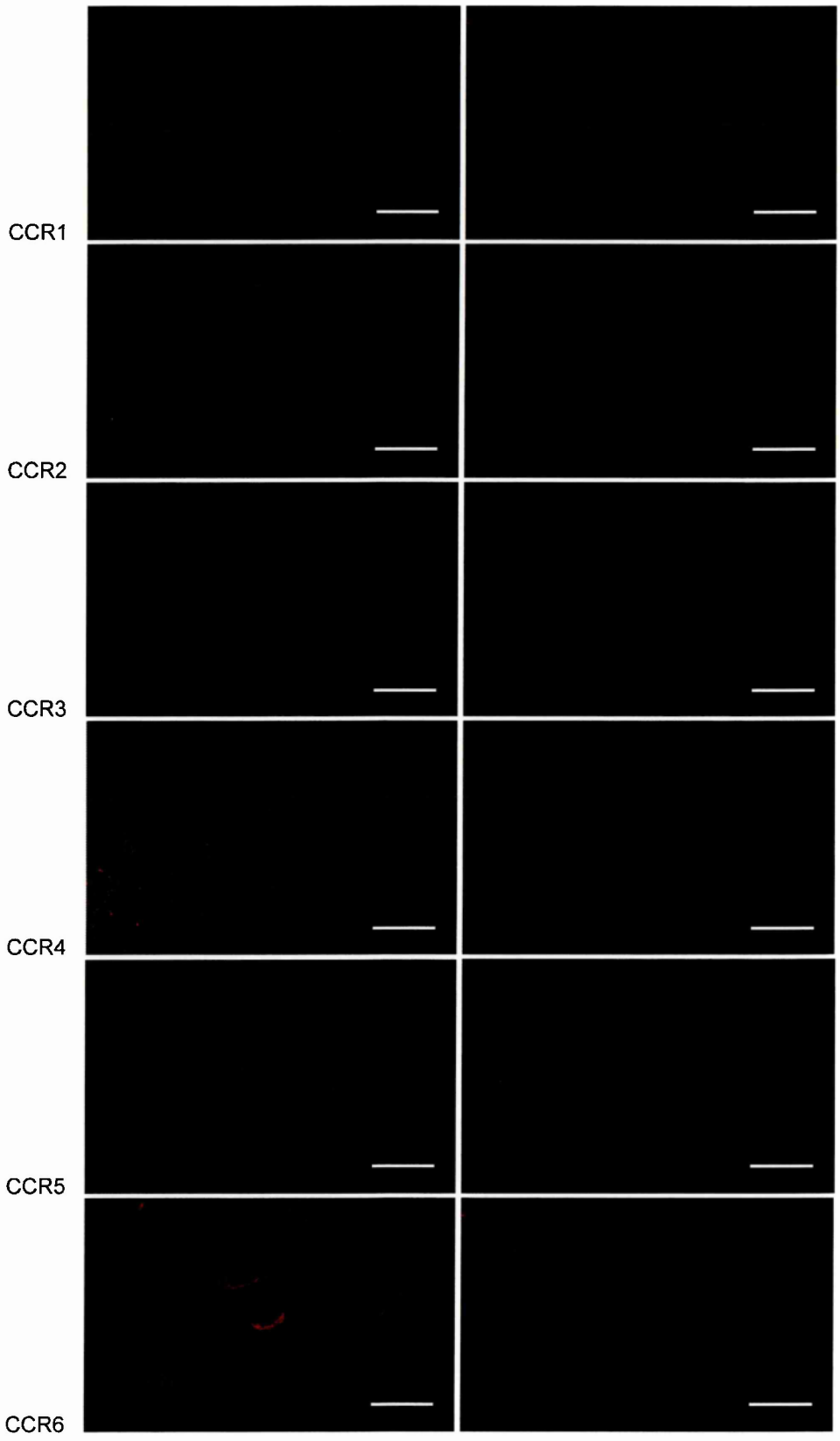


Figure 5.19 CC chemokine receptor staining of permeabilised LMVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls (CCR1-3,5 and 6) or secondary antibody alone (CCR4) were used as controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

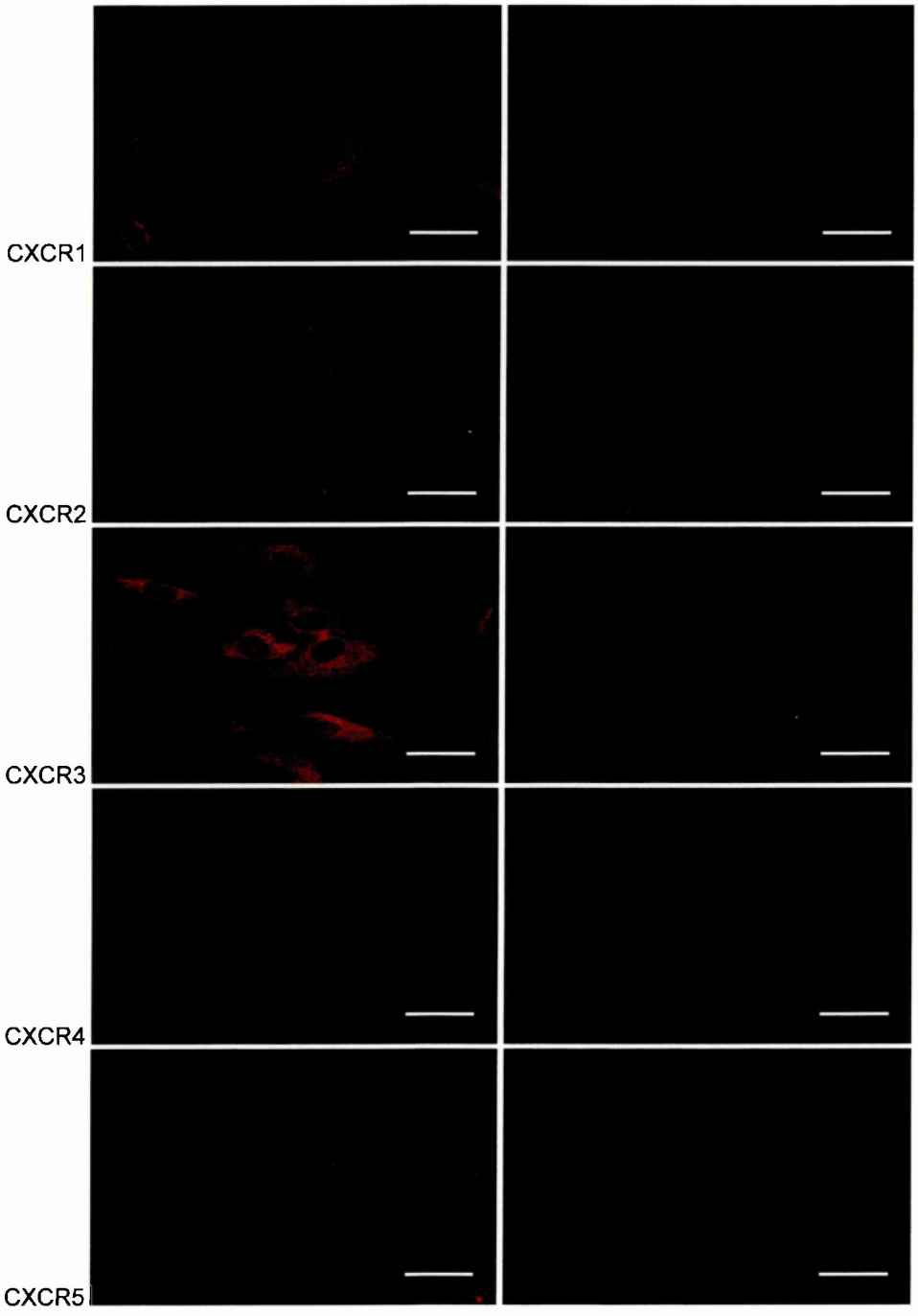


Figure 5.20 CXC Chemokine receptor staining of permeabilised HUVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls were used as negative controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

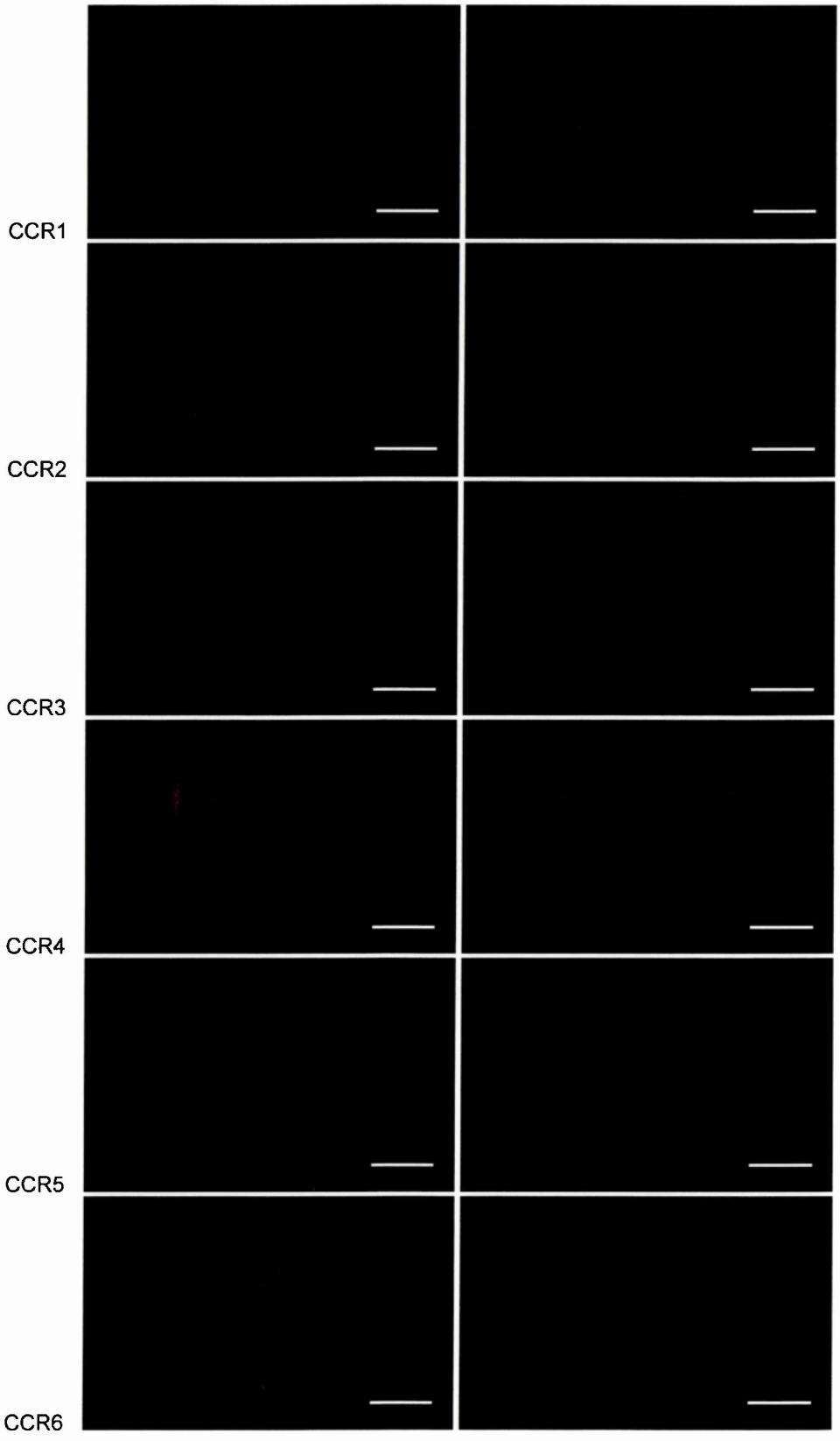


Figure 5.21 CC chemokine receptor staining of permeabilised HUVEC

Endothelial cells were grown on gelatin coated coverslips then chemokine receptors were detected using specific chemokine receptor antibodies (left column). Isotype matched controls (CCR1-3,5 and 6) or secondary antibody alone (CCR4) were used as controls (right column). Images were taken with a confocal microscope at x40. Scale bar = 40μm

5.4 Discussion

Expression of chemokine receptors by endothelial cells is still under much discussion. While it is now accepted that endothelial cells do express chemokine receptors, it is still much debated as to which receptors are expressed and why.

The results from research on CXC receptors is still conflicting, but seem to suggest that CXCR1 and CXCR4 are present in HUVECs (Schonbeck *et al.* 1995; Gupta *et al.* 1998), with CXCR2 mRNA being found by some researchers as well (Murdoch *et al.* 1999). In their results, both Gupta *et al.* and Murdoch *et al.* suggested that CXCR4 is the most abundant chemokine receptor on human EC. A more comprehensive study of all CCR1-6 and CXCR1-4 on HUVECs and DMVECs by RT-PCR has shown no CC receptors except for variable expression of CCR5 on subconfluent monolayers of HUVEC. They did however show the presence of all four CXC receptors with lower levels of CXCR1 and 2 (Feil and Augustin 1998).

In this study, we have used flow cytometry to determine the amounts of chemokine receptor present on our five different types of endothelial cell, we then used immunocytochemistry to determine the distribution of these receptors within the cell. We have shown distinct differences in chemokine receptor expression between endothelia from different vascular beds.

The subconfluent cultures of cells allow us to see each individual cell more clearly and determine where in the cell the receptors are, however at confluence, cells will be contact inhibited and will have stopped growing, so there could be slight differences in receptor levels between FACS results and immunocytochemical staining. The trypsinisation step could also have a potentially damaging effect on surface receptors hence the need for the two different methods.

5.4.1 CXCR1 and 2

CXCR1 and 2 are the only known mammalian receptors for ELR+ CXC chemokines (Belperio *et al.* 2000). This includes IL-8, which has a similar affinity for both receptors, however, CXCR1 is highly selective for IL-8 whereas CXCR2 is somewhat less selective for IL-8 compared to all the other ELR+ CXCR chemokines. The only other ligand which competes for CXCR1 is GCP-2, and this is an equipotent agonist at CXCR1 and 2 (Wolf *et al.* 1998). Following this, CXCR1 has been found to be the dominant receptor for IL-8 chemotaxis, whereas CXCR2 mediates neutrophil chemotaxis to

NAP-2 and Gro-α at relatively low concentrations (Wuyts *et al.* 1998; Feniger-Barish *et al.* 1999; Feniger-Barish *et al.* 2000).

One of the earliest papers investigating the specific receptors for IL-8 on HUVEC and DMVEC derived from human breast could not detect either CXCR1 or CXCR2 (Petzelbauer *et al.* 1995). However another paper published around the same time showed that endothelial cells (SVEC and HUVEC used indiscriminately) specifically bound IL-8 and that CXCR1, but not CXCR2 mRNA was present (Schonbeck *et al.* 1995).

Using DMVEC, Salcedo *et al.* (2000b) showed virtually no CXCR2 on the cell surface or in permeabilised cells, but they showed relatively high levels of CXCR1. HUVEC were shown to express both CXCR1 and 2, but at much lower levels than DMVEC. This group also demonstrated that after IL-8 stimulation, CXCR1 and 2 get internalised. Using blocking antibodies, they showed that by blocking CXCR2, migration was reduced by approx. 30% and blocking CXCR1 reduced migration by 70%. When both antibodies were used in combination, over 90% of migration was blocked. So although CXCR2 was shown to be less abundant on the cells, it still made a considerable contribution to migration. Murdoch *et al.* (1999) showed the presence of CXCR1 and 2 mRNA, however with flow cytometry, they showed no CXCR2 protein. Whether this is due to only the mRNA and not the protein of CXCR2 being present or whether this is due to incorrect primers (as there is 78% homology in CXCR1 and CXCR2 sequences) is still unclear.

5.4.2 CXCR3

CXCR3 is the receptor to three known ligands, IP-10, ITAC and Mig. These are all ELR- and hence angiostatic. IP-10 and Mig bind to CXCR3 with approximately the same affinity, whereas I-TAC has higher binding affinity. Murdoch *et al.* showed low levels of CXCR3 mRNA in HUVECs, however they did not investigate the levels of protein expressed. Protein expression has been shown in larger vessels, but not in cultured HUVECs (Garcia Lopez *et al.* 2001). Other groups have shown CXCR3 to be present on brain microvascular endothelia (Berger *et al.* 1999) and at low levels on DMVEC (Salcedo *et al.* 2000b) and expression has been found to be cell cycle dependent (Romagnani *et al.* 2001). My experiments show relatively high levels of CXCR3 in SVEC and HUVEC, and low in DMVEC, similar to those found by Salcedo *et al.* and Murdoch *et al.*

5.4.3 CXCR4

CXCR4 is very widely expressed on most haematopoietic cell types and is expressed by vascular endothelial cells. The ligand for CXCR4 is SDF-1, which is alternately spliced to form SDF-1α or SDF-1β. These two variants only vary by a four amino acid extension at the C-terminus. Being such an important receptor in development as well as a co-factor in HIV (Feng *et al.* 1996; Locati and Murphy 1999; Murdoch 2000), CXCR4 has been studied much more than any other chemokine receptor.

In this study, I have shown moderate levels of CXCR4 in SVEC and HUVEC, with virtually none present on the three other EC types. Many groups have shown substantial levels of CXCR4 both by mRNA and FACS on several different endothelial cell types. In HUVEC, Murdoch *et al.* (1999) showed high levels of CXCR4 mRNA and levels of protein present equivalent to that shown in my experiments. Gupta *et al.* showed by RT-PCR that CXCR4 was present on HUVEC as well as on coronary artery endothelium. Using flow cytometry, CXCR4 has been shown to be present at high levels on DMVEC (Salcedo *et al.* 2000b) and HUVEC (Berger *et al.* 1999; Salcedo *et al.* 1999). The presence of CXCR4 mRNA has been shown in many different arterial endothelial tissues by *in situ* hybridisation and by PCR on cultured HUVEC as well as immunocytochemistry (Volin *et al.* 1998).

5.4.4 CXCR5

This chemokine receptor is important in the development of normal lymphoid tissue and is found on most B cells as well as some subsets of T cells where it is dynamically regulated. Its only known ligand is BCA-1. CXCR5 has been found in my experiments to be present at a low level only on SVEC. As far as I can tell from the literature, this is the first examination of CXCR5 expression in endothelium.

5.4.5 CC receptors

Until now, there has been little evidence to suggest that any CC receptors are found at high levels on endothelium – however low levels of certain receptors (CCR1, CCR3, CCR4 and CCR5) have been found in some studies (Gupta *et al.* 1998; Murdoch *et al.* 1999). One group has found that CCR3 is present on coronary artery endothelial cells (CAEC) and that CCR5 is expressed at low levels in both brain microvascular endothelial cells (BMVEC) and CAEC (Berger *et al.* 1999).

In this study I have shown, on all except lung endothelium, high levels of CCR3, a chemokine receptor which is found on eosinophils and is important in their homing via eotaxin especially during allergic inflammation such as asthma.

CCR4 is a selective marker for T cells, in particular T_H2 cells. It binds to TARC and MDC (Imai *et al.* 1997; Imai *et al.* 1998). It is involved in several different processes including dendritic cell trafficking, T cell recirculation and several other processes, but it has been found to be vital in the homing of T cells to human skin, though not to the gut (Campbell *et al.* 1999). CCR4 has not previously been found on endothelial cells, and in our experiments, we found that it is present at extremely variable levels on our primary cells. This chemokine receptor was the only one where a directly conjugated antibody was not available from R & D systems. Hence we used a non-conjugated antibody from Santa Cruz. This had problems associated with it as during the second centrifugation step, many cells appeared to break up, leaving us with more debris in the tube than the other receptors. This could partially explain the variable results found, however when we look at the immunocytochemistry data, we find that there appear in certain cell types to be large vesicles which stain very brightly for CCR4.

One of the problems that we encountered was that the dermal and lung microvascular endothelial cells were very much larger and more fragile that the SVEC, HUVEC and BMEC. Consequently some of the variability in CCR4 expression could be due to these physical differences affecting recovery of cells in the FACS.

Many of the differences between my results and those discussed here could be due to differences in antibodies used. For example; we have tried two different antibodies to CXCR3, the first one we tried was not directly conjugated to FITC and gave high, but quite varying results. So when R & D introduced the directly conjugated, we switched to that as the results were more consistent. Yet Murdoch *et al.* using several different antibodies to CXCR3 including those with the same specificities as our two, were unable to show any expression of CXCR3 on HUVEC (Craig Murdoch, personal communication). It is also possible that there could be slight differences due to individual patients, however repeats of these experiments with different patients yield similar patterns of expression from the flow cytometry (data not shown).

5.4.6 Summary

In this study we have shown that chemokine receptors are present on endothelium and that there are differences between endothelia derived from different parts of the body. Since chemokines have been implicated in the process of angiogenesis, we will also address the question of whether the variability in chemokine receptor expression on different endothelium contributes to differences in endothelial responses to specific chemokines. This will be examined in more detail in chapter 7.

Chapter 6
Oligosaccharide expression on different endothelial monolayers

6. Oligosaccharide expression on different endothelial monolayers

6.1 Introduction

In Chapter 4 we showed that there are differences in the binding of chemokines to endothelial cells derived from different vascular beds, and that HSPG in the proteoglycan layer is involved in this binding We then wanted to further investigate the differences within the glycocalyx between endothelial cells. In this chapter, we investigate in more detail the composition of the proteoglycan layer, in particular the sugar residues present. To do this, we have used lectins to determine the levels of particular sugar structures on the endothelial cell surface.

Lectins are sugar-binding proteins which have been found in plants, viruses, micro-organisms and animals (reviewed in Singh *et al.* 1999). They all incorporate one or more sites which have highly specific, but reversible binding for carbohydrates. Some lectins contain a second type of binding site that binds with ligands other than sugars, this gives the lectins more specificity as they can bind to acetylaminocarbohydrates, aminocarbohydrates, sialic acid and other ligands.

Table 6.1 illustrates the binding specificities of the lectins used in this study. All those used belong to the legume family of lectins and are derived from one part or another of different plants. Several of the lectins we used have been shown to bind to the same primary monosaccharide, however there are subtle differences between each lectin as they also can recognise certain other moieties close to the primary binding sequence and they often show preferential binding to oligosaccharides rather than monosaccharides (Chatterjee and Ahmed 1998). Components of the family of legume lectins have shown many useful functions; they were first used in the agglutination of different blood cells (Sharon 1983), but they also serve as a useful marker for endothelium. Human endothelial cells specifically display certain α-L-fucose residues, which are reactive with UEA-1. Hence this lectin is often used as a marker of human endothelial cells and is useful in their isolation (Hormia *et al.* 1983; Jackson *et al.* 1990). GSL-1 stains endothelial cells in many different species, but only approx. 10% of human endothelium by binding terminal α-galactose (Bankston *et al.* 1991).

There are many studies, which have previously investigated lectin binding to different endothelia. Although some studies have used SVEC or HUVEC, the majority have been performed on different

species of mammals and there has been a great deal of difference found from one species to another.

The aim of this study is to determine whether there are any differences in the basal levels of expression of different oligosaccharides on different endothelium and to then investigate the effects of digestion of proteoglycans on this expression. We show several differences between endothelial cell types.

Table 6.1 Lectins used in cell surface ELISA, properties and binding specificities

LECTIN	ABBR.	ISOLATED FROM	SPECIFICITY	COMMENTS
Concanavalin A	Con A	Canavalia ensiformis (Jack bean) seeds	α-linked mannose	A common cell surface structure
Datura Stramonium Lectin	DSL	Thorn Apple or Jimsom Weed seeds	(β-1,4) linked N-acetylglucosamine and N-acetyllactosamine	Prefers oligomers (chitobiose or chitotriose)
Dolichos Biflorus Agglutinin	DBA	horse gram seeds	α-linked N-acetylglucosamine	
Erythrina Cristagalli Lectin	ECL, ECA	Coral tree seeds	Galactose residues, high binding to galactosyl (β-1,4) N-acetylglucosamine	Can be used to isolate NK cells
Griffonia (Bandeiraea) Simplificolia Lectin I	GSL I, BSL I	Griffonia simplificolia seeds	α-N-acetylgalactosamine and α-galactose residues	Mixture of 5 isolectins, binds several glycoproteins including laminin
Griffonia Simplificolia Lectin I isolectin B4	B4	Griffonia simplificolia seeds	α-galactose residues	Useful marker for endothelial cells
Griffonia (Bandeiraea) Simplificolia Lectin II	GSL II, BSL II	Griffonia simplificolia seeds	α- or β-linked N-acetylglucosamine	
Jacalin	Jacalin	Artocarpus integrifolia (Jackfruit) seeds	O-glycosidically linked oligosaccharides preferentially galactosyl (β-1,3) N-acetylgalactosamine	Binds same residue as PNA, but can still bind when residue is sialylated
Lens Culinaris Agglutinin	LCA	Lentil	α-linked mannose residues, an N-acetylchitobiose-linked α-fucose residue in the receptor increases affinity	Recognises additional sugars, so has a narrower specificity than Con A
Lycopersicon Esculentum (Tomato) Lectin	LEL, TL	Tomato	N-acetylglucosamine oligomers	Prefers trimers and tetramers, shares some specificities with DSL, STL and WGA
Peanut Agglutinin	PNA	Arachis hypogaea (peanuts)	Galactosyl (β-1,3) N-acetylgalactosamine	In many membrane associated proteins, normally sialylated which prevents binding
Phaseolus Vulgaris Erythroagglutinin	PHA-E	Red kidney beans	Complex oligosaccharides	Strong haemagglutinin activity, poor mitogen
Phaseolus Vulgaris Leucooagglutinin	PHA-L	Red kidney beans	Complex oligosaccharides	Potent mitogen
Pisum Sativum Agglutinin	PSA	Garden peas	α-linked mannose oligosaccharides with an N-acetylchitobiose-linked α-fucose residue in receptor	Almost identical in structure and specificity to LCA
Ricinus Communis Agglutinin I	RCA_{120} RCA I	Castor beans	Terminal galactose, maybe N-acetylgalactosamine	Desialylation maybe required to remove terminal sialic acid residues
Solanum Tuberosum (Potato) Lectin	STL, PL	Potatoes	N-acetylglucosamine	Similar binding to DSL and WGA, but with some differences
Sophora Japonica Agglutinin	SJA	Japanese pagoda tree seeds	Terminal N-acetylgalactosamine and galactose residues	Preferential binding to β anomers

Soybean agglutinin	SBA	Glycine max seeds (soybeans)	Terminal α- or β- linked N-acetylgalactosamine, weakly to galactose residues	
Ulex Europaeus Agglutinin I	UEA I	Furze gorse seeds	α-linked fucose residues	Marker for endothelial cells
Vicia Villosa Lectin	VVL, VVA	Hairy Vetch seeds	Terminal α- or β- linked N-acetylgalactosamine particularly linked to serine or threonine	May require specific amino acid sequences at the receptor site
Wheat Germ Agglutinin	WGA	Triticum vulgaris (wheat germ)	N-acetylglucosamine, terminal N-acetylglucosamine	In this native form can interact via sialic acid residues
Succinylated Wheat Germ Agglutinin	sWGA	Triticum vulgaris (wheat germ)	N-acetylglucosamine,	Does not bind to sialic acid residues

6.2 Methods

6.2.1 Lectin binding assay

Cells were set up and rested as described in section 2.2.2. They were washed, fixed and blocked as described in section 2.2.3 before washing three times in 0.1% tween 20 in PBS. The binding of oligosaccharides to specific lectins was detected by adding 65 μl per well of 10 μg/ml biotinylated lectin (Vector laboratories, Peterborough, UK) in ELISA diluent as listed in table 6.1. 10 μg/ml of MHC class I antibody and E-selectin antibody (Serotec) and 1:1000 of anti HSPG antibody (Chemicon) were also added in ELISA diluent to help standardise between experiments and determine the activation state of the endothelium. All treatments were randomised across the plate in triplicate, then incubated for 1 hour at 37°C as previously described. At the end of 1 hour, the plate was washed and 100 μl of the biotinylated rabbit anti mouse immunoglobulins (Dako) added at 1:700 dilution in ELISA diluent for those wells containing MHC class I, E-selectin or HSPG primary antibody with just ELISA diluent added to those with lectins. The levels of binding of each antibody or lectin were visualised as described in section 2.2.

6.2.2 Enzyme digestion

Enzyme digestions of the proteoglycan layer were performed to investigate its composition. In this experiment, a confluent monolayer of BMEC was washed three times in HBSS. Then enzymes were added as described in section 2.3 prior to commencing the lectin binding assay as described in section 6.2.1.

6.2.3 Statistics

To test significance, two way ANOVAs were performed on each experiment followed by a post hoc t-test comparing pairs of results or all results as necessary.

6.3 Results

6.3.1 Lectin binding to different endotheiial monolayers under resting conditions

We determined the levels of binding of various different lectins to their specific oligosaccharide ligands found within the proteoglycan layer of different endothelial monolayers. The lectins used and their specificities are described in Table 6.1. Figures 6.1 and 6.2 show the levels of binding of all the lectins tested to each of the different endothelial cell types tested. We have used the bone marrow endothelial cell line as a standard in this comparison. This is because as it is an immortalised cell line, it is likely to vary less between experiments than any of the other endothelial cell types. We have then standardised the lectin binding against MHC class I binding at 100 % as in previous experiments to aid in the comparison of different endothelial cell types. The results in figures 6.1 and 6.2 are one representative example out of three experiments performed, however as the results are not always consistent between experiments (SVEC were the most consistent suggesting less differences between individuals), we can only consider those results that are consistently statistically different. The level of significance used is $P<0.05$. Significant results discussed here are those where in a majority of experiments performed, the data showed the same trend and was significant. It is these results that we will examine in more detail. If a result is not significant in a majority of experiments performed, or is significant, but shows variability (i.e. different pattern of expression in different experiments) it is not discussed here.

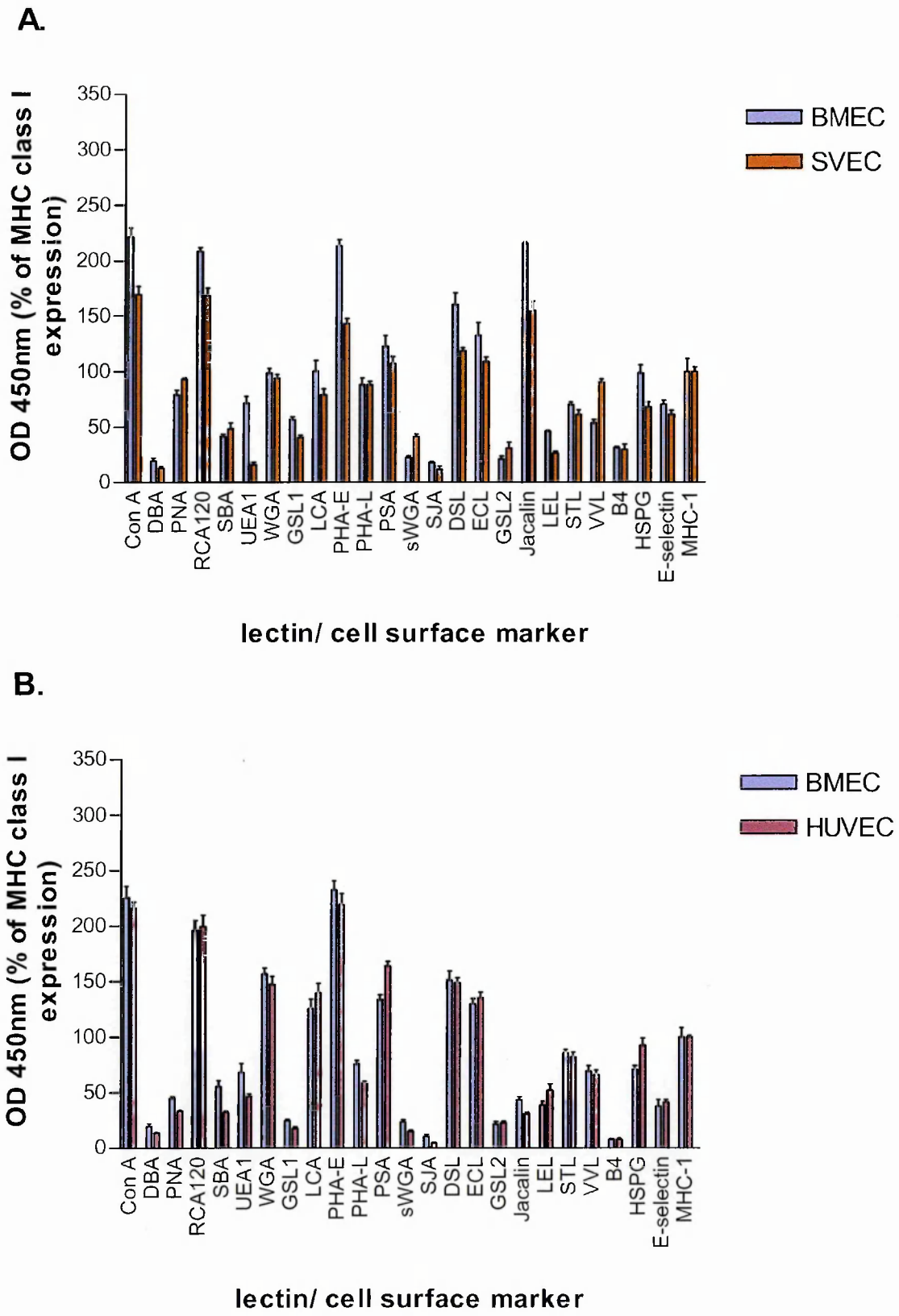


Figure 6.1 Lectin binding to monolayers of primary macrovascular endothelium compared to the transformed bone marrow cell line.

Biotinylated lectins were used to detect differences in the carbohydrates on the endothelial cell surface of A. SVEC and B. HUVEC. The experiments shown are one representative of at least 3 performed. Significance was tested by ANOVA with a post hoc t-test.

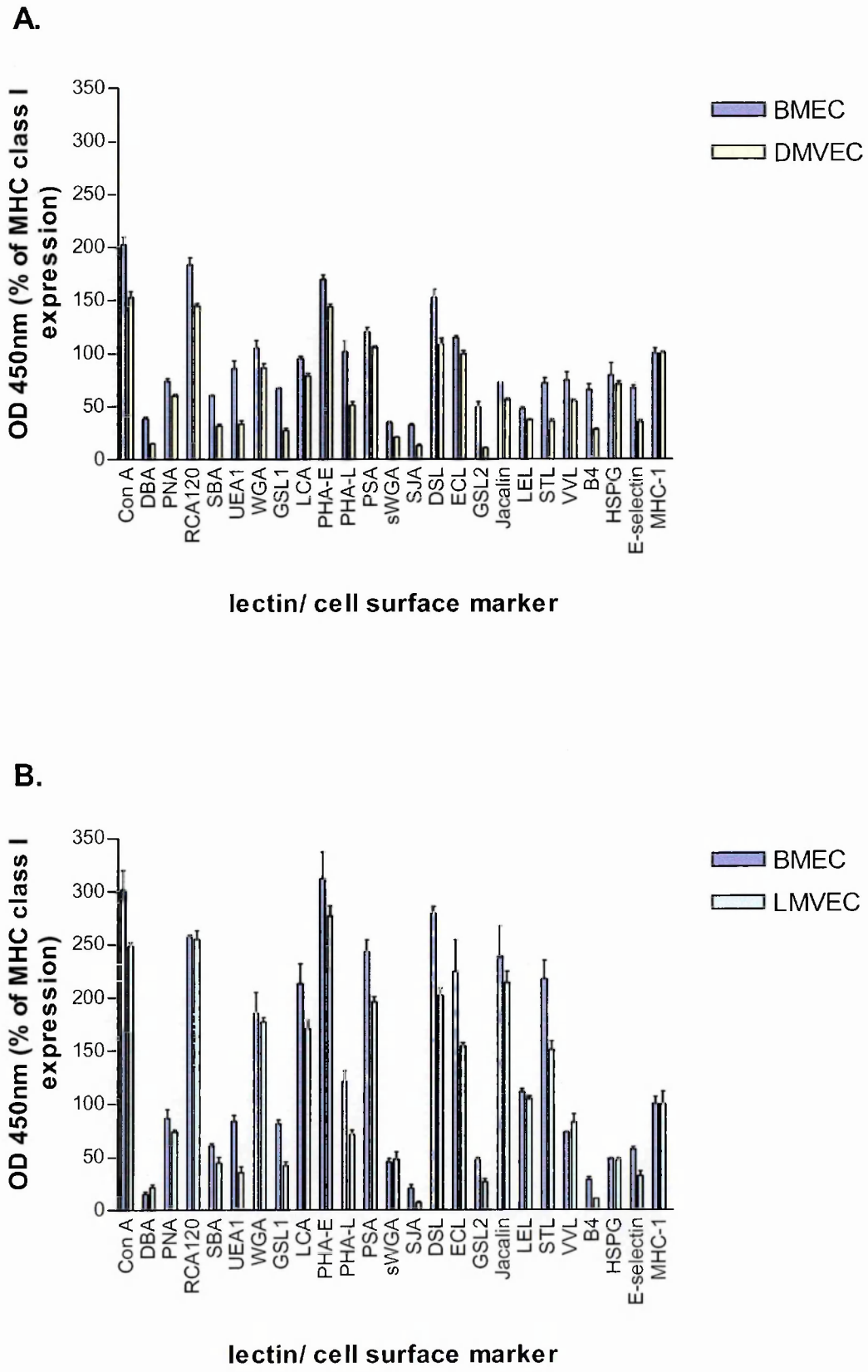


Figure 6.2 Lectin binding to monolayers of primary microvascular endothelium compared to the transformed bone marrow cell line

Biotinylated lectins were used to detect differences in the carbohydrates on the endothelial cell surface of A. DMVEC and B. LMVEC. The experiments shown are one representative of at least 3 performed. Significance was tested by ANOVA with a post hoc t-test.

All of the 22 lectins tested bound to all endothelia with the exception of two galactose binding lectins SJA and B4, which did not statistically bind any higher than the baseline to HUVEC and LMVEC and showed very low binding on other cell types. The levels of reaction of lectins with the different endothelia are similar in most cases, however there are some interesting differences. The binding levels are presented as a summary in Table 6.2.

Table 6.2 Summary of results of binding of lectins to different endothelial cells

The table also shows the variations observed between experiments. The results are classified according to amount of binding detected with + being less than 25 % of MHC class I levels, ++ is 25 – 75 % binding, +++ is 75 – 125 % binding, ++++ is 125 – 175 % binding and +++++ is over 175 % binding.

LECTIN	CELL TYPE				
	BMEC	SVEC	HUVEC	DMVEC	LMVEC
Con A	+++++	++++	+++++	++++ / +++++	+++++
DSL	++++ / +++++	+++	++++	+++ / ++++	++++
DBA	+	+	+	+	+
ECL	+++ / ++++	+++	+++ / ++++	+++	+++ / ++++
GSL I	++	++	+	++	++
B4	+ / ++	++	- / +	+	- / +
GSL II	++	++	+/++	+	- / +
Jacalin	++ - +++++	++++	+ / ++	++	+ - +++++
LCA	+++ / ++++	+++	++++	+++	++++
LEL	++	+ / ++	++	++	+++
PNA	++	+++	+	++	++
PHA-E	+++++	++++	+++++	++++ / +++++	+++++
PHA-L	+++	+++	++	++ / +++	++
PSA	++++	+++	++++	+++	++++
RCA_{120}	+++++	++++	+++++	++++ / +++++	+++++
STL	+++	++ / +++	++ / +++	++	++ / +++
SJA	+	+	- / +	+	-
SBA	++	++	++	++	+ / ++
UEA I	++ / +++	+ / ++	+ / ++	++	+ / ++
VVL	++	+++	++	++	++ / +++
WGA	++++	+++	++++	+++	++++
sWGA	++	++	+	+ / ++	++

All EC presented a strong reaction with Con A, RCA_{120} and PHA-E. They also all had moderately strong reactions with WGA, LCA, PSA, DSL and ECL, but low levels of binding to PNA, SBA, UEA-1, GSL-I, sWGA, GSL-II, LEL, STL and VVL. DBA, SJA and B4 bound at extremely low levels and in some cell types were not significantly different from the control.

The binding to BMEC to Con A, RCA_{120} and PHA-E varied somewhat between experiments, however, SVEC showed consistently lower binding compared to BMEC, while binding to the other cell types was statistically no different over several experiments compared to BMEC or to each

other. This is largely due to the inconsistencies between experiments, which were particularly evident for DMVEC and LMVEC.

A similar pattern of binding was observed for the lectins LCA, PSA, DSL and LEL although at a reduced level. SVEC was the only cell type that across three experiments bound at a significantly ($P<0.05$) lower level.

The binding of WGA to BMEC appeared to vary between experiments, and in each experiment, there was no statistical difference demonstrated between it and any other cell type. So although there were noticeable differences in the binding of WGA to different cell types, it was largely due to the variation experienced between cultures. WGA and succinylated WGA both bind the same residue, the modified form (sWGA) binds to a more restricted ligand and showed lower levels of binding. This is because WGA can also interact with sialic acid residues. The reduction from WGA binding to sWGA binding levels was particularly noticeable on HUVEC and LMVEC – suggesting that these cell types were perhaps more heavily sialylated on N-acetylglucosamine.

In a similar way, Jacaiin bound the same residue as PNA (galactosyl (β-1,3) N-acetylgalactosamine), with the exception that Jacalin can also still bind when the residue is sialylated, this is reflected in the lower binding levels of PNA on BMEC and SVEC. Jacalin binding showed considerable variability between experiments, in particular on BMEC and LMVEC. However within the same experiment, the differences were little, and the same overall pattern of binding was observed, but with levels of binding for Jacalin.

Likewise, LCA, PSA and Con A share similar receptor sites, but each has very slightly different specificities for residues around the specific receptor site. Con A recognises a smaller receptor site than LCA and PSA, and hence has a wider range of binding and so binds more.

SBA and PHA-L binding was not statistically different between the cell types tested, with the exception of LMVEC, which showed significantly reduced binding in comparison to the other cell types.

UEA-1 binds to α linked L-fucosyl residues and is used as a marker for human endothelium. It was expressed at a high level by BMEC, with significantly lower levels of expression on SVEC, HUVEC and LMVEC and inconsistencies on DMVEC.

DBA bound between 10 and 25% of the level of MHC class 1 and showed no consistent differences in binding between cell types.

GSL-I exhibited higher binding than B4 as it contains all 5 isolectins rather than just the one. SVEC and BMEC bound at a similar level (approx. 50%) while there was significantly lower binding of B4 found on HUVEC, DMVEC and LMVEC.

6.3.2 Binding of lectins to endothelium after enzyme digestion

The use of enzymes to digest different components of the proteoglycan layer showed very interesting results as the digestions with heparinase or chondroitinase had no effect. The removal of sialic acid residues from the layer by using neuraminidase showed massive increases in the binding of certain lectins. Of the lectins examined, PNA, VVL and PHA-L showed significant increases in binding.

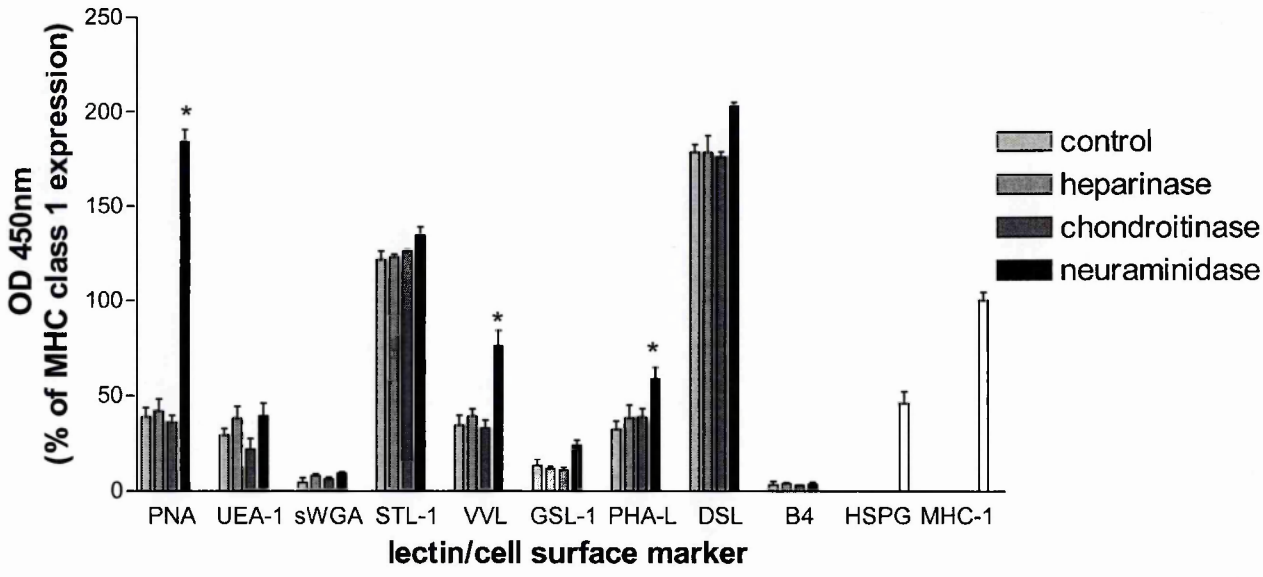


Figure 6.3 Effects of enzyme digestion on the proteoglycan layer of BMEC

Biotinylated lectins were used to detect differences in the carbohydrates on the endothelial cell surface of BMEC after digestion with 10 U/ml heparinase, 1 U/ml chondroitinase, 1 U/ml neuraminidase or no enzyme for exactly 1 hour at 37°C. The experiment shown is one representative of at least 3 performed. Significance was tested by ANOVA with a post hoc t-test (*=P<0.05).

6.4 Discussion

We have tested the binding of 22 different lectins to five different types of human endothelia in culture, which provides a comparison of oligosaccharide expression. We have found that there is a great deal of heterogeneity in the levels of specific oligosaccharides present on these different endothelial cells. The cells used in this assay were at confluence and were contact inhibited. They were seeded at a high density, then grown overnight to confluence and rested for 24 hours to allow development of the glycocalyx before performing the assay. The overall results including experimental variations are summarised in table 6.2.

6.4.1 Lectin binding to resting endothelium

UEA-I is used as a marker of human endothelial cells. Holthofer *et al.* (1982) have shown specific binding to many endothelial cell types from different organs and different sized vessels. These include the kidney, liver (with the exception of the sinusoids), pancreas, lung, skin, brain and placenta as well as cultured HUVEC. This was also shown by another study that investigated UEA-I binding to EC in culture – demonstrating that it specificaliy bound to isolated endothelium. It was also shown to be a useful tool in the isolation of pure endothelial cultures (Jackson *et al.* 1990).

There are very few studies in the literature investigating the expression of oligosaccharides on human endothelium, apart from the binding of UEA-I. Much more literature is available on a wide variety of other mammalian species including rats, mice, dogs, cats, cows, horses, sheep, pigs and monkeys (Alroy *et al.* 1987; Abdi *et al.* 1995; Smolkova *et al.* 2001).

GSL-I binding to terminal α-galactose is used as a marker for endothelial cells in many different species, but it only binds approximately 10% of human endothelium (Alroy *et al.* 1987). WGA was found to bind to endothelial cells from many different species, whereas succinylated WGA, which is the modified form, only binds to some human specimens and not those of any other animals. Alroy *et al*'s study found that RCA_{120} bound to all the endothelia from all the species tested, but Con A, DBA, LCA, PNA and SBA did not stain in any species studied. This is very different to our study where we have shown high levels of Con A and LCA binding to endothelia, with lower levels of binding of all the other lectins used in their study. Some of the differences could be due to the methods used. As all their sections were paraffin processed, it is possible that some of the smaller oligosaccharides were destroyed in the processing.

Another possible source of variation between experiments is changes in physiological conditions or stress as variations in glycosylation of some glycoproteins can then be induced (Spicer and Schulte 1992) e.g. during hypoxic stress (Weinhouse *et al.* 1993).

In a study investigating the heterogeneity of liver endothelium a similar affinity binding of Con A and RCA_{120} was found throughout the different types of endothelium found in the liver (Dini and Carla 1998). However PHA and WGA binding was found to be higher in the periportal zone and UEA-I binding was low, but present consistently on the sinusoidal endothelium.

In pigs, endothelium does not label with UEA-1. Heterogeneity of binding of other lectins has been shown along the vascular tree – both microheterogeneity, where there are differences in lectin binding to the same EC cell type and macroheterogeneity where there are differences between different types of EC and between different organs (Brouland *et al.* 1999). They showed little binding to liver EC and high binding to umbilical vein of all the lectins that they studied (B4, DBA, EEA-1, HPA, MAL-1 and PNA) although the binding of PNA was very low to all endothelial cell types. They also demonstrated differences in vWF binding to different EC types. It was suggested that this shows the plasticity or unstability of endothelial cells. In another study using foetal pig endothelium from different organs there was much variety in the levels of binding including the very strong labelling of the brain with PNA. This suggested that tissue specific heterogeneity in oligosaccharide expression is already determined to some extent in the foetus (Plendl *et al.* 1996).

Using a rat model Smolkova et al (2001) showed differences in endothelial cell binding of lectins between organs. They found that LCA, ConA, LEA, WGA and RCA_{120} bound to rat vascular EC, while PNA and UEA-I did not. The lectins all bound to the luminal plasma membrane and heterogeneity was observed with HPA and GSL-I reacting with most endothelial cell types with the exceptions of kidney glomerular EC, liver sinusoidal EC and adrenal gland EC.

We have shown virtually no binding of DBA to any of our endothelial cell types and there is no mention in the literature of it binding to human endothelial cells. Ponder and Wilkinson (1983) have however shown heterogeneity in DBA binding between different strains of mice which was not found by Alroy *et al.* (1987). It would appear that DBA staining differs according to species, organ and segment of the vascular tree (Ponder and Wilkinson 1983; Spicer and Schulte 1992)

The affinity of binding of 39 different lectins was tested to sheep lung endothelium (Abdi *et al.* 1995). It was found that GSL-1, DSA and LEA bound to the pulmonary artery, but that differential binding occurred in different vessel types within the lung e.g. there was less binding to the small vessels. These results agree with what we have observed in cultured human endothelium, but they

also found no binding of DBA, ECL and UEA-I. The binding of DBA is similar to that which we have demonstrated, however we have shown binding of ECL and UEA-I to all our endothelial cell types including the lung which suggests a difference between species.

From the results in this study, we showed that there were only small differences in the levels of binding of individual lectins to different endothelial monolayers. Hence it was not possible to relate the levels of particular oligosaccharides to the different levels of chemokine binding to endothelial monolayers shown earlier. To determine more clearly whether particular oligosaccharides are required for binding of specific chemokines, it would be possible to use lectins to block the oligosaccharides and to then test the chemokine binding to see if the lectins have any blocking effect.

6.4.2 Effects of enzyme digestion

In this study we also showed that neuraminidase digestion of the glycocalyx allowed increased binding of certain lectins including PNA, VVL and PHA-L to their specific sugars. Due to the expense of the enzymes, this was only tested on BMEC and only on a select panel of lectins. We could expect to see similar results for other lectins in particular RCA_{120} and SBA, which have previously been shown to bind more when the sialic acid is removed and their binding site is exposed.

An early experiment treating myoblasts with neuraminidase was found to increase the amount of RCA_{120} detected (Holland *et al.* 1984). This appears to work by cleaving the sialic acid residues from the proteoglycan layer, hence exposing more binding sites for the lectin to react with. This has since been shown to also apply to binding of lectins to endothelial cell from various different species, It is particularly effective on SBA and PNA as was demonstrated on Bovine Aortic EC, (Augustin-Voss and Pauli 1992). The removal of sialic acid residues was actually found to reduce binding of WGA to rat endothelium (Smolkova *et al.* 2001), it would appear that the positively charged WGA perhaps requires the negative charge provided by the sialic acid to bind with high affinity.

In the same study they also showed that HPA and GSL-I binding were increased after treatment with the enzyme, but no detectable effects were observed on PNA or LEA which is in contrast to the earlier study (Augustin-Voss and Pauli 1992). In investigating most mammalian species (with the exception of the rat), Alroy *et al.* (1987) found that PNA bound after treatment to most species with the exception of the cat. The effects of neuraminidase on SBA binding were only striking in bovine and murine specimens although there was binding observed to some endothelial cells in

cats, dogs, goats, horses and humans. The differences demonstrated in sWGA and WGA binding as well as the fact that PNA binds well after neuraminidase digestion of the proteoglycan layer suggests that it is heavily sialylated on all the species that they tested.

Heparinase and chondroitinase, which remove heparan sulphate and dermatan sulphate respectively had no effect on the binding of PNA, UEA-1, sWGA, STL-1, VVL, GSL-1, PHA-L, DSL or B4 to endothelial cells. This showed that the lectins do not show specificity for either of these molecules.

6.4.3 Summary

In the last few chapters, we have shown many differences between endothelia – in their chemokine production, receptor expression, composition of the proteoglycan layer and binding to it. In the next chapter, we will investigate whether chemokines have any functional effects on endothelial cells in culture.

Chapter 7
Endothelial cell responses to chemokines

7. Endothelial cell responses to chemokines

7.1 Introduction

In Chapter 5 we showed that all the endothelial cell types tested expressed chemokine receptors. In this chapter, we will investigate whether chemokines acting on their receptors are involved in the processes of angiogenesis.

Angiogenesis, the formation of new blood vessels is a basic process in inflammation, reproduction, deveiopment, tumour growth and tissue repair. It is a sequence of complex and tightly controlled events which first of all involves the release of matrix metalloproteinases by endothelial cells, these can act on the basement membrane to break it down, which then allows the proliferation and migration of endothelial cells into tissue. The final step in the formation of a new blood vessel is the reconstruction of the matrix and formation of a capillary tubule from the endothelial cells present.

The process depends on the balance of promotors (angiogenic factors) and inhibitors (angiostatic factors). During wound repair, the balance shifts towards angiogenic factors. Once new blood vessels are established, the equilibrium is re-established. In contrast, lack of regulation of angiogenesis favours increased vascularisation and can have pathological significance in certain diseases such as rheumatoid arthritis, psoriasis and ocular diseases as well as cancer.

VEGF and FGF (both acidic and basic forms) are well established angiogenic and endothelial proliferative factors. CXC chemokines and some CC chemokines have also been implicated in the process of angiogenesis. Platelet factor 4 (PF-4) was the first angiostatic chemokine found and was also found to attenuate angiogenesis during tumour growth (Maione *et al.* 1990; Sharpe *et al.* 1990). IL-8 was then discovered to be angiogenic *in vivo* and to promote endothelial proliferation *in vitro* (Koch *et al.* 1992).

The angiogenic properties of CXC chemokines are dependent on the expression of the highly conserved ELR (Glu-Leu-Arg) motif which immediateiy precedes the first cysteine residue (Strieter *et al.* 1995b; Belperio *et al.* 2000). The lack of the ELR motif indicates chemokines that are angiostatic; these include CXCR3 binding chemokines as well as PF-4. Table 7.1 shows the CXC chemokines that are known to regulate the process of angiogenesis (Moore *et al.* 1998).

Table 7.1 Known angiogenic and angiostatic CXC chemokines

Classified by the presence or absence of the ELR (Glu-Leu-Arg) motif preceding the first cysteine residue.

ANGIOGENIC CXC CHEMOKINES CONTAINNG THE ELR+ MOTIF	ANGIOSTATIC CXC CHEMOKINES THAT LACK THE ELR MOTIF (ELR-)
IL-8	IP-10
ENA-78	PF-4
Gro-α, -β, -γ	Mig
GCP-2	
NAP-2	

This is an exploratory study and as such has many problems associated with it. The first step in this study was to investigate whether chemokines could induce proliferation of endothelial cells and to see whether there are any differences in responses between endothelial cells isolated from different tissues.

7.1 Methods

7.1.1 Thymidine incorporation assay

Proliferation assay medium: M199 with 2 mM glutamine supplemented with 1 % FCS, 100 U/ml Penicillin, 100 µg/ml streptomycin.

Cells were plated into 0.2% gelatin coated 24 well plates at a density of 4 x 10^4 cells per well in 600µl of proliferation assay medium. The plates were grown overnight for 16 hours before stimulation this achieves approximately 30% coverage of the area of the plate with EC at the start of the assay. Concentrations of 0 – 50 ng/ml of chemokines and cytokines diluted in assay medium were then added to triplicate wells to stimulate the endothelial cells. Standard growth medium (M199 with 10% FCS) was used as a positive control. The endothelial cells were then incubated for 24 hours before the addition of 2 µCi ^{3}H thymidine per well for a further 24 hours. The cells were then trypsinised and collected on filter paper using an automatic cell harvester (Skatron combi cell harvester). The radioactivity of the filters was read by liquid scintillation on a Wallace microbeta trilux counter. Experiments were repeated three times.

7.1.2 Optimisation of conditions for the assay

The first experiment was performed on SVEC without the addition of chemokines, but with different concentrations of FCS, to determine the optimum concentration of FCS to perform a proliferation assay (fig. 7.1). We showed a significant increase in proliferation of SVEC after the addition of 5 or 10 % FCS to M199, but not after the addition of 1%. We then chose to use 1% FCS for the majority of our assays as this would interfere less with the proliferation of the cells. We chose to use 10% FCS as our positive control as this induced proliferation.

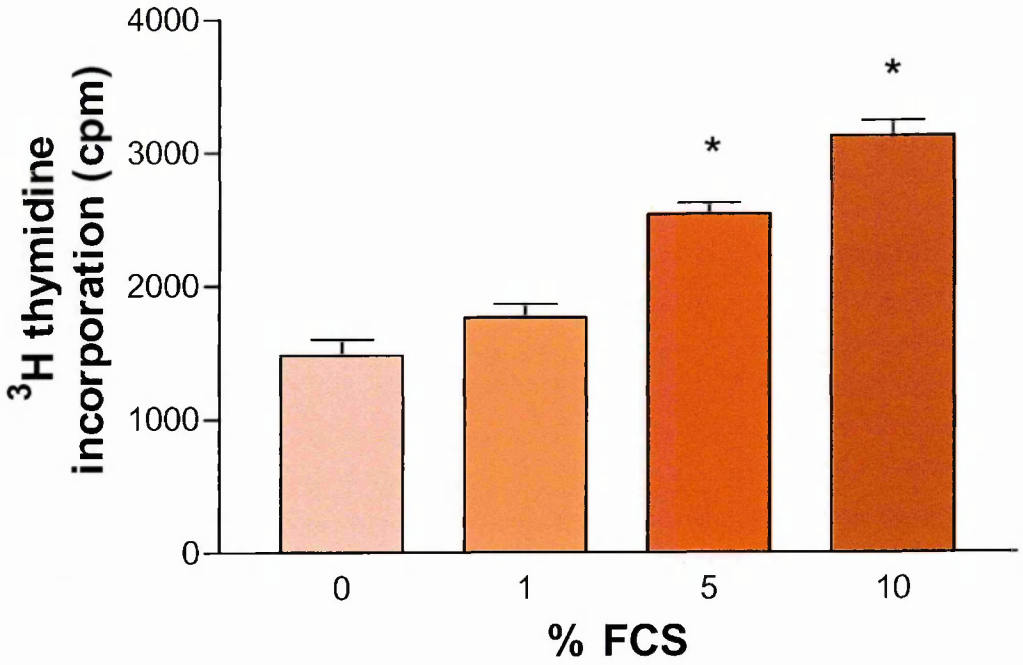


Figure 7.1 SVEC proliferation with differing amounts of FCS

^{3}H-thymidine incorporation was used to measure the proliferation of SVEC in a preliminary experiment to determine the optimum concentration of FCS for a proliferation assay. Results are the average of triplicate wells. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$).

7.2 Results

We have investigated the proliferation of endothelial cells after a 48 hour incubation with different chemokines, and with ^{3}H thymidine added to the culture for the last 24 hours. Overall, we have shown very few significant differences in these assays as compared to the control of assay medium with no chemokine added (see figs. 7.2 – 7.5).

However we have shown that Gro-α (a known angiogenic chemokine) does cause endothelial cells to proliferate – at both 5 and 50 ng/ml in SVEC and DMVEC, but only at 5ng/ml in LMVEC. We showed no significant effects of Gro-α on HUVEC proliferation. TGF-β showed no significant effects on SVEC, DMVEC or LMVEC proliferation and no consistent results on HUVEC – in some experiments, TGF-β increases proliferation, and in others it decreases it (as shown in fig. 7.5b).

We have shown no effect of either IL-8 or MCP-1 on SVEC, DMVEC or HUVEC, however both chemokines show a significant decrease in thymidine incorporated (and hence cell number) in LMVEC (figs. 7.4c, e). This could be due to fewer cells as they lifted off at higher doses of these two chemokines – an effect that was aiso observed on DMVEC, but to a lesser extent.

IP-10 is a known angiostatic chemokine; however, we have not been able to show this effect on SVEC, LMVEC or HUVEC and only in some experiments performed on DMVEC (as shown in fig. 7.3d). The concentration of 50 ng/ml of IP-10 reduced the cell numbers (but reduced them below the background level).

We have shown an increase in proliferation of SVEC after the addition of over 5 ng/ml of Eotaxin to the culture medium (fig. 7.2f), but no effects on any other cell type. We have also tested the effect of these 6 different inflammatory mediators on cells in culture with 5% serum. To provide a direct comparison, we used the same initial concentration of cells, however this did not appear to be the ideal density as when we came to read the plates after 48 hours of stimulation, all the wells on the plate were almost confluent. The only difference between wells that we could consistently demonstrate was that Eotaxin was causing proliferation of SVEC (fig. 7.6f)

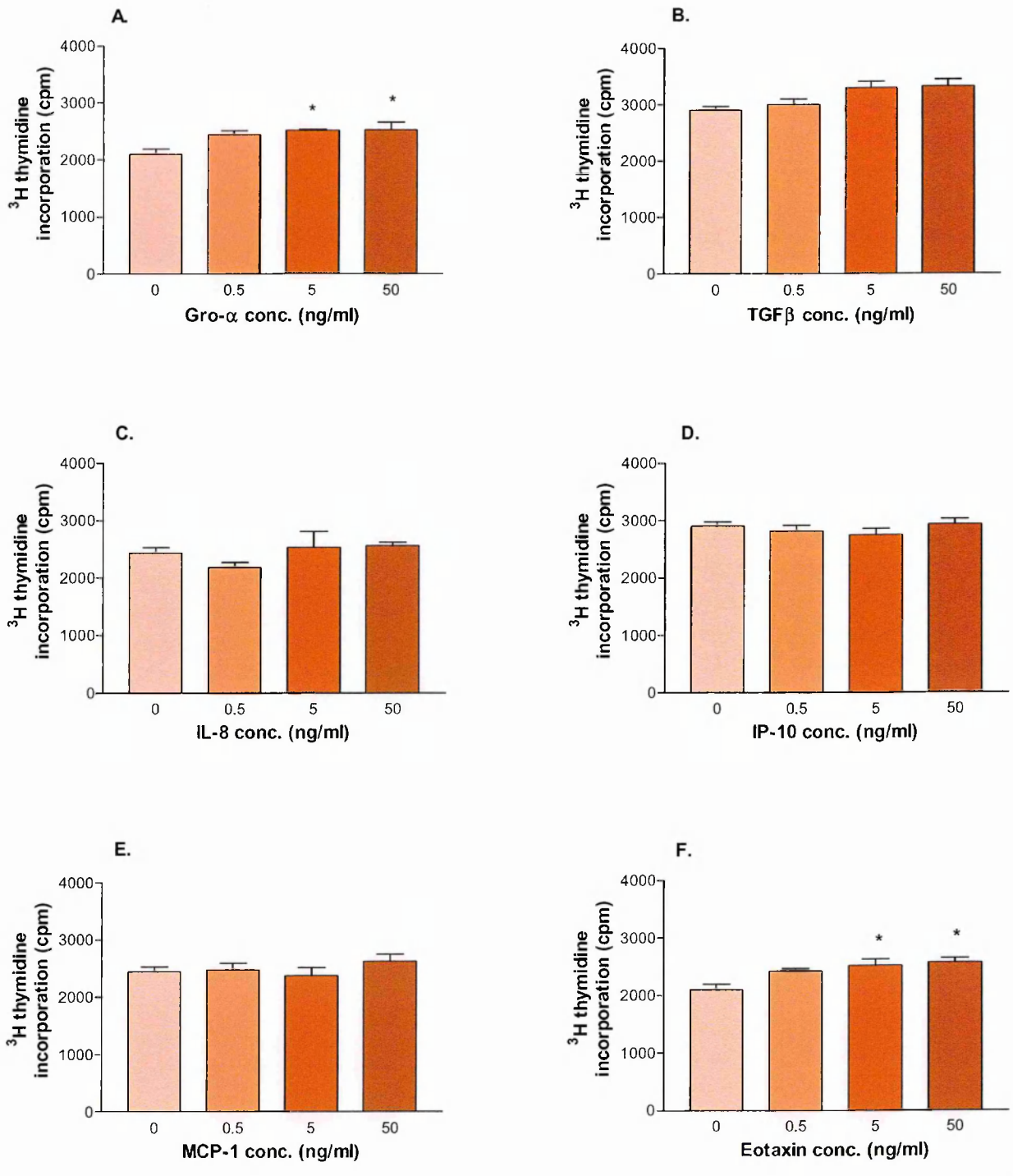


Figure 7.2 Effects of chemokines on proliferation of SVEC

SVECs were cultured in proliferation assay medium and stimulated with different chemokines at different concentrations for 24 hours followed by 24 hours of ^{3}H thymidine incorporation to measure proliferation. Chemokines used were A. Gro-α, B. TGF-β, C. IL-8, D. IP-10, E. MCP-1, and F. Eotaxin. Results presented are the mean ± S.E. of triplicate wells from one representative experiment. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$) where necessary.

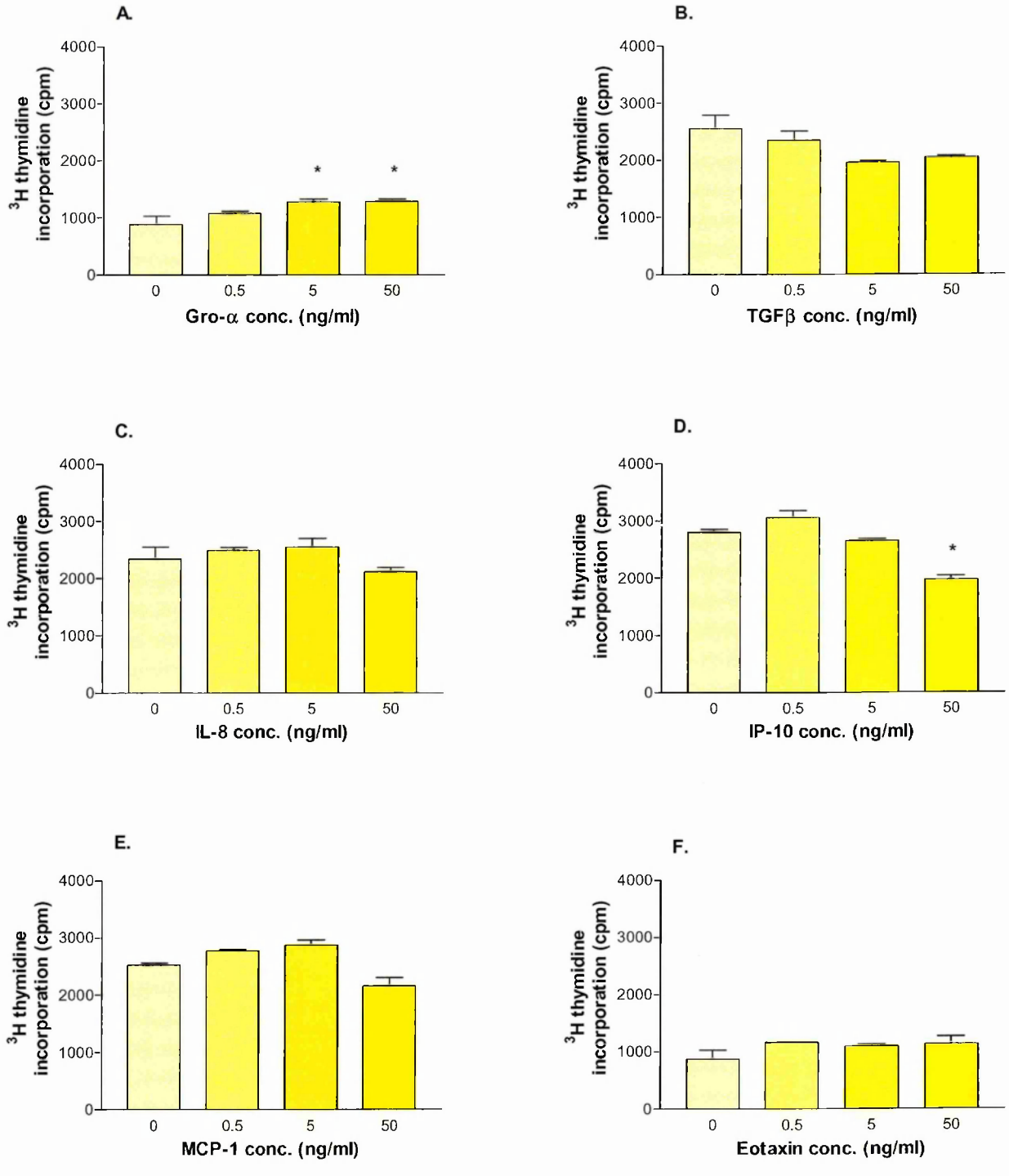


Figure 7.3 Effects of chemokines on proliferation of DMVEC

DMVECs were cultured in proliferation assay medium and stimulated with different chemokines at different concentrations for 24 hours followed by 24 hours of ^{3}H thymidine incorporation to measure proliferation. Chemokines used were A. Gro-α, B. TGF-β, C. IL-8, D. IP-10, E. MCP-1, F. Eotaxin. Results presented are the mean ± S.E. of triplicate wells from one representative experiment. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$) where necessary.

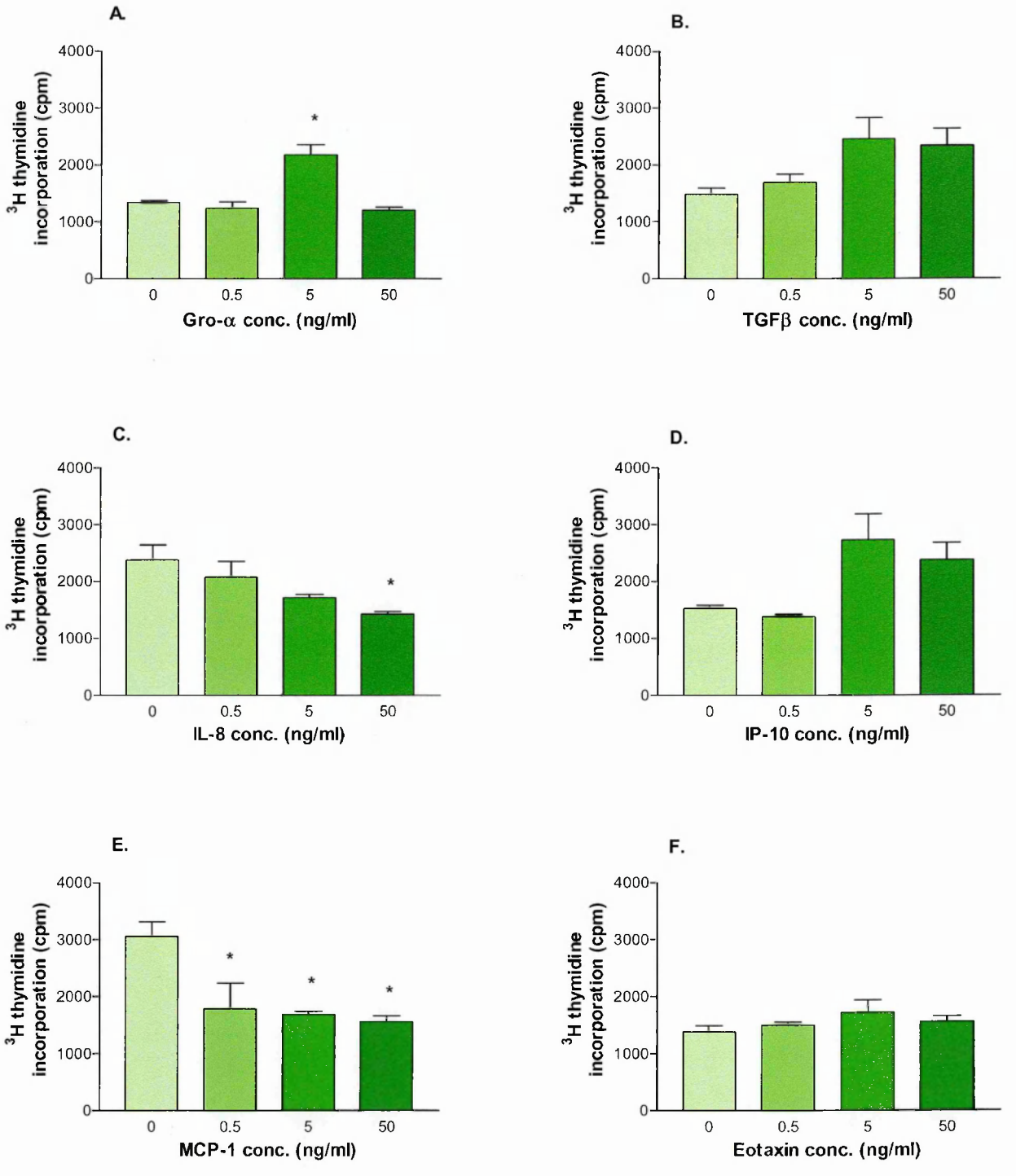


Figure 7.4 Effects of chemokines on proliferation of LMVEC

LMVECs were cultured in proliferation assay medium and stimulated with different chemokines at different concentrations for 24 hours followed by 24 hours of ^{3}H thymidine incorporation to measure proliferation. Chemokines used were A. Gro-α, B. TGF-β, C. IL-8, D. IP-10, E. MCP-1, F. Eotaxin. Results presented are the mean ± S.E. of triplicate wells from a representative experiment. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$) where necessary.

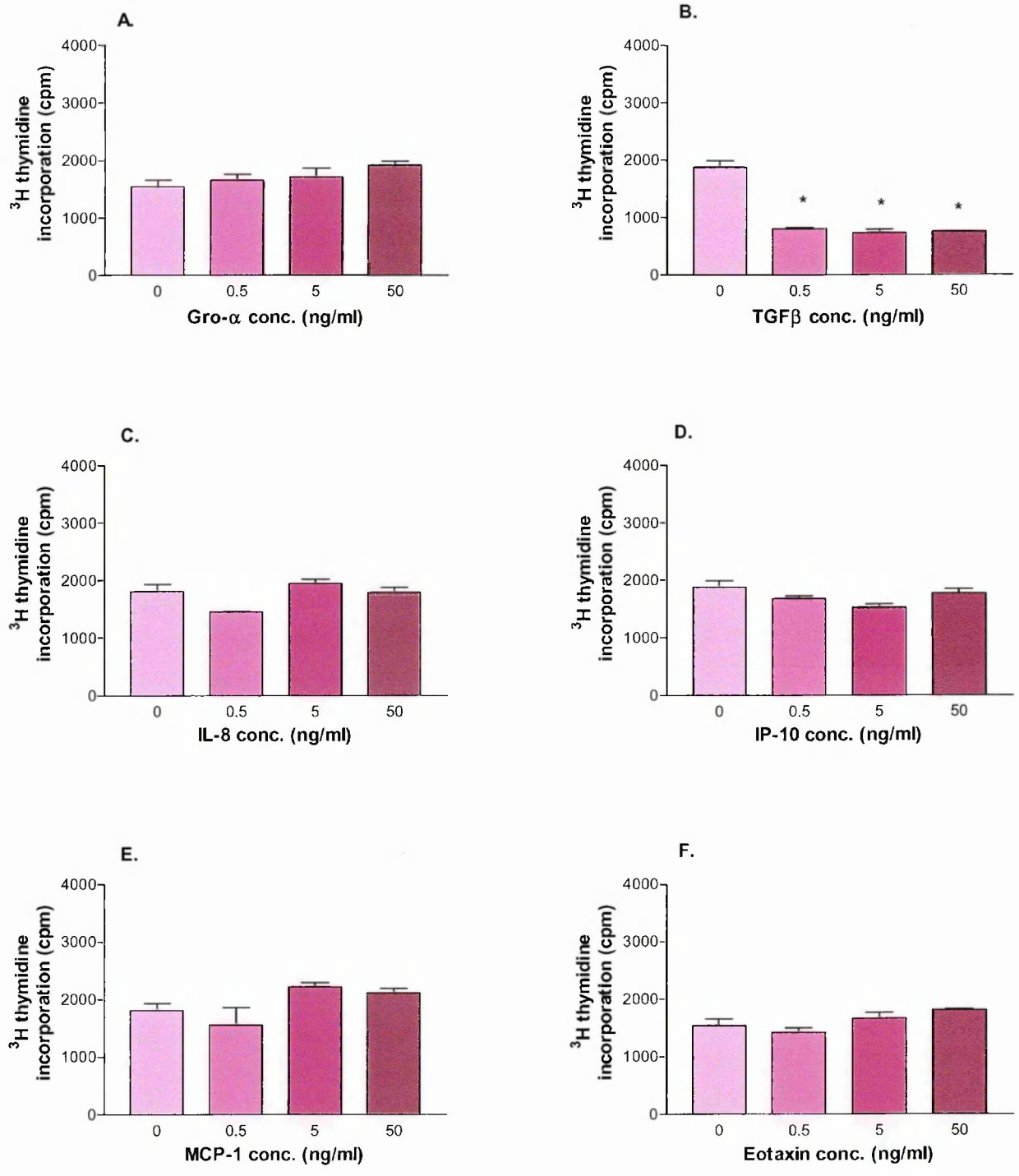


Figure 7.5 Effects of chemokines on proliferation of HUVEC

HUVECs were cultured in proliferation assay medium and stimulated with different chemokines at different concentrations for 24 hours followed by 24 hours of ^{3}H thymidine incorporation to measure proliferation. Chemokines used were A. Gro-α, B. TGF-β, C. IL-8, D. IP-10, E. MCP-1, F. Eotaxin. Results presented are the mean ± S.E. of triplicate wells from a representative experiment. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$) where necessary.

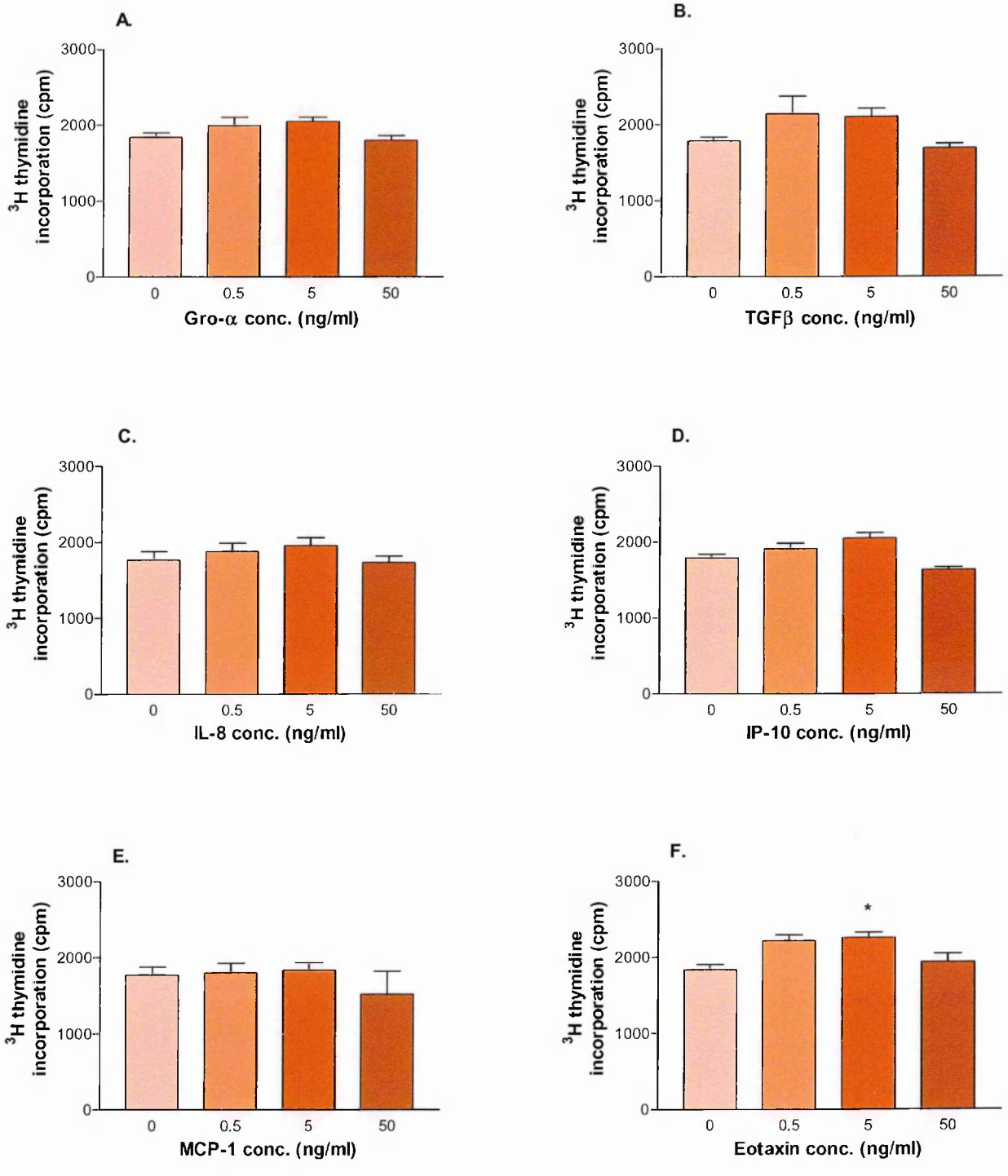


Figure 7.6 Effects of chemokines on proliferation of SVEC cultured in 5 % serum

SVECs were cultured in M199 with 5 % FCS instead of 1 % FCS and stimulated with different chemokines at the concentrations shown for 24 hours followed by 24 hours of ^{3}H thymidine incorporation to measure proliferation. Chemokines used were A. Gro-α, B. TGF-β, C. IL-8, D. IP-10, E. MCP-1, F. Eotaxin. Results presented are the mean ± S.E. of triplicate wells from one representative experiment. Significance was tested by ANOVA with a post hoc t-test (* = $P<0.05$) where necessary.

7.3 Discussion

In the literature, the concentrations of chemokines, the length and type of the assays, cell densities and serum levels used in assessing proliferation vary considerably. The parameters chosen for our assays were based on the most common conditions encountered for thymidine incorporation assays, however we have not been able to reproduce many of the results found in the literature.

We had problems with optimising the assay, as to compare proliferation between cell types, we needed to keep as many conditions as possible the same for all cell types. SVEC and HUVEC grow very much faster than LMVEC and DMVEC, which caused difficulties, as these were the cell types that the assay was set up with due to their availability at the time. This is also shown in the results obtained by Petzelbauer *et al.* (1995) where no proliferation was demonstrated in response to IL-8. One possible solution would be to increase the serum levels as DMVEC and LMVEC survive much better in higher serum levels. We tried this, but found that SVEC and HUVEC were achieving confluence before the end of the assay. Ideally if there had been more time, we would have liked to reduce the cell density and optimise the assay length. The other option, which makes it slightly less easy to compare between cell types, would be to optimise the assay for each cell type.

In these assays, we once again encountered a problem with a slight reduction in cell numbers at high doses of chemokine, which wasn't apparent when the cells were viewed under the microscope before trypsinisation. It appears that the cells become loose and are then easily removed when the culture medium is aspirated to add trypsin to digest the cells. It would be more beneficial to use a different method of harvesting - perhaps as some groups have used, it would be better to freeze and thaw the cultures several times rather than remove the medium and hence lose some of the cells and some of the radioactivity. This phenomenon was evident in cells cultured with most chemokines – particularly the ELR+ CXC, which are known to have quite potent angiogenic properties, but it did significantly affect the results.

Figure 7.7 Summary of results obtained from thymidine incorporation assays

Showing endothelial cell proliferation (+), no statistical significance (NS), or a reduction in thymidine incorporation into endothelial cells in comparison to control samples (-).

	SVEC	DMVEC	LMVEC	HUVEC
Gro-α	+	+	+	NS
TGFβ	NS	NS	NS	-
IL-8	NS	NS	-	NS
IP-10	NS	-	NS	NS
MCP-1	NS	NS	-	NS
Eotaxin	+	NS	NS	NS

7.3.1 Angiogenic factors

It has been shown that Gro-α is angiogenic for HUVEC, (Fujisawa *et al.* 1999) however, HUVEC is the only cell type in which we have shown no proliferation in response to Gro -α. It is possible that this is due to the different methods used. In their study, Fujisawa *et al.* serum starved their endothelial cells, then added chemokines (10 – 100 nM) for 24 hours before doing an MTT assay. They also showed that proliferation could be inhibited by the addition of antibodies against Gro. The mechanisms by which angiogenesis occurs are still being investigated. In these experiments we have not serum starved our cells as the microvascular endothelium are particularly sensitive to this treatment and also under physiological conditions, endothelium are continually bathed in plasma, so we felt that having some serum present would be preferable.

As has already been mentioned, IL-8 is a known mediator of angiogenesis in endothelial cells inducing proliferation and promoting chemotaxis *in vitro* and inducing neovascularisation *in vivo* (Koch *et al.* 1992; Norrby 1996). In these experiments, we have shown no significant proliferative activity in response to IL-8 at the concentrations tested.

This could be because of different conditions, Koch *et al.* used 10% serum to grow their cells overnight before washing and using 1% for the assay, whereas we have used 1% serum for the plating stage and the whole assay. They also plated their cells at one quarter the density that we did before growing them for 72 hours and counting cells. But, their results also show a decrease in proliferative activity at higher chemokine doses, with a maximal at 10nM (approx. 4ng/ml). This agrees with the reduction in proliferation observed at 50 ng/ml that we see on SVEC with 5%

serum and on all other cell types with 1% serum, though it is only significant on LMVEC (see table 7.2).

In another study, it was shown that IL-8 added at high doses (over 0.25 nM or 0.1 ng/ml) did not induce proliferation in 5% serum, whereas lower doses induced proliferation and thymidine incorporation (Langeggen *et al.* 2001). Since the concentrations that we have used are 1.25 – 125 nM, this could explain the lack of proliferation observed. Strieter *et al.* (1995a) in their first study *in vivo* found that doses of 2 – 40 ng/ml induced angiogenesis when administered intracorneally in rabbits, while lower and higher doses had no effect. Similar results were also obtained in rat corneas (Koch *et al.* 1992).

We and others have shown the presence of CXCR1 and 2 on HUVEC and other cell types (Chapter 5 and Murdoch *et al.* 1999; Salcedo *et al.* 2000b). These two receptors are the binding sites for IL-8, but Gro-α binds only to CXCR2, as do all the other ELR+ CXC chemokines. This is one of the reasons among many others that Addison *et al.* (2000) proposed that CXCR2 is the receptor through which angiogenic activity is mediated. Studies on CXCR2 knockout mice demonstrated a delayed wound healing response including reduced neovascularisation in comparison to the wild type mouse, this was postulated to be due to a reduced angiogenic response to MIP-2, the murine homologue of IL-8 (Devalaraja *et al.* 2000). IL-8 and Gro-α have also been detected in studies on wound healing in the first few days after wounding, their expression correlating with increasing vascularisation (Engelhardt *et al.* 1998).

The effects of the cytokine TGF-β on endothelial cells still seem to be controversial. It appears that it is important in angiogenesis, however *in vitro* it has been found to inhibit proliferation and migration of endothelial cells (Heimark *et al.* 1986). It is thought that TGF-β is not as important in the early stages of angiogenesis, as it is later on in the remodelling and tubule formation. The inhibition of endothelial proliferation is associated with increases in ECM and proteoglycan synthesis. It could be the 'off' signal for proliferation and migration before formation of the new capillary.

MCP-1 was originally thought to have an indirect effect on angiogenesis via its attraction of monocytes to wound sites (Goede *et al.* 1999), and the subsequent production of IL-8 and classical angiogenic factors by these cell types, however this has been challenged in several recent studies, which suggest that MCP-1 is directly implicated via CCR2 on EC (Weber *et al.* 1999a; Salcedo *et*

al. 2000a). In our studies, we have not been able to detect CCR2 on endothelium and we have not detected any proliferation in response to MCP-1. This result from this present study agrees with that observed by Weber *et al.* who suggest that although MCP-1 is not proliferative for endothelium, it is still acting through its CCR2 receptor (which they have detected on HUVEC) and is important in angiogenesis during the migratory stage. Salcedo *et al.* also found that *in vivo*, the angiogenic effects were most potent at 5 – 100 ng/ml and it was also shown that this was not due to any inflammatory infiltrate, but due to the direct effects of MCP-1 acting via CCR2. They also showed chemotaxis *in vitro* of HUVEC and DMVEC towards MCP-1, with maximal migration occurring at 0.5 – 5 ng/ml.

Eotaxin is a potent chemoattractant for eosinophils and has also been shown to be angiogenic and to act via its specific CCR3 receptor on endothelial cells (Salcedo *et al.* 2001), which we have detected at high levels on endothelial cells (chapter 5). Eosinophil products include TGF-β, which did raise the question as to whether eotaxin acted indirectly, however, using a rat aortic ring assay, which has no eosinophils, Salcedo *et al.* were able to show that the effect is directly induced using Eotaxin.

Eotaxin was also shown to chemoattract DMVEC, however it was less potent than SDF-1, MCP-1 or IL-8 and required a higher concentration (1 – 10 nM, 0.4 – 4 ng/ml showed maximal migration). Salcedo *et al.* showed no proliferative effects of eotaxin on DMVEC, which is in agreement with our study, as SVEC is the only EC type in which we have shown that eotaxin can cause proliferation.

7.3.2 Angiostatic chemokines

CXC chemokines lacking the ELR motif are inhibitors of angiogenesis. These chemokines include IP-10, Mig and I-TAC which all bind CXCR3 (Loetscher *et al.* 1996; Cole *et al.* 1998) and PF-4 for which the receptor is unknown (Gentilini *et al.* 1999). They have been shown not only to not induce vascularisation *in vivo* or proliferation *in vitro*, but to actually act as angiostatic agents in the presence of ELR+ chemokines or proliferative cytokines such as bFGF or VEGF (Angiolillo *et al.* 1995; Gengrinovitch *et al.* 1995; Luster *et al.* 1995; Strieter *et al.* 1995a). We have shown the presence of CXCR3 on endothelium as have other studies (Chapter 5 and Salcedo *et al.* 2000b; Garcia Lopez *et al.* 2001). In this study, we have shown no significant effect of IP-10 on SVEC, LMVEC or HUVEC, but we have shown a reduction in proliferation after the addition of 50 ng/ml IP-10 to DMVEC. This particular experiment does not clearly demonstrate whether IP-10 is anti –

angiogenic. This would be better shown with a higher level of initial proliferation – perhaps by using a higher level of serum or by adding VEGF or bFGF followed by IP-10 and noting the differences between cell types in their response to IP-10. Unfortunately we didn't have time to fully work out the conditions for the initial experiments, as this would have been an interesting experiment to perform.

7.3.3 Disease implications

Anti-angiogenic therapy has important implications in many disease states – most obviously cancers, but also rheumatoid arthritis and certain ocular diseases. Possible candidates for this therapy include chemokines themselves or chemokine antagonists, however because of their varied activities on different cell types, it is important to localise them to the site where they are required. Although it appears that chemokines exert different effects at different concentrations (e.g. Gro-β). The addition of a chemokine at a high dose would not be advisable *in vivo* as it could have many other effects which are often also dose dependent – in particular at higher concentrations, leukocytes tend to be activated which could cause more problems than they solve!

Chemokines are particularly of interest in cancer as inhibiting the formation of blood vessels to supply the tumour has many potential therapeutic benefits. It has been shown that IL-8 and its receptors CXCR 1 and 2 are present in carcinomas (Richards *et al.* 1997) and it is thought that they may be acting to promote angiogenesis and hence tumour survival. IL-8 has been found to be significantly elevated in non-small cell lung cancer. It was also determined to be a major angiogenic factor contributing to tumour survival. However, in a mouse model of human non-small cell lung cancer the addition of neutralising antibodies to IL-8 has been shown to reduce tumorigenesis via reduced angiogenesis (Arenberg *et al.* 1996a). Patient survival and metastatic potential could be related to the type of chemokine produced by different types of carcinoma. For example, squamous cell carcinoma produces high levels of IP-10 and has fewer blood vessels present compared to adenocarcinoma, which has poorer survival rates and higher metastatic potential (Arenberg *et al.* 1996b; Arenberg *et al.* 1997).

Gro-β has been reported to inhibit angiogenesis (Cao *et al.* 1995), but the concentrations used in this study were approx. one thousand times higher than found for angiogenic activity (1 – 10 nM) suggesting that perhaps high doses significantly reduce the sensitivity of the receptors to specific chemokines. The gro chemokines are normally angiogenic at physiological concentrations and their expression is also associated with tumours and tumorigenesis in much the same way as IL-8.

Using a neutralising antibody against Gro has been shown to reduce tumorigenesis associated with reduced angiogenesis (Luan *et al.* 1997).

7.3.4 Summary and future work

These assays were the pilot for a potentially very interesting study, which was put into a grant for further funding. Unfortunately we have been unable to show much in the way of significant findings, mostly due to the preliminary nature of these experiments. To continue this work, these assays need to be optimised more carefully for chemokine concentrations, serum concentrations, length of assay and cell density. Then the effects of Gro-α, IL-8 and IP-10 on different endothelial cell types could be investigated in more detail; in particular the effects of IP-10 on growth factor stimulated endothelial cells and the effects of neutralising antibodies.

Other assays that would give clearer indications of the importance of these chemokines in angiogenesis in different tissues include chemoattraction assays to identify chemokines that endothelial cells will migrate towards as well as *in vitro* angiogenesis assays.

Chapter 8
Discussion

8. Discussion

8.1 General Discussion

The aim of this study was to investigate differences between endothelia isolated from different vascular beds in particular in relation to leukocyte migration and inflammation. We have focussed on the chemokines, as we were interested in their role in controlling selective migration of leukocytes across endothelium to different sites of damage or infection. We have compared chemokine production and receptor expression across several different vascular beds and then investigated the role of the p

roteoglycan layer in the binding of chemokines to these different endothelia. The final exploratory study investigated the effects of different chemokines on proliferation of endothelial cells derived from different vascular beds. A summary of all these results can be found in table 8.1.

We have shown that there are differences between different endothelial cells in the chemokines produced, chemokine receptors expressed and in the carbohydrates of the proteoglycan layer and chemokines that bind to them. We have also shown preliminary data showing differences between endothelial cells in their proliferative responses to chemokines. Other studies in our group have shown differences in adhesion molecule expression under similar stimulation protocols (personal communication E. Mordelet). Confluent monolayers were assayed by the cell based ELISA method as described earlier, both without stimulation and after stimulation for 24 hours with TNF-α or IFN-γ. It was found that LMVEC and DMVEC expressed higher levels of adhesion molecules overall, and that after TNF-α stimulation ICAM-1 and VCAM were increased in all cell types, as was ICAM-1 after IFN-γ stimulation. One interesting point was that selectins (E and P) were increased at 24 hours in DMVEC, LMVEC and HUVEC only. Since selectins are maximally expressed at 4 to 8 hours after stimulation, this is not the ideal time at which to measure their responses to cytokines, but it is certain that at 24 hours BMEC and SVEC do not show increased levels of selectins in response to either TNF-α or IFN-γ. This is likely because they have been induced and by 24 hours have decreased back to baseline, not that they have not been induced at all. Another notable difference is that HUVEC express very low levels of both ICAM-2 and PECAM in comparison to other cell types. BMEC overall express the lowest levels of adhesion molecules, however as is

discussed elsewhere, the microenvironment of the bone marrow contains many constitutively expressed cytokines, which causes continually increased levels of adhesion molecules on bone marrow EC. Hence cytokine stimulated BMEC would represent more closely the *in vivo* phenotype. Although there were several differences observed in adhesion molecule expression between different endothelial cell types, these were found to be much less than differences in chemokines produced.

There are several different factors that contribute to the positioning of leukocytes in different tissues. The first is the difference found,at the rolling stage of recruitment, in adhesion molecules present on the endothelial cell surface which dictate whether a particular leukocyte can interact with the endothelium to slow down and roll along the vessel wall. Once it is rolling along the vessel wall, the leukocyte needs to be activated if it is to migrate into the tissues. This requires that the chemokines are presented on the proteoglycan layer of the endothelial cell and that there is a specific receptor that they can interact with on the leukocyte surface. If this does not occur, it appears that the leukocyte continues rolling and may detach from the vessel wall and return to the circulation. If this does occur, then the activated leukocyte can firmly adhere to the vessel wall via binding of integrins on the leukocyte to cellular adhesion molecules of the Ig family on the vessel wall. Again, this will only occur if pairs of molecules that can bind each other are present on the leukocyte and endothelial cell. Finally, the leukocyte can undergo migration through the vessel wall along a chemokine gradient. *In vivo*, these reactions are complex and there would probably be more than one chemokine present at different concentrations held on the ECM. It is by a combination of different chemokine gradients, that the leukocyte will get to its final position and exert its effects (Foxman *et al.* 1997).

Chemokines present on the endothelial cell surface *in vivo* are likely to have been produced by a combination of endothelium, tissue cells and infiltrating leukocytes. The proportions of chemokines produced by each cell type will vary according to the tissue. For example endothelum from the dermis has a more prominent role under resting conditions than that found in the lung due to the proportionally larger number of alveolar macrophages and higher production of chemokines by this cell type than by endothelium. The proportions will also vary according to the state of the tissue – whether it is responding to injury or infection or is in the process of an allergic response to an allergen. Under resting conditions, I would expect the chemokines produced by tissue cells and endothelium to dominate, but during an inflammatory response, the leukocytes would probably play

a more prominent role. Tissue cells and endothelia become stimulated on production of cytokines by antigen presenting cells such as langerhans cells in the skin and higher chemokine levels are induced, recruiting leukocytes, which in turn produce more cytokines and chemokines aiding further recruitment.

Chemokine production upon cytokine stimulation is dose dependent – at low doses, the cells are not activated, but at high concentrations, the cells become desensitised to the cytokine and also are not activated. There is an optimum cytokine concentration range for stimulation of chemokine production, which can vary according to cell type. IFN-γ and TGF-β are immunomodulatory cytokines, sometimes enhancing and sometimes reducing chemokine production. In this way, optimal chemokine concentrations to recruit leukocytes can be maintained, as if concentrations become too high, leukocytes could be activated in the 'wrong' place (before they have reached the site of infection) and cause more damage.

In all of the studies reported here, we have used *in vitro* culture systems. In culture, there are necessarily differences from the *in vivo* state. There are several measures that can be taken to try to increase the similarities *in vitro* to those *in vivo*, but there are still several points that need considering.

We use gelatin to provide a substrate in all of our assays to simulate the basement membrane and ECM *in vivo*. During normal culture, growth factors are present in the different media. As they would *in vivo*, the cells respond by proliferating, and as they do so, they lay down their own basement membrane, they are also contact inhibited and express many other characteristics of the primary endothelium. The distinctive phenotype of endothelium is gradually lost over many passages in culture, so that the sooner the cells are used after being placed into culture, the closer they will be to the *in vivo* situation. As we were investigating differences between endothelia, we need to use the cells before they lose their distinctive phenotype, and also before there is any potential loss of heterogeneity. For this reason, we have used our own primary cells at passages 2 – 5 while those isolated by Clonetics which we receive at passage 4 are used between passage 5 and 7. However, several components of the microenvironment in culture are missing from our *in vitro* systems; one is the element of shear force, which has been discussed in relation to chemokine expression already, another is the contribution of other cells, plasma, ECM and other molecules that would normally be found surrounding the endothelium *in vivo*. The endothelium can also be activated in culture, particularly in the presence of growth factors, so to try to overcome

this, we have rested the cells for 24 hours at the start of each of our assays in medium lacking growth factors. Although *in vitro* systems are not ideal, they are useful tools to investigate endothelial cells and have indeed provided great insights into their functions.

Primary bone marrow endothelium is unstable in culture due to the differences between culture conditions and those found *in vivo*. Cytokines play an important role in the bone marrow microenvironment and this is difficult to simulate *in vivo*, but it appears that the cytokine stimulated endothelium is likely to be closer to that found *in vivo* than the resting BMEC. This issue is made slightly easier by the production of the bone marrow cell line that we have used as this stabilises the phenotype, and although as yet the transformation process has not been found to have affected the phenotype significantly, this is still a possibility.

The liver endothelium were also found to de-differentiate at higher passages (personal communication A. Linke), but at the passage used here, they maintain much of the phenotype of sinusoidal endothelium.

In this final section, we will explore how our results relate to the *in vivo* situation in each of the different vascular beds that our endothelial cells are taken from.

Table 8.1 Summary of important findings

ND = Not done, - = nothing detected

	BMEC	SVEC	DMVEC	LMVEC	HUVEC	LIVER EC
Constitutive chemokines produced	none	none	IL-8 > MCP-1	IL-8 > MCP-1	IL-8 > MCP-1	IL-8
Chemokines produced after TNF-α stimulation	MCP-1 > IL-8 > RANTES > IP-10	IL-8 > MCP-1 > RANTES	IL-8 > RANTES > MCP-1 > IP-10	IL-8 > MCP-1 > RANTES > IP-10	IL-8 > MCP-1 > RANTES	IL-8 > MCP-1 > RANTES
Chemokines produced after IFN-γ stimulation	IP-10 > MCP-1	MCP-1 > IP-10	IP-10 > IL-8 = MCP-1	IP-10 > MCP-1 > IL-8	MCP-1 > IL-8 > IP-10	IL-8 = IP-10
Effect of TGF-β on constitutive chemokine expression	-	↑ IL-8, MCP-1	↓ IL-8 & MCP-1	↓ IL-8 & MCP-1	None on IL-8, small ↓ in MCP-1	ND
Effect of TGF-β on TNF-α stimulated chemokine expression	↓ IL-8, MCP-1, IP-10	↑ IL-8, ↓ RANTES,	↓ IL-8, RANTES, IP-10	↓ IL-8, MCP-1, IP-10	↓ IL-8 ↑ RANTES	↓ IL-8, MCP-1
Do TNF-α & IFN-γ act in synergy?	RANTES & IP-10	RANTES, MCP-1 & IP-10	RANTES & IP-10	RANTES & IP-10	RANTES, MCP-1 & IP-10	RANTES & IP-10
Effect of TGF-β on IFN-γ stimulated chemokine expression	↑ MCP-1 ↓ IP-10	↑ IP-10	v. little effect	↓ IL-8, MCP-1, IP-10	↑ MCP-1, IP-10	↓ IL-8, IP-10
Which chemokines bind to the cell surface?	MIP-1α & RANTES	RANTES > MIP-1α	MIP-1α > RANTES	MIP-1α > RANTES > IP-10	MIP-1α > RANTES	ND
Chemokine receptors expressed on the cell surface	CXCR1, 3, CCR3, variable CCR4	CXCR1, 3, 4, 5, CCR3, variable CCR4, low CXCR2 & CCR6	CXCR1, 3, CCR3, variable CCR4, low CCR6	CXCR1, 3, variable CCR4, low CCR6	CXCR1, 3, 4, CCR3, 4, low CXCR5	ND
Proliferation enhancers	ND	Gro-α, eotaxin	Gro-α	Gro-α	-	ND
Proliferation inhibitors	ND	-	IP-10	IL-8, MCP-1	TGF-β	ND

8.2 HUVEC

Umbilical vein endothelium is often used as a standard endothelial cell type, however, in this study we have found that it produces high constitutive levels of IL-8 and MCP-1 in comparison to the other endothelial cell types tested. It responds highly to TNF-α by producing IL-8 at approx. 10 times the constitutive level and MCP-1 at approx. twice the constitutive level. It does not produce IP-10 in response to TNF-α and in this respect is also different to other endothelial cell types tested here. IFN-γ stimulation reduces constitutive levels of IL-8 and induces IP-10 production. TGF-β does not reduce constitutive IL-8 production, but does reduce MCP-1 production. The addition of TNF-α and IFN-γ in combination reduce the high levels of IL-8 observed with TNF-α stimulation alone, but act in synergy to produce high levels of RANTES, MCP-1 and IP-10. TGF-β and TNF-α acting together show similar effects on RANTES, IL-8 and MCP-1 production as those observed with TNF-α and IFN-γ, but to a lesser extent. Two interesting results are the production levels of MCP-1 and IP-10 observed after TGF-β and IFN-γ stimulation together. It appears that TGF-β and IFN-γ act in synergy to produce levels higher than those observed for each cytokine individually.

HUVEC have been found to express CXCR1, 3, 4, CCR3, 4 and low levels of CXCR5. This suggests that they could respond to several of the chemokines that we have been examining including IL-8, RANTES and IP-10. We showed that MIP-1α bound well to the endothelial cell surface, while RANTES bound to a lesser extent. We have not shown significant levels of the MIP-1α receptors CCR1 or CCR5 present on HUVECs, although different groups have found low levels of mRNA for CCR1 and 5 present (Feil and Augustin 1998; Gupta *et al.* 1998; Weber *et al.* 1999a). It is possible that MIP-1α was binding via either of these specific receptors which were present at a low level as digestion of the proteoglycan layer had little effect and MIP-1α was not internalised during the time course of the experiment.

The umbilical vein EC have proven extremely useful as a tool to investigate endothelial function, however they are derived from a transport vessel for waste from the placenta and foetus. It does have to be remembered that it is a large vessel and would not normally be the site of leukocyte recruitment. The microvascular endothelium is where leukocyte migration normally occurs and since it is becoming easier to isolate, will be a better model. However, the constitutive expression of IL-8 and MCP-1 does suggest that there could be recirculation of neutrophils and monocytes

occurring and the well-documented responses of HUVEC to cytokines show that recruitment could occur *in vivo* if necessary.

8.3 Bone marrow endothelium

Bone marrow endothelium is situated at the interface between the stroma and the blood and regulates the transport of naïve and mature blood cells into the circulation. This is not normally an inflammatory site, but is concerned with the normal recirculation of leukocytes. We have shown that bone marrow endothelium does not constitutively produce any chemokines, however, a high level of MCP-1 is produced after TNF-α stimulation. IL-8, RANTES and IP-10 are also produced and unusually, bone marrow endothelium appears to produce IP-10 more in response to TNF-α than in response to IFN-γ. The endothelial cell line that we are using constitutively expresses low levels of adhesion molecules, but it is unknown as yet whether it expresses cytokines as primary bone marrow EC do. Primary BMEC constitutively express over 10 different cytokines including G-CSF, GM-CSF, TGF-β, IL-1, IL-6 and IL-11 (Candal *et al.* 1996; Li *et al.* 2000), this causes increased levels of adhesion molecules to be expressed. When IFN-γ is added in combination with TNF-α, a reduction in IL-8 and MCP-1 levels are seen, but a massive increase in the IP-10 level is observed. TGF-β reduces the production of IL-8, MCP-1 and IP-10 from the high levels observed after TNF-α stimulation, but has little effect on RANTES expression. Like all the other endothelia tested, synergy between TNF-α and IFN-γ causes both RANTES and IP-10 to be expressed very highly. BMEC have been shown to bind MIP-1α and RANTES to the cell surface and we have shown that RANTES binds via HSPG, and possibly by a second mechanism, whereas we have not been able to show how MIP-1α binds. Study of receptors detected on the endothelial cell surface show no expression of either of the MIP-1α specific receptors, CCR1 or CCR5. The receptors for RANTES are CCR1, CCR3 and CCR5. We have shown high levels of expression of CCR3 on the cell surface, this receptor has lower affinity for RANTES than for eotaxin, but this is a second possible mechanism by which RANTES might bind.

8.3.1 Implications *in vivo*

There is only a little known about the effects of chemokines on bone marrow endothelial cells, because as yet, only a few chemokines have been investigated. SDF-1 is known to be constitutively expressed in the bone marrow stroma; it is thought to be produced by endothelial

cells, but as yet this has not been demonstrated. CXCR4 is known to be required for survival as CXCR4 knockout mice have been found to die *in utero* due to the lack of development of B cells, but T cell development is not affected (Ma *et al.* 1998; Zou *et al.* 1998). We have shown a very low level of CXCR4 expression by BMEC. CXCR4 and its interaction with SDF-1 is important in the homing of haematopoietic progenitor cells to the bone marrow (Mohle *et al.* 2001; Lapidot and Petit 2002). SDF-1 has been shown to be associated with endothelium within the bone marrow and was recently shown to bind to heparan sulphate and dermatan sulphate on the proteoglycan layer (Netelenbos *et al.* 2001). CXCR4 was shown to be expressed on CD34+ progenitor cells and through binding to proteoglycans can induce their migration (Netelenbos *et al.* 2002). There is a lot of information available on the adhesion molecules expressed by bone marrow endothelium and transmigration appears to utilise the constitutively expressed E-selectin and VCAM-1 (Voermans *et al.* 2000). SDF-1 has been shown to activate integrins after immobilisation on HSPG and induce firm adhesion to ICAM-1 and VCAM-1. This process of SDF-1 mediated adhesion has been shown to be increased after the application of IL-1 to the medium. Despite the high levels of binding exhibited by MIP-1α to BMEC in our studies, it has been found to be irrelevant to the homing of progenitor cells, but is more important in other organs during inflammatory reactions (Cook 1996). There have been some differences in the findings, which address the expression of cytokines by BMEC. So far there is little information on this particular cell line, however other groups have shown that both TGF-β and IFN-γ can be constitutively expressed by bone marrow endothelial cell lines (Li *et al.* 2000).

IP-10 appears to play a role in the regulation of growth of haematopoietic progenitor cells in bone marrow. It inhibits colony formation in the presence of certain growth factors such as SCF and GM-CSF. Some studies suggest that IFN-γ is consistently produced by BMEC, this could continuously stimulate IP-10 production and act as a negative regulator of colony formation (Sarris *et al.* 1993).

In vitro assays performed using mouse cell lines have indicated that the MCP-1 and CCR2 interaction is important in recruitment of multiple myeloma cells to the bone marrow. In this disease, the bone marrow is heavily infiltrated with these monoclonal plasma cells, which gradually replace the normal haematopoietic cells present, inducing anaemia and reducing the numbers of circulating blood cells, bone absorption also occurs and the patient cannot react properly to infection (Vanderkerken *et al.* 2002).

8.4 Saphenous vein endothelium

Saphenous vein endothelium is isolated from the large vessels of the leg, in this study we have shown that very low levels of MCP-1 are constitutively produced by these ECs *in vitro*. IL-8 is the major chemokine produced upon TNF-α stimulation, this is in common with all other endothelia tested except bone marrow. After TNF-α stimulation, MCP-1 and RANTES are also upregulated, but only to a low level. When IFN-γ is added in combination with TNF-α, a large increase in IP-10, RANTES and MCP-1 production is observed, but IL-8 production is reduced from the levels shown by TNF-α alone. TGF-β increases all the IL-8 and MCP-1 from constitutive levels and increases IL-8 from the high levels observed after TNF-α stimulation. TGF-β also increases IFN-γ stimulated production of IP-10.

SVEC is different from all the other endothelial cell types in its chemokine binding to the cell surface. We have shown that RANTES binds highly to SVEC, while MIP-1α binds to a lesser extent. RANTES is important in the attraction of eosinophils, memory T cells and monocytes to sites of inflammation via its binding of CCR1, CCR5 and to a lesser extent CCR3.

Many chemokine receptors are expressed on the surface of SVECs, (see summary in table 8.1) suggesting that many different inputs could be integrated. We have shown that two of these receptors could be involved in the process of angiogenesis as both Gro-α (CXCR2) and eotaxin (CCR3) have significant proliferative effects. It is possible that if conditions were optimised, we might demonstrate similar or opposite effects from other chemokines.

8.4.1 Implications *in vivo*

There is very little in the way of specific data on SVEC, they have been used as a model for large vessel endothelium, and have sometimes been used interchangeably with HUVECs. From the data in this comparative study, however, it can be seen that there are several differences. One important difference is the lack of IP-10 production by HUVEC after TNF-α stimulation as well as the high constitutive secretion of MCP-1 and IL-8 by HUVECs. The effects of TGF-β are also different, TGF-β appears to be pro-inflammatory in SVEC as well as HUVEC. The chemokine receptor expression patterns of the two EC types are similar, with the exception of CCR6 which was found on SVEC (see table 8.1). CCR6 was not previously shown to be expressed on endothelial cells, but is known to bind LARC (also known as MIP-3β or exodus-1), which is mainly expressed in the liver and is an

attractant for immature dendritic ceils, Th2 cells and NK cells (Baba *et al.* 1997; Greaves *et al.* 1997).

8.5 Dermal endothelium

DMVEC, LMVEC and HUVEC show many similarities in this study, but the differences between them are particularly interesting. All three constitutively express both MCP-1 and IL-8, with IL-8 at a higher level at the time points investigated. All three express IL-8 as their major chemokine after TNF-α stimulation. The microvascular cells produce IP-10 highly after IFN-γ stimulation, which HUVECs do not, TGF-β also reduces constitutive expression of MCP-1 across all three endothelial cell types, but it is only in the microvascular endothelia that IL-8 is decreased by TGF-β stimulation. One of the most interesting differences about DMVEC is the high production of RANTES in comparison to the other endothelial cell types after TNF-α stimulation. In common with all other endothelial cell types tested, both IP-10 and RANTES are produced in a synergistic manner after TNF-α and IFN-γ stimulation. TGF-β has anti-inflammatory effects – reducing the production of all chemokines tested. Aiso interesting is the fact that MIP-1α was shown to bind more to the endothelial cell surface than RANTES, despite the high levels of RANTES detected in the supernatants of endothelial cells. This suggests that perhaps it is produced within the tissues from tissue cells and / or infiltrating cells and is used to recruit more cells from the circulation. RANTES was shown to bind as well to collagen as it did to HSPG and shows low binding to laminin and fibronectin, the ECM is a particularly important component of the dermal layer of the skin. DMVEC show CXCR1 and 3 and CCR4 on their cell surface in common with all other endothelial types tested. They also express CCR3, which is shown by all the endothelial cell types except LMVEC and CCR6 at a low level in common with LMVEC and SVEC. As would be predicted from studies on HUVEC (Luster *et al.* 1995; Fujisawa *et al.* 1999), DMVEC are proliferative in the presence of Gro-α and proliferation is reduced in the presence of IP-10. Since Gro-α only binds via CXCR2 and there is virtually no expression of CXCR2 on the DMVEC cell surface, it appears that only a few CXCR2 receptors could be enough to stimulate proliferation, however, there is a slight possibility that it couid be due to differences between individuals. This is a somewhat intriguing result that deserves further study as cultures for proliferation assays actually had less serum in than those for chemokine receptor expression, which could have been an activating factor.

8.5.1 Implications *in vivo*

In vivo, the endothelium in the skin is fundamental to the processes of wound healing and also in allergic reactions and inflammatory skin conditions such as psoriasis.

The process of wound healing is complicated, in particular in relation to chemokine expression and actions. There is still much to be investigated, but some chemokines have been shown to be important in this process (reviewed in Gillitzer and Goebeler 2001). Dermal endothelium has been shown to be a source of several of these chemokines (Goebeler *et al.* 1997). Neutrophils are the first cell type recruited and it appears that several different CXC chemokines are involved such as ENA-78, Gro-α and NAP-2. This acute stage of inflammation in skin appears to use CXCR2 to chemoattract neutrophils to the wound site followed by CXCR1 interactions with IL-8 to activate the respiratory burst. MCP-1 is expressed during the first week of wounding in humans, but in animal models, RANTES and MIP-1α are also important at this stage. These chemokines attract macrophages and lymphocytes to the wound site. In humans, IP-10 and Mig production stimulated by IFN-γ after day 4 after wounding are aiso important in the accumulation of lymphocytes. From days 1-4, the endothelial cells react to growth factors such as VEGF and angiogenic chemokines such as Gro-α and IL-8 to promote the revascularisation of the tissue. It is not yet known whether CXCR3 interactions with IP-10, I-TAC or Mig have any effects in wound healing, although their addition to wounds has been shown to disrupt neo-vascularisation (Luster *et al.* 1998). TGF-β appears to play an important role in the later stages of angiogenic processes as it seems to be involved in tubule formation, but it is anti-proliferative (Heimark *et al.* 1986; Pepper *et al.* 1993; Chen and Manning 1996). In our studies, we have also shown that it reduces chemokine production by the dermal endothelial cells, also mice lacking TGF-β show massively increased inflammatory infiltrates leading to organ failure (Shull *et al.* 1992). Hence it would appear that the modulation of chemokine production by TGF-β could an important regulator of leukocyte recruitment leading to healing of the wound and returning to 'normal'.

We have shown CCR6 expression on DMVEC, this receptor is usually expressed on Langerhans cells and its interaction with LARC directs their constitutive homing to the epidermis (Charbonnier *et al.* 1999). These cells are antigen presenting cells and are some of the first cells that antigens come into contact with.

Psoriasis is a chronic inflammatory skin condition associated with too much growth of epidermal cells causing scaling and redness. Large quantities of IL-8, activated neutrophils and T cells are present in this condition. CXCR1 and CXCR2 have both been found to be upregulated in the epidermis and drugs, which treat this condition, tend to reduce expression levels of IL-8 receptors.

Hypersensitivity reactions in the skin rely on T helper cells. Th1 cells express CXCR3 and CCR5 while Th2 cells express CCR3 and CCR4. In a normal delayed type hypersensitivity (DTH) reaction in the skin, Th1 are the predominant cell type, but in atopic individuals Th2 is the predominant cell type causing chronic inflammation (Wierenga *et al.* 1990; Romagnani 2002).

In DTH reactions such as contact dermatitis, TNF-α and IFN-γ released by Langerhans cells increase chemokine production as we have shown in DMVEC. This then attracts macrophages, and T cells. In normal individuals, Th1 cells dominate, but in an allergic individual, this is polarised towards Th2 and encourages the recruitment of eosinophils, basophils and the release of histamine and other vasoactive and inflammatory mediators and chemotactic factors. Mast cells are also thought to be important in this type of reaction and RANTES seems to cause their accumulation (Conti *et al.* 1997). The type of cellular infiltrate and disease progression are dependent on the relative levels of stimulation and inhibition of chemokine production by various cytokines produced by eosinophils, basophils, mast cells, macrophages, T cells and endothelium.

RANTES is important in both Th1 and Th2 dominated reactions as it is the only chemokine that can recruit both cell types – Th1 via CCR5, and Th2 via CCR3. We have shown high levels of RANTES produced by DMVEC, and it appears that these cells also could respond to RANTES via CCR3.

8.6 Lung endothelium

IL-8 and MCP-1 are constitutively expressed by LMVEC in the same way as DMVEC and HUVEC. IL-8 is also the major chemokine expressed after TNF-α stimulation. There are however some interesting differences between lung endothelium and the other endothelia tested in this study. MCP-1 is highly expressed in LMVEC in comparison with DMVEC. It is also the highest expressor of IP-10 after IFN-γ stimulation, however the synergistic effect of TNF-α and IFN-γ in this cell type appears to be maximal at 24 hours, and has not increased in the second 24 hours. TGF-β showed reduction of chemokine production after both TNF-α and IFN-γ stimulations; this is in common with SVEC, but not any other endothelial cell type. Two other interesting findings were that LMVEC

bound IP-10 to the cell surface as well as RANTES and MIP-1α and that there was virtually no expression of CCR3 by this cell type. There was expression of CXCR1, 3 and CCR4 as in all the other EC types tested, and low levels of CCR6 were also demonstrated. This endothelial cell type will respond to a range of different chemokines, including IL-8, IP-10, ITAC, Mig, TARC, MDC and LARC, but there are only low levels of receptors on its surface to respond to RANTES, MCP-1, MIP-1α, Gro-α or eotaxin. Despite this, it does appear to respond to Gro-α and to MCP-1, suggesting that maybe these receptors are present at levels below the detection limit of our antibodies. Proliferation was found to occur in response to Gro-α and an anti-proliferative effect was observed in response to MCP-1.

8.6.1 Implications *in vivo*

During acute lung injury, neutrophils are recruited followed by macrophages and lymphocytes in a similar way to wound healing in skin. IL-8 is the major neutrophil chemoattractant found in the lung and its levels are increased after damage. In the lung, the endothelium is not the major producer of chemokines and cytokines, there are many alveolar macrophages, which produce many different mediators and the smooth muscle cells, epithelial cells and infiltrating T cells also show some involvement (Gutierrez-Ramos *et al.* 2000).

Many of the cell types and chemokines found in allergic disease in the skin are also found in the lung. Asthma is a chronic inflammatory disease of the bronchioles, characterised by mononuclear cell, eosinophil and mast cell infiltration of the submucosa and sub epithelial fibrosis. It can be allergic or viral in origin and chemokines are involved in tightly controlled patterns. From the many different animal models of asthma and what can be discovered *in vitro*, much is now known about this disorder, however, there is still a lot which is unknown.

The first cells involved are mast cells and basophils, these are recruited by the MCPs, RANTES, MIP-1α and eotaxin and can be activated to produce other mediators including histamine. Th2 cells are the major helper cell type and are recruited by eotaxin and MDC via CCR3 and 4 on their ceil surface. We have not investigated the expression of these chemokine by endothelia, so we cannot determine whether there is any involvement of the endothelium in this recruitment. Th2 cells produce IL-4 and IL-5. IL-4 activates cells or potentiates effects of other cytokines on various leukocytes, while IL-5 is thought to be involved in the mobilisation of eosinophils from the bone marrow. MIP-1α and eotaxin cause chemotaxis of eosinophils into the lung, these then accumulate

and cause inflammation and damage by release of toxic proteins and lipid mediators. This leads to smooth muscle cell contraction, hyper-reactivity of the airway, increased vascular permeability and increased mucous secretion – the classical manifestations of asthma leading to shortness of breath. They also lead to further accumulation of leukocytes.

Throughout the disease progression, RANTES MIP-1α and the MCPs are present and appear to increase inflammation in a non-specific manner, while the other chemokines mentioned above appear to have defined roles (Ying *et al.* 1997; Gonzalo *et al.* 1998; Taha *et al.* 1999; Ying *et al.* 1999; Lloyd 2002). Macrophages act to amplify the response and also release further mediators.

8.7 Liver endothelium

The liver endothelium produces only low constitutive levels of IL-8, but is a high producer once stimulated with TNF-α. IFN-γ reduces the high levels of IL-8 seen after TNF-α stimulation alone as does TGF-β. RANTES and IP-10 are only produced to any physiologically significant extent after stimulation with both TNF-α and IFN-γ together. In comparison to IL-8, the levels of MCP-1 expressed by this cell type are low (approx. one sixth). The addition of TGF-β to cytokine stimulated liver endothelium reduced the production of chemokines.

8.7.1 Implications *in vivo*

In vivo, large numbers of lymphocytes and macrophages are positioned in the liver to protect it from foreign antigens that may arrive in the portal blood from the gastro-intestinal system. This resident population can be rapidly expanded during inflammation, hence the chemokines invoived in trafficking of lymphocytes and macrophages are most important for this endothelium. The lymphocytes found in this tissue include NK and NKT cells as well as CD4+ and CD8+ T cells. The hepatic vasculature is complex, but the type we are concerned with here is from the sinusoids. These endothelial cells are discontinuous and express ICAM-1 and PECAM on their surface at rest.

An inflammatory response in the liver can be activated to recruit lymphocytes in several different conditions, including hepatitis viruses and alcohol damage, but where the lymphocytes localise depends on the type of infection. Hepatitis virus inflammation occurs in the parenchyma, while other infections can localise to the biliary ducts.

Little chemokine has been detected on sinusoidal endothelium in comparison to other endothelial types within the liver, low levels of IP-10, Mig and ITAC can be found, which increase after stimulation. These chemokines selectively recruit activated T cells. Th1 cells express high levels of CXCR3 and are associated with inflammation in the liver (Shields *et al.* 1999; Lalor *et al.* 2002).

IL-8, MCP-1, MIP-1α and RANTES are usually associated with portal tract inflammation, but not sinusoidal inflammation. The cells we have been given show many of the features of sinusoidal endothelium, but it is possible that there are contaminating cells or that there has been de-differentiation in culture, a phenomenon that was common with BMEC. Hence the production of cell lines and our use of a BMEC cell line in this study.

8.8 Possible therapeutic targets

Leukocyte accumulation in inflammation is an essential part of host defence. In non-infectious disorders leukocyte accumulation could be an unwanted effect, leading to impaired organ function. Chemokines are beneficial in wound healing and in the resolution of infections, however, continuous expression causes leukocyte accumulation and activation including the release of potentially harmful levels of enzymes and oxygen radicals. In many disorders of allergic and chronic inflammation, such as arthritis, asthma, atherosclerosis and dermatitis, chemokines are expressed at increased levels. These chemokines are the focus of much research as blocking some or all of their actions can have beneficial effects by reducing the leukocyte infiltration and hence the damage caused. There are two potential sites at which interference could be targeted, one is in the interaction between the chemokine and its specific receptor, this uses the N-terminal domain of the chemokine for signalling and in CXC chemokines the ELR motif too. Blocking this interaction can block the signalling pathways and hence chemotaxis and mediator release. The other site where there is potential for therapeutic agents to act is in the interaction between the proteoglycan layer and the heparin binding site of the chemokine at the C-terminus (Witt and Lander 1994; McFadden and Kelvin 1997).

8.8.1 Blocking receptor binding

There have been several encouraging studies showing that neutralising antibodies to chemokines and chemokine receptors can be used to decrease or block recruitment and activation of leukocytes. For example; a monoclonal antibody to IL-8 was shown to prevent neutrophil

accumulation and tissue damage in lung reperfusion injury in rabbits (Sekido *et al.* 1993). Similar effects have also been observed in models of acute glomerulonephritis, dermatitis and arthritis (Harada *et al.* 1993; Wada *et al.* 1994). Antibodies to MCP-1 reduced recruited monocytes in glomerulonephritis too (Wada *et al.* 1996).

Another method of blocking the receptors is to use mutated chemokines that no longer signal. By truncating the N-terminus, chemokines can still bind to the proteoglycan layer, but cannot signal via the specific chemokine receptor. For example; the deletion of five amino acids from the N-terminus of IL-8, if used at a sufficiently high concentration can block enzyme release by neutrophils (Moser *et al.* 1993), similarly truncation of MCP-1 reduces monocyte chemotaxis and other MCP-1 mediated functions (Zhang *et al.* 1994; Gong and Clark-Lewis 1995).

Perhaps the chemokine that has attracted the most attention is RANTES since it was discovered that one of its receptors, CCR5, is a co-receptor for HIV-1 (Alkhatib *et al.* 1996; Deng *et al.* 1996; Dragic *et al.* 1996). An N-terminal truncated form of RANTES has been found to prevent HIV-1 infection by blocking viral entry via CCR5, and CCR5 knockout mice show reduced infection by HIV. In response to these findings, several different companies are now using high throughput screening to find small molecule antagonists for both CCR5 and CXCR4 (another HIV co-receptor) that could help in the treatment of HIV.

So far, there have been several different small molecule antagonists identified that have specificities against chemokine receptors. One, called SB225002 is specifically inhibitory towards CXCR2, but not CXCR1, hence it affects Gro-α and angiogenesis, but doesn't have much effect on IL-8 as this chemokine can still bind via CXCR1 (White *et al.* 1998).

Small molecule antagonists to CCR1 could prove useful in the therapy of patients with rheumatoid arthritis and multiple sclerosis as these disorders are characterised by increased levels of MIP-1α and RANTES, followed by macrophage infiltration. A combination of neutralising antibodies to MIP-1α, RANTES and IL-8 was found to significantly reduce leukocyte infiltration into the synovium in animal models of rheumatoid arthritis (al-Mughales *et al.* 1996). Met-RANTES, a mutated form of RANTES was found to delay the onset and severity of a collagen induced arthritis model (Plater-Zyberk *et al.* 1997). Anti MIP-1α was found to have beneficial effects in the EAE model of multiple sclerosis, as this treatment reduced the number and severity of relapses (Karpus *et al.* 1995).

Increased levels of MCP-1 and monocyte infiltration have been found in several disorders including rheumatoid arthritis, glomerulonephritis, asthma, stroke and atherosclerotic lesions. The use of anti MCP-1 antibody showed reduction in an animal model of inflammation and fibrosis that characterises glomerulonephritis (Lloyd *et al.* 1997). It is hypothesised that a small molecule antagonist of CCR2, might be useful in the resolution of this disease.

In asthma, mast cells are triggered to degranulate and release constrictor agents followed a few hours later by an increase in levels of eosinophils recruited to the peribronchial region of the airway. Their activation is implicated in much of the pathogenesis of this disorder as described earlier. CCR3 is found solely on eosinophils, Th2 cells and basophils, this receptor binds eotaxin strongly, but also binds at a lower level to RANTES and MCP-3. CCR1 is also present on the surface of eosinophils, and via this receptor, MIP-1α, RANTES and MCP-3 also bind. *In vivo*, depletion of MIP-1α by neutralising antibodies decreases the amount of eosinophils recruited by 50%, while anti RANTES antibody reduces it by 60% (Lukacs *et al.* 1996). Blocking these 2 chemokines, eotaxin or all together, could considerably reduce the pathogenic effects of asthma.

In rheumatoid arthritis, many chemokines are found in the synovium including IL-8, ENA-78, Gro-α, MCP-1, MIP-1α and RANTES. MIP-2 (which is similar to human IL-8) and MIP-1α levels in mouse models were found to parallel the incidence and magnitude of arthritis (Thornton *et al.* 1999). Depletion by neutralising antibodies significantiy reduced the incidence of arthritis.

8.8.2 Blocking the heparin binding site

In a similar way to those described above, molecules can be introduced that can interfere with chemokine binding to the proteoglycan layer. This can be done by the prevention of GAG synthesis (specific for one type of GAG or blanket across all GAGs) or by the prevention of sulphation. Using enzymes (as we have used in chapter 4) the proteoglycan layer can be digested, or the final possibility is that soluble synthetic mimetics of GAGs can be produced that can bind the chemokines in solution and so competitively inhibit their binding to the proteoglycan layer.

Viruses have been found to encode secreted binding proteins that can interact with chemokines, these are non-specific, binding many different chemokines (reviewed in Lalani *et al.* 2000). One recent observation is that *in vivo* eotaxin can be blocked by the presence of vCkBP-II (from

vaccinia virus), a binding protein with specificity for CC chemokines and IL-8 across many species (Alcami *et al.* 1998).

Soluble heparin has in many studies been shown to compete with cell surface GAGs for chemokines, but as yet its potential is limited due to its large size. A smaller molecule, with specificity only for the heparin binding site of the chemokine may have more potential as an *in vivo* therapeutic agent.

Also in a similar way to the blocking of the chemokine receptor binding site, other molecules could act at the heparin binding site on chemokines including anti chemokine antibodies specific for this region of the molecule, small molecule antagonists, and non-signalling chemokine peptides.

8.8.3 Possibilities for the future

Each method has its own distinct advantages and disadvantages, but for penetration deeper into the appropriate tissues, smali molecule antagonists need to be developed. The blocking of specific receptors could inhibit specific sub sets of leukocytes from being recruited, but as there is much redundancy within the chemokine / chemokine receptor system, it is possible that more than one receptor/chemokine interaction will need to be considered in each disease type. An inhibitor of binding to HSPG, could have less specific effects and generally block most recruitment into a specific tissue. From our studies, it has been difficult to determine the specificities of chemokines for distinct carbohydrate groups, but with the technology for determining glycoprotein sequences advancing all the time, it will soon be possible to have a better idea of which chemokines bind best to which residues or combinations of residues.

8.9 Overall conclusions

Our hypotheses stated that:

1. endothelial chemokine production
2. inflammatory chemokine binding to endothelia
3. endothelial chemokine receptor expression
4. proteoglycan layer carbohydrate composition
5. effects of chemokines on endothelia

will all depend on the tissue of origin of the endothelial cells.

We showed significant differences in the production of the inflammatory chemokines IL-8, RANTES, MCP-1 and IP-10 between endothelia under both resting and cytokine stimulated conditions, but MIP-1α was not produced by any endothelia tested.

We also showed that MIP-1α, RANTES and IP-10 have the ability to bind cultured endothelia, but that the binding of these chemokines depends on the tissue of origin of the endothelium. The binding of RANTES could be attributed to binding to HSPG, but MIP-1α appeared to bind via a different mechanism, perhaps to the specific chemokine receptors.

We showed variability in the expression of chemokine receptors CCR1 – 6 and CXCR1 – 5 between endotheiia derived from different tissues although as yet it has not been established what the purpose of many of these receptors may be. There were also differences found in the composition of the proteoglycan layer, though not such significant differences as seen in the production of chemokines or in the binding of chemokines. The final study began to investigate the purpose of the chemokine receptors on the endothelial surface, by examining proliferation of EC in response to different chemokines and cytokines. This study needs to be optimised further before any firm conclusions can be drawn.

Our studies have shown that although HUVEC have been a good model endothelium, there are subtle differences between endothelia from different vascular beds in their interactions with chemokines, which have important consequences in the control of leukocyte migration to different tissues. In other studies, our group has also shown differences in adhesion molecules produced by

these same endothelia. The most significant contribution to differences in recruitment between vascular beds appears to come from the differences in chemokine production, which when combined with chemokines produced by each of the tissues involved will enable recruitment of very distinct sets of leukocytes under inflammatory or allergic conditions. A significant contribution to the diversity of leukocyte recruitment also comes from the variation in binding of chemokines to the endothelial surface with a potential contribution from the smaller differences in adhesion molecules present.

8.10 Future studies

One of the most important points that remain to be elucidated is the responses of endothelial cells to chemokines. The study that was begun in chapter 7 needs optimising for serum levels, chemokine concentrations, assay length and cell density before investigating the effects of chemokines in further detail. This could then lead to further investigations into the process of angiogenesis by investigating whether endothelial cells show differences in their migratory properties. Eariy results in our lab indicate that there are differences for example; SVEC are more migratory towards both IP-10 and IL-8 than DMVEC (personal communication D. Male). A final assay, to investigate the overall angiogenic potential of different chemokines would be an *in vitro* angiogenesis assay in which capillary tubule formation is assessed. This would confirm that it is not simply a growth effect of the endothelial cells. Interesting questions have also arisen about the apparent differential effects of TGF-β on endothelia. In most endothelial cell types, it reduces chemokine production by endothelial cells, but in some cases, it appears to increase the chemokine production. It would be interesting to find if this is related in any way to its proliferative / anti-proliferative properties on different endothelia. It would also be useful to investigate which chemokines can activate which endothelial cells and via which receptors. This could be done using a calcium mobilisation assay with blocking antibodies to different receptors. An assessment of the effects of cytokines and chemokines on the levels of chemokine receptors present on different endothelia would also prove useful in further determining the effects of chemokines produced under inflammatory conditions.

Chapter 9
References

9. References

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