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THE MECHANISMS AND REGULATION OF NEUTROPHIL TRANSENDOTHELIAL MIGRATION

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ABSTRACT

The Mechanisms and Regulation of Neutrophil Transendothelial Migration

Neutrophil migration into tissues is an integral and essential feature of acute inflammation. The neutrophil first must traverse the endothelial barrier, and while the initial adhesive interaction between the intravascular leukocyte and the endothelial surface has been well studied, the subsequent step of migration through the endothelial monolayer is the subject of this thesis.

Methods were developed to observe and quantify neutrophil transendothelial migration, both together with or independently of neutrophil-endothelial adhesion. Two mechanisms of neutrophil transmigration were identified- 1) Transmigration in response to a chemotactic gradient applied across the endothelial monolayer, and 2) neutrophil transmigration in response to endothelium which has been activated by cytokines, in which no exogenous chemoattractant is present. The neutrophil chemotactic cytokine interleukin-8 (IL-8) was found to play a role in both of these mechanisms, insofar as a gradient of IL-8 was a potent inducer of transmigration, and furthermore, endothelial cells activated by cytokines were found to produce IL-8, measured by enzyme linked immunosorbent assay, and IL-8 mRNA, by northern blot. Endothelial cell production of IL-8 was characterised in terms of concentration/response and time course, and inhibition of IL-8 by neutralising antibodies was found to inhibit transmigration through cytokine activated endothelium. Desensitisation of neutrophils to IL-8 and other chemotactic factors confirmed a role for IL-8 in induction of transmigration by activated endothelium, but also suggested other IL-8 independent pathways.

The cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 as well as bacterial lipopolysaccharide have previously been identified as activators of endothelium and induce IL-8 production and transmigration. TNF-α has two receptors on endothelial cells, and TNF-α mutant molecules were used to determine that the major receptof mediating the TNF-α signal is the p55 receptor, but the p75 receptor plays a significant role in enhancing TNF-α potency. Studies in our laboratory identified receptors for interleukin-3 (IL-3) on

activated endothelial cells, and IL-3 was shown to significantly enhance IL-8 production at mRNA and protein levels, as well as induction of neutrophil transmigration, by activated endothelium. Cytokines previously shown to inhibit endothelial adhesion, TGF-β and IL-4, were found also to inhibit production of IL-8 and induction of transmigration by activated endothelium.

Therefore, two mechanisms of neutrophil transmigration were identified, and the role of one of the major mediators of this process, IL-8, was described. Production of IL-8 by endothelial cells was characterised, and factors which regulate its production, both positively and negatively, and therefore regulate neutrophil transmigration itself, were identified.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if applicable if accepted for the award of the degree.

William B. Smith

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ABBREVIATIONS

aa	amino acid
Ab	antibody
BM	basement membrane
BSA	bovine serum albumin
βTG	β thromboglobulin
CD	cluster determinant
CHO	chinese hamster ovary
Con-A	concanavalin-A
CR3	complement receptor 3
CSF	colony stimulating factor(s)
d	day(s)
EC	endothelial cells
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EM	electron microscope
f-MLP	n-formylmethionylleucylphenylalanine
FAb	antigen-binding portion of immunoglobulin
FC	flow cytometry
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
G (protein)	guanine nucleotide binding
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour(s)
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HEV	high endothelial venule
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin

K_D	dissociation constant
LAD-I (II)	Leukocyte adhesion deficiency disease type I (II)
LFA-1	lymphocyte function-associated antigen-1
LPS	(bacterial) lipopolysaccharide
LT	leukotriene
MAb	monoclonal antibody
Mac-1	macrophage antigen-1
MFI	mean fluorescence intensity (flow cytometry)
MGSA	melanoma growth stimulating activity
MHC	major histocompatibility complex
min	minute(s)
NAP(-1)(-2)	neutrophil activating protein
NCS	newborn calf serum
NS	not significant
OD_X	optical density at X nm
PAF	platelet activating factor
PBS	phosphate buffered saline
PECAM	platelet-endothelial cell adhesion molecule
PF4	platelet factor 4
PG	prostaglandin
PHA	phytohemagglutinin
PKC	protein kinase C
PL (C)(A_2)	phospholipase C, A_2
PMA	phorbol myristate acetate
PT	pertussis toxin
R	receptor
RT	room temperature
s	seconds
SD	standard deviation
SEM	standard error of the mean
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule 1
VLA-*n*	very late antigen-*n*
WT	wild type

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Neutrophils in acute inflammation

Neutrophils predominate in the cellular infiltrate at sites of acute inflammation. The role of neutrophils in the inflammatory reaction is complex. They have effector functions such as phagocytosis and killing of micro-organisms, which fulfil the aim of inflammation, *ie.* ridding the body of pathogenic invaders. However, the proteolytic enzymes and toxic oxygen metabolites released for this purpose also cause tissue damage, and lead to tissue breakdown and scarring. Neutrophils are also now known to release classical mediators of inflammation such as eicosanoids (*ie.* prostaglandins and leukotrienes), platelet activating factor (PAF), and even cytokines. Although previously thought to be end stage cells with little protein synthetic capacity, they have recently been shown to be able to synthesise small but significant amounts of the classical pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)[1,2,3] as well as the chemokine interleukin-8 (IL-8)[4,5]. These mediators are not only responsible for some of the classic features of the inflammatory lesion (*ie.* prostaglandins cause vascular dilatation and increased vascular permeability, leading to erythema and swelling) but the cytokines released perpetuate and amplify inflammation by attracting more leukocytes, by mechanisms which will be outlined in this thesis. Therefore, neutrophils at inflammatory sites are not just the end effector of the inflammatory response, but are also actively participating in the development and maintenance of the inflammatory process.

The migration of leukocytes, and their localisation at inflammatory sites, was first observed more than a century ago by Metchnikoff and others. Although the phenomenon of chemotaxis, the guidance of cell migration by chemical gradients, has been used as an explanation for the tissue localisation of leukocytes, it does not fully explain how cells flowing in the bloodstream can stop and migrate across the vascular endothelium into the tissues. The mechanism of leukocyte infiltration into developing inflammatory sites must conform to certain requirements. Infiltration must occur only at the appropriate site and

remain localised. Cells in the circulation clearly cannot target any given area, therefore factors arising locally must determine the capture of cells which pass through the site in the microvasculature. Leukocyte accumulation must be transient, so that inflammation ceases when the inciting stimulus has been controlled. There is also a requirement for selection of appropriate leukocyte types, which may vary with time, *eg*. neutrophils at acute inflammation, eosinophils in allergic inflammation, and mononuclear cells in delayed type hypersensitivity reactions or in chronic inflammation, following the neutrophils. Inappropriate inflammation is responsible for many diseases, which suggests that the above properties become disordered in pathological states. Conversely, disorders of leukocyte migration can lead to immunodeficiency and opportunistic infection. In this chapter, I will review recent concepts of leukocyte adhesion and migration which are relevant to this area, before presenting my own research which highlights the active role of the endothelium in leukocyte transmigration.

1.2 Neutrophil adhesion to endothelium

The interface or barrier between blood and tissue is the vascular endothelium, and before migrating across this barrier, it is clear that the neutrophil must first be immobilised from the flow of blood, and adhere to the endothelial surface. Normally circulating leukocytes and endothelial cells are non-adhesive for each other, but this can be regulated in circumstances of inflammation. In fact, it appears that a loose interaction may occur normally, whereby a subset neutrophils at any one time are rolling along the endothelial surface(6). These cells are therefore able to be influenced by signals at the endothelial surface, whereas leukocytes in the centre of the stream of flowing blood cannot be exposed to such control. Rolling interaction probably commences when leukocytes in post capillary venules are displaced from the axial flow by interactions with erythrocytes and erythrocyte aggregates(7), and the velocity of flow reduces as the leukocyte approaches the wall. The definition of a rolling leukocyte is one which is in contact with the vessel wall, whose velocity is well below the flow of blood (*ie*. $<50\mu m/s$ *cf*. $>300\mu m/s$), and therefore clearly impeded by an adhesive interaction with the vessel wall, yet is not stationary(7). Once

rolling, the leukocyte may either be induced to stronger adhesion and immobilised, continue to roll briefly, or detach and continue circulating.

Both neutrophils and endothelial cells may exist in either resting or activated states. Activation can be induced by a variety of stimuli- products of invading micro-organisms, cytokines, coagulation factors, or even hypoxaemia. There are also factors which inhibit activation and tend to maintain the resting state, exerting an homeostatic effect. Activation is not a stereotyped response; different functions may be activated in response to different stimuli. A common result of cell activation is an increase of cell adhesiveness, either to other cells, or to surfaces or matrix proteins. Activation of increased adhesiveness of neutrophils within the circulation, by stimuli which are released into the bloodstream, may lead to their adhesion downstream from the site of release, or indeed may act systemically, and is clearly not going to result in the desired effect of localising cells to the inflammatory site. Stimuli which activate endothelial cell adhesiveness for neutrophils, however, can act locally on endothelium within the tissue bed at the inflammatory site, and can capture neutrophils which are rolling along the endothelial surface, and immobilise them there, prior to their migration. Likewise, the surface display or local release by endothelial cells of factors which activate neutrophil hyperadhesiveness will also act upon the rolling cell, leading it to adhere at that site. Thus it can be seen that the endothelial cell plays a central controlling role in the localisation of leukocytes in inflammation by directing the first step of adhesion.

The ability to culture endothelial cells (EC), developed in the 1970's, enabled studies which revealed specific adhesive interactions between neutrophils and EC, although surface charge effects were initially thought to be the important regulating factors[8]. Increases in the adhesiveness of neutrophils had been noted for many years, and in the early 1980's were attributed to the neutrophil complement receptor CR3 (more recently referred to as CD18/CD11b). A report in 1985[9] was the first description of cytokines increasing EC adhesiveness for neutrophils, as well demonstrating adhesion due to cytokine stimulation of the neutrophil. The mechanism of this was inferred to be the synthesis of a

new EC surface molecule, since adhesion took 4h to develop, and was inhibited by the protein synthesis inhibitor cycloheximide.

1.3 Adhesion molecules

It is now recognised that adhesion between cells is mediated by specific sets of molecules on the cell surface, which are often referred to in terms of receptor and ligand, although both molecules contribute to the adhesive function. Monoclonal antibody (MAb) technology has allowed these molecules to be individually characterised and isolated. Adhesion molecules were found to fall into families based on homology, and have been further classified based on their expression patterns and interactions. Adhesion may be regulated by an increase in the expression of adhesion molecules at the cell surface as a result of new synthesis or the release of preformed stores, or functional activation of previously latent surface molecules.

Although I do not intend to extensively review adhesion molecules, those with an important role in neutrophil-endothelial interaction and transmigration in particular will be introduced.

1.3.1. Integrins

This family of cell surface heterodimeric glycoproteins were the first adhesion molecules to be described (reviewed in(10,11)). Each β-chain may combine with several different α-chains, to form dimeric molecules with different ligand specificity, and different cellular distribution. Most integrins have been shown to bind matrix molecules such as collagen, fibronectin or laminin, and may be involved in either firm adhesion to or migration across these substrates. The leukocyte integrins[(12)] also have major roles in intercellular adhesion. As the name suggests, they are expressed only on cells of myeloid and lymphoid origin. There are three different heterodimers, all of which use the β_2 (CD18) chain (mw 95kD), paired with a unique α (CD11) chain: lymphocyte function-associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1), and p150,95. Further properties of these molecules are described in table 1.1.

Table 1.1 Leukocyte (β_2) integrins- distribution and specificity

Leukocyte integrin	Synonyms-	Cellular distribution-	Ligand(s)-
LFA-1	$\alpha_l\beta_2$ CD11a/CD18	All leukocytes	ICAM-1,2,3
Mac-1	$\alpha_m\beta_2$ CD11b/CD18 CR3	monocyte/macrophages, granulocytes, large granular lymphocytes, immature and CD5$^+$ B cells	C3bi, fibrinogen, factor X, ICAM-1
p150,95	$\alpha_x\beta_2$ CD11c/CD18	as for Mac-1, also activated lymphocytes	Fibrinogen, ?C3bi

Integrin activation

The surface expression of Mac-1 can be quantitatively upregulated after cell stimulation by release of stores in intracellular secretory granules(13,14,15), the membranes of which fuse with the cell plasma membrane. However, the adhesive function of integrins is regulated mainly by functional activation of integrin molecules already expressed on the surface(16,17), probably by molecular conformational alteration. This event can occur rapidly, within seconds to minutes, and is usually transient, lasting minutes to a few hours. Integrin activation follows cell stimulation, and probably results from signals passed to the cytoplasmic part of the molecule from an intracellular signalling cascade (referred to as "inside-out" signalling(11)). Integrins may also be activated through their extracellular portions directly, by an alteration in the extracellular divalent cation milieu(18,19,20) (which may occur physiologically in some circumstances) or as a result of the binding of certain special subsets of antibodies against the integrin molecule(21,22,23,24). These conformational changes result in an increase in the avidity of the molecule for its ligand(25), and the transformation of a non-adhesive to an adhesive cell. Activation of adhesive function may also be related to aggregation of integrin molecules in the plane of the membrane(26).

The pathway of "inside-out" signalling is not known, but may involve phosphorylation of cytoplasmic domains of the integrin molecules. Whilst the leukocyte integrin α-chains

were found to be phosphorylated constitutively[27,28], the β-chain became phosphorylated in response to phorbol myristate-acetate (PMA), and this correlated with adhesion[27,28,29]. Adhesion in response to PMA was inhibited by the protein kinase C (PKC) inhibitor staurosporine[30], confirming that phosphorylation is necessary for activation of (in this case) Mac-1 by PMA. However, activation of Mac-1 also occurs after stimulation of the leukocyte with the chemotactic peptide N-formylmethionylleucylphenylalanine (f-MLP), (albeit with a different time course), which results in only a minimal and transient phosphorylation of β_2. Importantly, this was not inhibited by staurosporine, suggesting two pathways of integrin activation, phosphorylation dependent and independent.

Ligand binding

The ligand binding region of leukocyte integrin molecules appears to be the I (insert) domain of the α chain, so-called because it appears to be a region which is present in only a subset of integrin α-chains (α_1 and α_2 as well as the leukocyte integrins), and is therefore thought to have been inserted during the evolutionary development of these molecules[31]. Evidence for this domain being responsible for binding is provided by mapping epitopes of inhibitory and non-inhibitory MAb, and also by chimæric and mutated molecules, where substitutions in this region interfered most with binding[32]. However, there is other evidence which suggests that integrins may have multiple ligand binding domains, each with different specificity, and in some cases, independently regulated[33].

Leukocyte adhesion deficiency I

The importance of integrins in neutrophil-endothelial interactions and in neutrophil accumulation at inflammatory sites *in-vivo* is demonstrated by a highly pertinent "experiment of nature", in the form of leukocyte adhesion deficiency-I (LAD-I), in which the gene for the β-chain of the leukocyte integrins (CD18) is defective. In this autosomal recessive condition, all of the leukocyte integrins are absent or expressed at a reduced level. Patients with this condition have an elevated neutrophil count in their peripheral blood, but the neutrophils are unable to localise at inflammatory sites. Monocytes and lymphocytes show defective or delayed migration, but may eventually appear in the tissues. These patients are manifestly immunodeficient, and usually die of infection at a young age. The

precise part that integrins play in transendothelial migration of leukocytes will be discussed in subsequent chapters.

Other integrins

In addition to the leukocyte (β_2) integrins, neutrophils bear small amounts of some of the β_1 or very late antigen (VLA) family of integrins, which in general act as matrix receptors-VLA-5 ($\alpha_5\beta_1$) can act as a neutrophil receptor for fibronectin, while VLA-6 ($\alpha_6\beta_1$) mediates adhesion to laminin. Another novel integrin found on neutrophils has been called the "leukocyte response integrin", and has a role in phagocytosis[(34)], although its role in adhesion is not clear. The β_2 integrin VLA-4 ($\alpha_4\beta_1$, CD49d/CD29) is not present on neutrophils, but is expressed by monocytes, lymphocytes and eosinophils. It can act as a receptor for fibronectin, but also plays an important role in the adhesion of leukocytes to endothelium via the inducible endothelial ligand vascular cell adhesion molecule-1 (VCAM-1).

1.3.2 Immunoglobulin gene superfamily adhesion molecules

Intercellular adhesion molecule-1, 2 and 3

Intercellular adhesion molecules (ICAM)-1, 2 and 3 are the ligands or counter-receptors for the leukocyte integrin LFA-1, and ICAM-1 is also a ligand for Mac-1[(35)]. They are all members of the immunoglobulin (Ig) gene superfamily and share close homology, containing 5, 2, and 5 immunoglobulin-like domains respectively[(36,37)]. The domains may have specialised functions, insofar as the binding regions on ICAM-1 for LFA-1 and Mac-1 are distinct (the first and third Ig domains, respectively[(35)]). ICAM-1 and -2 are expressed on endothelial cells and leukocytes, whereas ICAM-3 is expressed on leukocytes only. EC constitutively express ICAM-1 and ICAM-2, but ICAM-1 only is up-regulated after stimulation with TNF-α, IL-1 and bacterial lipopolysaccharide (LPS), through synthesis and expression of new molecules. Interaction between either ICAM-1, 2 or 3 and T lymphocyte LFA-1 can serve as a costimulatory signal for antigen-receptor mediated T cell activation[(38,39)].

Vascular cell adhesion molecule-1

Vascular cell adhesion molecule-1 (VCAM-1) is also a member of the Ig gene superfamily, expressed on cytokine-activated endothelial cells[(40)]. It is a specific ligand for the integrin

VLA-4, which is not present on neutrophils but is on other leukocytes. It plays an important role in the adhesion of monocytes[41], lymphocytes[40,41] and eosinophils[42] to activated endothelium. Interestingly, the cytokine combinations which best induce VCAM-1 on EC are different to those which induce ICAM-1; in particular, IL-4 has a special role in VCAM-1 induction. VCAM-1 is also expressed on some non vascular cells[41] and perivascular cells *ie*. vascular smooth muscle cells (Gamble, JR, submitted).

PECAM (CD31)

PECAM (platelet-endothelial cell adhesion molecule) (CD31), also known as EndoCAM (endothelial cell adhesion molecule) is also of the Ig gene superfamily. The expression of PECAM on EC was first described in 1989[43], although it had earlier been described on leukocytes[44]. It is a 130-140kD transmembrane protein, cloned in 1990[45,46], and has 6 Ig-like domains[45]. PECAM is found in the intercellular junction between EC *in vivo* and *in vitro*[43,47], and is thought to have a role in junction formation, since antibodies prevented initial formation of endothelial cell-cell contacts[47]. PECAM is thought to act in EC by interacting directly with other PECAM molecules on the neighbouring cell. Fibroblasts transfected with PECAM formed PECAM-enriched junctions with EC, whereas PECAM did not concentrate at points of contact between EC and non-transfected fibroblasts[48]. However, PECAM transfected L-cells were able to aggregate with non-transfected cells, suggesting that there is another ligand for PECAM[49]. It appears therefore that PECAM can act by both heterophilic (binding to another different molecule) and homophilic (binding to PECAM on other cells) adhesion mechanisms, and mediate homotypic (adhesion between identical cell types) as well as heterotypic (adhesion between different cell types) interactions. The heterophilic PECAM ligand may be a heparin or chondroitin sulphate type glycosaminoglycan (GAG), although its exact nature has not been defined[50].

The presence of PECAM on leukocytes as well as EC, and its putative propensity for homophilic adhesion, suggest the possibility of a role in leukocyte/endothelial interactions. However, the regulation of PECAM does not indicate any obvious mechanism for this. PECAM expression on neutrophils is not altered by chemotactic stimulation (personal unpublished observations), and it is down-regulated on activated lymphocytes[51]. 4h incubation of EC with TNF-α at reasonable pro-inflammatory concentration did not

significantly alter PECAM expression (personal unpublished observations), but prolonged (48h) incubations with very high concentrations of TNF-α (2000U/ml) and interferon-γ (IFN-γ) (1000U/ml) led to a redistribution of PECAM from the cell junctional concentration to a diffuse surface distribution[52]. Such treatment has previously been shown to increase EC permeability, but whether redistribution of PECAM is a cause or a result of this is not clear. PECAM is subject to phosphorylation on the cytoplasmic domain, which in platelets, results from activation, and leads to its redistribution and association with the cytoskeleton[53]. It is also phosphorylated in T cells after activation, and its phosphorylation is increased from baseline in EC stimulated for 5min with histamine or thrombin (again suggesting a link with monolayer permeability) but not for 4h with TNF-α[51]. Phosphorylation or cell activation has not yet been shown to affect ligand binding activity. However, PECAM can cross-modulate integrin affinity, since antibodies which bind to PECAM can activate β_1 integrin mediated adhesion in lymphocytes[54] and can activate binding of the β_2 integrin LFA-1 in murine lymphokine-activated killer cells[55]. No direct role for PECAM in leukocyte-endothelial adhesion has yet been demonstrated, but it has recently been shown to be required for leukocyte transmigration[56], which will be discussed in a later section.

1.3.3 Selectins

Structural features

E-, P- and L-selectin are cell surface glycoproteins involved in intercellular adhesion, and all have been particularly implicated in the initial rolling interactions of leukocytes on the endothelial surface, as well as their stronger adhesion at inflammatory sites. Each of these molecules consists of three major structural extracellular domains- a number of membrane-proximal complement binding protein-like (CBP) elements, a region with homology to epidermal growth factor (EGF), and a N-terminal C-type (calcium dependent) lectin domain. The lectin domain has a clear adhesive function and indeed selectin ligands are known to feature specific carbohydrate elements. The EGF domain has no direct functional relationship to EGF itself but may interact with the lectin domain to play a role in determining ligand specificity, while the CRP domains have as yet no specific function ascribed to them other than acting as a spacer from the cell surface.

Expression of P and E-selectin

E- and P-selectin are expressed on endothelial cells, and are independently regulated. P-selectin is expressed constitutively on resting EC at low levels. Stores of P-selectin are kept within intracellular rod-shaped vesicles known as Weibel-Palade bodies and are released rapidly to the cell surface after stimulation with thrombin and histamine, with expression peaking in approximately 10-15 minutes and diminishing to background by 90 minutes(57). Exposure of EC to oxygen radicals upregulates P-selectin expression over a longer period of several hours(58). These rather brief periods of up-regulation did not seem commensurate with the demonstrated role of P-selectin in longer term inflammatory events *in-vivo*(59), but recently it has been found in our laboratory that the T cell product IL-3 increases mRNA for P-selectin, and up-regulates its expression over a 24-72 hour period, suggesting a potential role in chronic inflammation (Y. Khew-Goodall *et al.*, manuscript in preparation).

E-selectin is not expressed on resting EC and is not stored. Transcription of the E-selectin gene is induced by stimulation of EC with pro-inflammatory cytokines such as TNF-α, IL-1 and also LPS. It is therefore expressed on the cell surface after 1-2h of stimulation, and peaks at 4-6h. Expression on EC *in vitro* is transient, with a reduction to undetectable levels again at 24-48h. This transience would seem to be out of keeping with the demonstration of E-selectin on EC *in vivo* in inflammatory lesions of many days duration, but it has also been shown that IFN-γ has the effect of prolonging the expression of E-selectin on EC activated by one of the above cytokines(60,61).

Adhesive function of P and E-selectin

Both P- and E-selectin mediate the adhesion of neutrophils, eosinophils and monocytes to endothelium. While E-selectin was shown to bind a subset of circulating lymphocytes (skin-homing memory T cells, defined by expression of the cutaneous lymphocyte-associated antigen (CLA)(62)), P-selectin bound only chronically activated and not fresh resting lymphocytes(63). Initial assays to characterise leukocyte adhesion to endothelium were held under static conditions, but *in vivo*, leukocytes are subject to shear stress forces at the vessel wall from the flow of blood. Recently, assays have been developed which examine adhesion under conditions of flow. These have revealed that the selectins mediate

rolling interactions with leukocytes. Both P- and E-selectin have been shown to mediate leukocyte rolling[64,65], although attachments to P-selectin were looser, leading to more rapid rolling, whilst E-selectin mediates a stronger and more shear-resistant bond, with slow rolling at high E-selectin density, even at high shear stress[65]. Integrin adhesion to ICAM does not occur under conditions of shear stress, unless selectin-mediated rolling occurs first[64].

P-selectin gene deletion

The relevance of selectin mediated rolling in normal physiology and host defence is well illustrated by the lack of this function, in P-selectin gene deletion mice[66]. These animals are normal in development, but leukocytes do not roll in their mesenteric venules, and there is an increase in circulating leukocytes (a part of the "marginated pool" of leukocytes may actually represent those that are rolling on the walls of small vessels[6]). The recruitment of neutrophils to an inflammatory stimulus is delayed, occurring 1-2 hours later than control animals. This is likely to be due to the lack of a rolling pool ready to respond rapidly to migratory signals present at the endothelial surface. The late infiltrate correlates well with the expected induction time of E-selectin, which would normally be responsible for a strong second wave of rolling with subsequent emigration, but in these mice, provides the only such mechanism.

Carbohydrate ligands for P and E-selectin

The ligands for P- and E-selectin on leukocytes have been the subject of intense interest. The lectin portion of the selectin molecules is the active binding domain, and commensurate with this, most evidence suggests that the ligands are carbohydrates. However, the exact carbohydrate structures which are specifically bound by the selectins, and the nature of the molecules which support and present these carbohydrate moieties to the selectins (*ie.* whether glycolipid or protein) has been difficult to determine, and remains controversial. Sialylation is an essential feature of carbohydrate ligands for both P- and E-selectin, since neuraminidase treatment (which de-sialylates cell surface structures) eliminates binding[67]. P-selectin was initially found to bind to leukocyte CD15, which is lacto-*N*-fucopentaose III[68], but the active portion of this was a trisaccharide structure called the Lewis^x (Le^x) antigen, or Galβ1$\rightarrow$4(Fucα1$\rightarrow$3)GlcNAc. Subsequently, it was

shown that the sialylated form (sialyl-Le^x) was a much higher affinity ligand, and this is now known to bind to both P- and E-selectin[69]. Antibodies against sialyl-Le^x, as well as liposomes containing glycolipids bearing this determinant, inhibited binding of HL-60 cells (a myeloid cell line) to E-selectin[70]. At least two other carbohydrate determinants, sialyl-lewis^a (Siaα2$\rightarrow$3Galβ1$\rightarrow$3(Fucα1$\rightarrow$4)GlcNAc)[71] and sulfatide (galactose-4-sulfate ceramide)[72] have been implicated as selectin ligands.

Presentation of the carbohydrate ligands

The small carbohydrate determinants that are bound by the selectins do not exist on the cell surface as isolated entities, but are displayed on glycosylated proteins or glycolipids. Tunicamycin (which inhibits glycosylation of proteins) treatment of cell lines prevented their binding to P-selectin, but had less effect on binding to E-selectin, suggesting that the carbohydrate ligand for P-selectin is borne by a protein. This was confirmed by protease digestion of HL60 cells, which abrogated their binding to P- but not E-selectin. Soluble E-selectin prevented binding of HL60 cells to P-selectin completely, but soluble P-selectin only partially inhibited binding to E-selectin[67]. Taken together, these results indicate that P-selectin binding is restricted to carbohydrates borne by a glycosylated protein(s), whilst E-selectin can bind to the same ligands, but also more broadly, to similar carbohydrate structures present on membrane glycolipids. This may explain the greater avidity and stronger binding seen on E- *vs.* P-selectin[65]. A glycoprotein ligand for P-selectin (GLPS-1) was recently cloned[73], and a 150kD glycoprotein ligand for murine E-selectin has also been isolated[74]. One specific type of glycolipid ligand for E-selectin was recently isolated from neutrophils, and was a terminally sialylated lactosylceramide[75]. Some recent data[76] indicate that eosinophils as well as neutrophils bind to E-selectin via both protein associated and non-protein associated sialyl-Le^x determinants, but suggest that a dimeric form, sialyl-dimeric-Le^x, may be the dominant ligand. Interestingly, although a subset of lymphocytes bind P- and E-selectin, lymphocytes do not bear significant amounts of sialyl-Le^x or sialyl-Le^a. The lymphocyte surface ligand for E-selectin is identified by the MAb HECA-452 and has been named CLA (cutaneous lymphocyte associated antigen) but is not yet fully characterised[77].

Leukocyte adhesion deficiency-II

Patients have recently been reported who lack any expression of sialyl-Lewis^x on leukocytes (LAD-II)[78], providing further information on the *in vivo* ligands for selectins, and the significance of selectin mediated events. The cause is likely to be a defect in fucose metabolism. As in LAD-I, the patients have elevated blood leukocyte counts, and are subject to recurrent pneumonia and other bacterial infections. Their neutrophils do not bind to IL-1 activated EC, and did not roll on endothelium under conditions of shear force, however, under static conditions, neutrophils were seen to adhere in response to chemoattractant stimulation[79]. Unlike LAD-I, the patients do not develop normally, but are mentally retarded with short stature, suggesting a more widespread defect.

Distribution and regulation of L-selectin

L-selectin is distinct from P- and E-selectin in its distribution, since it is borne by leukocytes[80]- the majority of neutrophils, monocytes and B-lymphocytes, subsets of T lymphocytes, and probably also eosinophils[81]. Its regulation is also different, since it is present on resting cells, and rapidly down-regulated by cell activation (*ie.* by cytokines, peptides, PMA or even incubation at 37°C *in vitro*[82,83]) or after cross linking[84] through shedding from the cell surface into the medium. Not surprisingly, soluble L-selectin is detectable in normal serum, and may act as an inhibitor of the function of cell borne L-selectin by competing for its ligands[85]. Regulation of the function of L-selectin occurs immediately after activation and prior to shedding, when it undergoes an increase in affinity for a putative ligand, and increases its adhesiveness[86].

Adhesive function of L-selectin

L-selectin has a role in the adhesion of leukocytes to EC, although again its precise ligand is not clear. It was originally identified (in the mouse, known as MEL-14 Ag) as a lymphocyte homing receptor, directing the migration of lymphocytes by binding to specialised vessels present in lymphoid organs known as high endothelial venules (HEV)[87]. Adhesion of neutrophils and monocytes to cytokine activated endothelium is in part mediated through L-selectin on the leukocytes; this was best demonstrated in assays which incorporated an element of shear stress (*ie.* carried out on an oscillating mixer)[88,89]. In intravital video-microscopy studies of rabbit or rat mesenteric vessels, neutrophil rolling

along the endothelial surface was observed, and this was inhibited by antibodies to L-selectin[90,91] or soluble L-selectin[92]. Like rolling on the endothelial selectins, firm integrin mediated adhesion could not occur if rolling was inhibited. Indeed, neutrophils activated by cytokine or peptide stimulation, which have activated integrins (and are hyperadhesive in static adhesion assays) but have shed L-selectin, show reduced or absent rolling on activated EC[91,93], and are unable to adhere.

Carbohydrate ligands for L-selectin

L-selectin has also been demonstrated to have direct lectin activity, and *in vitro* binds to mannose-6-phosphate, fructose-1-phosphate, sulfatide, and fucosylated polysaccharides[94], and indeed, like P- and E-selectin, also binds sialyl-Le^x[95]. These findings highlight the somewhat artifactual nature of *in vitro* carbohydrate binding assays, since unlike P- and E-selectin, L-selectin (either purified or transfected into CHO cells) does not bind to neutrophils or other leukocytes, which bear ample sialyl-Le^x (Y Khew-Goodall, unpublished observations). Sialylation is also necessary for binding of L-selectin, since it is inhibited by neuraminidase treatment[96].

Presentation of the carbohydrate ligands

It has been inferred that the inducible EC ligand for L-selectin is in fact E-selectin, since antibodies to both of these molecules which inhibit adhesion to endothelium do not show additive effects when combined, suggesting that they operate through the same pathway[97]. One hypothetical mechanism for this might be the binding of carbohydrates by L-selectin, which then presents them for binding to E-selectin[98]. An extension of this might be that L-selectin also binds P-selectin, which provides a mechanism for rolling in non-inflamed vessels, however no evidence yet exists for this (in fact, the binding of P-selectin to PMA-activated neutrophils, which have shed L-selectin, was not diminished[99]). Putative L-selectin ligands would have to meet the requirements of inducibility on activated EC, and/or presence on lymphatic HEV. Two sulfated glycoproteins (of 50 and 90 kd) which specifically bound to L-selectin were isolated from lymph nodes. One of these was found to be a mucin-like molecule, and named GlyCAM-1[100], while the 90 kd molecule was found to be identical to CD34[101]. Whilst GlyCAM-1 is expressed specifically on HEV endothelium, expression of CD34 was not known to be localised to any specific organs[102],

or to be up-regulated by inflammatory mediators[(103)]. It is possible that tissue-specific modification of the glycosylation of CD34 may confer specificity for L-selectin binding. Another L-selectin ligand defined by the MAb MECA-79 (the epitope for which may be present on more than one core molecule, *ie*. GlyCAM-1) has been described to be present at sites of long-standing chronic inflammatory involvement as well as lymphatic HEV[(104)].

Table 1.2 Selectins and their ligands

Selectin	Cell type	Carbohydrate ligand	Protein/lipid ligand	Cell type
P-selectin	Endothelial cells, platelets	Sialyl-Le^x, sialyl-Le^a, sulfatide	PSGL-1	neutrophils, monocytes
E-selectin	Endothelial cells	Sialyl-Le^x, sialyl-Le^a, sialyl-di-Le^x	CLA sialyl-lactosyl-ceramide (glycolipid) 150kD (mouse)	lymphocytes neutrophils
L-selectin	Neutrophils, mono-cytes, eosinophils, lymphocyte subsets	Sialyl-Le^x, mannose-6-PO_4, sulfatide	?E-selectin GlyCAM-1 CD34 MECA-79	activated endothelium HEV all endothelium HEV, chronic inflammation

In summary, a clear role has been established for the selectins in the initial contact and rolling of leukocytes on endothelium. Adhesion of neutrophils to endothelium involves integrin-ICAM interactions. The relationship between rolling, adhesion and the subsequent transmigration of neutrophils, and the roles of the adhesion molecules in transendothelial migration will be discussed in subsequent sections.

1.4 Chemotaxis of neutrophils

After adhering to the endothelial surface, leukocytes migrate through the endothelial monolayer to enter the tissues. This step is likely to involve the process of chemotaxis, which is the directed migration of cells along a chemical concentration gradient. This process has been studied extensively over many years (reviewed(105,106)). Chemotactic migration depends on three separate steps- 1) the ability of cells to sense the presence of a stimulant and the direction of the gradient; 2) transduction of signals from the sensory receptors, in a topographical manner; and 3) effector mechanisms to mediate the mechanical and motile events, which produce cell polarity and locomotion[105]. It is important to distinguish chemotaxis from an increase in random motility, or chemokinesis. Some factors may stimulate locomotion in a non-directional manner, regardless of whether a gradient is present; these factors are said to be purely chemokinetic. Also, factors which clearly have chemotactic activity, when present at a uniform concentration, (*ie.* no gradient), stimulate non-directional locomotion, thus can also have chemokinetic effects.

Gradient sensing

Neutrophils use a spatial mechanism to sense the direction of a gradient (unlike bacteria, which use a temporal mechanism). In order to respond to a gradient of factor, neutrophils must be able to sense a difference in the concentration of the factor between one side of the cell and the other. It has been shown that, in the optimal concentration range, neutrophils are able to respond to a 1% difference in concentration of a chemotactic peptide across their dimensions[107]. Since chemotactic factors interact with neutrophils via specific membrane receptors, differences in receptor occupancy across the cell must be the basis of this discrimination. It follows that the cell should be best able to distinguish differences at the concentration of factor equal to its dissociation constant (K_D), when about 50% of the receptors are bound, and this is indeed the case[105,107]. Optimal concentrations for stimulation of chemokinetic motility in uniform concentrations of factor are somewhat below the K_D[106]. Recycling of receptors, with continuous internalisation and re-expression of fresh receptors is necessary for migration[108].

Chemotaxis receptors and signalling

Neutrophil chemoattractants include bacterial formylated peptides (bacteria initiate protein synthesis with *N*-formyl methionine), lipid mediators such as PAF and leukotriene B_4 (LTB_4), the complement component C5a, and peptides of the chemokine family, *eg*. IL-8. These interact with the neutrophil via surface receptors, which have now been cloned(109,110,111,112) or purified(113) and may be clustered together in the genome(114). All of these are members of the G (guanine nucleotide binding) protein linked family of receptors, which also have the characteristic of seven membrane spanning domains. The initial event on the binding of chemoattractant to its receptor is likely to be the activation of G proteins, which results in the hydrolysis of GTP. This can be blocked by pertussis toxin (PT), which irreversibly ADP ribosylates the G_i protein α-subunit, and inhibits chemotaxis(115). Subsequent events in chemoattractant signal transduction are the activation of phospholipase C (PLC) which hydrolyses membrane phosphatidylinositol biphosphate (PIP_2) releasing inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 results in the release of calcium from intracellular stores and DAG activates protein kinase C (PKC), which phosphorylates (and thereby may activate or deactivate) a number of proteins. Elevation of cytoplasmic calcium activates calcium-calmodulin dependent protein kinases, phospholipase A_2 (PLA_2) and actin binding proteins. PLA_2 releases arachidonic acid from the membrane, which may serve as a substrate for production of LTB_4 and PAF, thereby completing an amplification loop. A frequently measured readout of chemoattractant stimulation is the flux in intracellular calcium concentration.

Some other factors which act as neutrophil chemoattractants (and factors attracting other cell types) do not have receptors similar to the classical chemoattractant receptor family. Transforming growth factor-β (TGF-β) has recently been noted to have potent chemotactic activity for neutrophils(116,117) (although not all studies agree(118)), yet TGF-β receptors do not associate directly with G proteins. Nevertheless, TGF-β does activate G proteins, and chemotaxis to TGF-β is PT inhibitable, yet there is no subsequent phosphoinositide or DAG production, and no calcium flux(119). Chemotaxis to TGF-β, as with other factors, was associated with polymerisation of actin. Therefore, there are alternative transduction pathways in cell locomotion.

Polarisation and locomotion

The response of neutrophils to chemoattractants is initially manifested by polarisation[105], in which the normally rounded and axially symmetrical cell becomes elongated, with specialised functional elements asymmetrically distributed. Polarisation is not gradient dependent, and will occur in uniform solutions of activators. The polarised neutrophil projects a pseudopod (or lamellipodium) which is a thin layer of cytoplasm which excludes organelles but is rich in actin[120]. The mid region contains granules, organelles, and towards the rear, the nucleus. At the opposite pole to the pseudopod is the tail or uropod, which is the rear of the locomoting cell. Locomotion occurs by flow of the cytoplasm and cell contents into the adherent pseudopod, while the uropod retracts and detaches from the substrate. Membrane lipid flows forward with the cell in the direction of locomotion[121]. Adhesion proteins may be transported forward on the dorsal surface of the pseudopod to participate in new surface contacts[122]. Chemotactic peptide receptors are also asymmetrically distributed, with the majority situated in the mid region of the cell, some on the pseudopod, and very few on the uropod[123]. In the presence of a gradient of a chemotaxin, there is a bias (amongst a population) for pseudopod formation, and hence locomotion, towards the higher concentration, whereas chemokinetic migration is directionally random.

Role of the cytoskeleton

The basis for polarisation and motility is the neutrophil cytoskeleton. The polymerisation of actin is involved in extension of the pseudopod[120]. Contacts between the actin meshwork and the substratum, through the membrane via adhesion molecules (probably integrins), anchor the pseudopod. Integrins binding to matrix may signal into the cell, increasing polymerisation of actin. Extension of the pseudopod may lead to tension between the front and the rear of the cell. The actin-myosin meshwork at the rear of the cell does not undergo further polymerisation, but becomes contractile, resulting in a retraction of the rear section of the cell, with increased tension on the cell-substratum adhesions which eventually release.

The connection between ligands binding to surface receptors, the generation of second messengers within the cell, and alterations in the cytoskeleton is incompletely understood,

particularly with respect to the spatial resolution of such signals. Slight differences in receptor occupancy on different sides of the cell must lead to partitioning of cytoskeletal assembly and disassembly regions. The role of calcium ions (Ca^{2+}) has been investigated[124]. Migrating neutrophils consistently show periodic whole-cell transient elevations in [Ca^{2+}]. Buffering Ca^{2+} intra- and extracellularly did not inhibit chemotactic mobility on low adhesion substrates, indicating that cytoskeletal rearrangements could still occur in the absence of calcium. However, on high adhesion substrates such as fibronectin and vitronectin, neutrophils were unable to migrate in the absence of calcium transients, and it was found that this was due to an inability to detach integrin adhesion points at the rear of the cell[125]. It seems therefore that elevations in intracellular Ca^{2+} are involved in the release of integrins from matrix, which is necessary for movement. Phosphoinositides, also generated by chemoattractant signalling, have also been implicated in actin assembly[126].

Chemotactic desensitisation

It has been found that chemotactic responses can be desensitised by prior exposure to the chemotactic stimulus[106]. Desensitisation may be specific, *ie.* responsiveness to the same factor is abrogated but other stimuli evoke the same response as they would have without the prior exposure[127], or non-specific, in which case responses to other or all stimuli are lost. The mechanism that is likely to account for agonist specific desensitisation is loss of receptors for that particular agonist, probably by internalisation of receptor-ligand complexes[128], such that few receptors remain on the surface to respond to a second stimulation by the same agonist. Receptors for other factors should not be affected, so if signalling pathways remain intact, responses should be normal. It is possible that the binding of one agonist may affect the receptors for another[129]; this is referred to as cross-desensitisation (or receptor cross-down modulation), but the cell should still be able to respond to other factors whose receptors remain present. Non-specific desensitisation is likely to be due to post receptor mechanisms, such as exhaustion of intracellular second messenger mediators or of effector function. Generally, incubation of cells in moderate concentrations of agonists leads to specific desensitisation, while high concentrations lead to nonspecific desensitisation.

Chemotaxis assays

Several assay systems have been developed to study leukocyte chemotaxis. The best known is the Boyden chamber and its variations, whereby migration of cells across or into a filter, towards a higher concentration of stimulus, is measured[130]. Nitrocellulose filters are approximately 50μm thick and are a complex matrix structure, so cells migrate into the filter, and the distance of migration can be determined microscopically. Polycarbonate filters are a "unit membrane" 10μm thick, with well defined holes through which cells migrate to the other side, where they may remain adherent to the underside of the filter, or drop off into the medium. These assays test the locomotion of a population of cells, and count the number of cells which respond to a stimulus rather than a degree of response. It is necessary to correct for chemokinesis, since many factors are able to stimulate migration across the filter in the absence of a gradient. The true chemotactic response may be expressed as gradient-stimulated migration minus chemokinetic migration, or as an index, of gradient/non-gradient migration. An alternative to this type of assay is to measure the migration of cells through an agarose gel towards a well filled with a chemotactic factor. This assay is dependent on the diffusibility of the chemotaxin through the gel, and also may be more affected by adhesion of the cells to the gel matrix than is the polycarbonate filter assay. An alternative method is to microscopically observe the movements of individual cells on a slide subjected to a chemotactic gradient, with video recording and analysis, which gives information about the rate and direction of cell movement, as well as the variation in behaviour within a population of cells. Not surprisingly, these different assays may occasionally produce disparate results even with the same cell type and chemotactic stimulus.

Haptotaxis

An important theory regarding the mechanism of chemotaxis has arisen which challenges previous concepts of soluble chemotactic factors and gradients. The word "haptotaxis" was coined in 1965 by Carter[131] to describe the response of cells to gradients of surface adhesiveness, in which cells migrate towards regions which provide a better substrate for adhesion. The classical neutrophil chemoattractant casein was demonstrated to be able to induce migration when bound to a nitrocellulose filter, when soluble factor had been

washed out of the system[132]. Indeed, soluble casein was in this system considered possibly to inhibit migration, although this was not conclusively proved. Haptotaxis has re-emerged as an important concept recently with the demonstration that the chemokine family of peptide leukocyte chemoattractants are able to bind to tissue proteoglycans and to the lumenal surface of vascular endothelial cells. It was recently demonstrated that filters to which IL-8 was bound induced equal or better levels of neutrophil migration than was seen when soluble IL-8 was present without pre-binding to the filter[133], and this was also shown for monocyte migration to RANTES[134] (the chemokines will be discussed in detail in the next section). It was suggested that haptotaxis is solely responsible for cell migration in Boyden chamber assays[132,133], however another chemotactic factor, f-MLP, does not bind to filters, does not induce migration under conditions of haptotaxis, yet acts well as a soluble chemotaxin. The conclusions to be drawn from these studies are that surface bound gradients can induce cell motility (haptotaxis), but not all cell motility is due to surface bound factors. Migration may in some cases be the sum of responses to surface bound and soluble factor. Therefore, *in vivo*, haptotaxis may be one mechanism by which cell migration occurs.

Additional effects of chemotactic stimulation

Factors which stimulate chemotactic migration of leukocytes usually have other effects in addition to cell locomotion. Most factors induce increases in cell adhesiveness due to activation of β_2 integrins (which may in fact be a part of the migratory mechanism), and also cause production of superoxide, and degranulation with release of granule enzymes and other products. The latter effects are clearly part of the host defence response, but their relationship to migration is not clear. Granule enzymes may be necessary to digest tissue matrix to allow migration to occur, but degranulation is not necessary for chemotaxis itself[108]. Concentrations required to produce these associated effects are generally 2-10 fold higher than those optimal for chemotaxis[105]. Some factors show dissociation of responses, *eg*. IL-8 does not induce superoxide release, suggesting that responses to all chemotactic factors are not stereotyped, and that their signal pathways are complex and divergent.

In summary, in relation to neutrophil transendothelial migration, soluble and/or surface bound gradients of chemoattractant substances need to be considered as possible mediators of cell migration across the endothelial monolayer, once the neutrophil has made contact with the EC surface. Assays of chemotaxis (and chemokinesis) will be used to explore these possibilities in subsequent chapters. Desensitisation to specific chemoattractant factors will be used as a tool to explore the role of these factors in transmigration.

1.5 Interleukin-8

The presence of a neutrophil chemotaxin in the supernatants of activated lymphocytes and neutrophils was noted as long ago as 1969(105). It was not until 1987-88 that reports of the purification, cloning and gene sequence of this factor appeared(135,136,137,138,139). Several groups isolated a specific neutrophil chemotaxin, leading to a variety of different names- LYNAP (lymphocyte derived neutrophil activating peptide), MONAP (monocyte derived neutrophil activating peptide), MOC (monocyte derived chemotaxin), NAF (neutrophil activating factor), GCP (granulocyte chemotactic peptide), MDNCF (monocyte derived neutrophil chemotactic factor) and NAP-1 (neutrophil activating peptide-1). This factor was later given the less descriptive but unifying name interleukin 8, although NAP-1 is still used.

IL-8 is a 72 amino acid, 8,400 dalton peptide. The main feature of IL-8 is its potent and specific chemotactic effect on neutrophils. It is not known to be produced constitutively by any cell, but is produced by a wide variety of cells when activated by inflammatory stimuli (table 1.3). Transcription of the IL-8 gene is at least partially under the control of the nuclear transcription factors AP-1, NF-κB and NF-IL6 or C/EBP(140,141,142) which bind to specific DNA sequences in the IL-8 gene promoter region.

Table 1.3 Cellular sources of IL-8

cell type producing IL-8	stimulus	ref.
monocytes	LPS, IL-1, TNF-α, anoxia/hyperoxia	135,136,137,139,143,144
lymphocytes	PHA, Con-A	138
endothelial cells	IL-1	145
neutrophils	LPS,IL-1, TNF-α, phagocytosis	4,5
eosinophils	calcium ionophore	146
alveolar macrophages	LPS, zymosan	147
fibroblasts	IL-1, TNF-α, viral infection	148,149,150
keratinocytes	IL-1	150
bronchial epithelium	IL-1, TNF-α	151,152
pulmonary epithelial cell line	IL-1, TNF-α	153
mesothelial cells	IL-1, TNF-α, IFN-γ	154
NK cells	PMA, IL-2+anti-CD16	155
colonic epithelial cell lines	IL-1, TNF-α, IFN-γ, LPS	156
hepatocytes	IL-1, TNF-α	157
chondrocytes	IL-1, TNF-α	158

Effects of IL-8 on neutrophils

IL-8 has additional effects on neutrophils beyond stimulation of chemotactic migration. It causes release of primary (azurophilic) granule contents (β-glucuronidase) from cytochalasin B pretreated neutrophils[138] and was a direct mediator of specific granule lactoferrin[159] and gelatinase[160] release. It has been reported to stimulate neutrophil adhesion to various substrates through activation of the β_2 integrin CD11b/CD18 (Ch. 3, (161,162)), as well as an increase in β_2 integrin expression[162]. IL-8 causes neutrophil shape change[163] and stimulates phagocytosis[164]. The production of superoxide anion (O_2^-) by IL-8 stimulated neutrophils was controversial; some early studies reported production of O_2^- after IL-8 alone[163], but later studies found that very high concentrations of IL-8 were required, and the production of O_2^- was of very low magnitude[165]. (I could

demonstrate no production of O_2^- over the concentration range 1-1000nM (data not shown)). The current consensus after several careful studies is that IL-8 is a poor stimulus for O_2^- release on its own, but stimulates O_2^- release in neutrophils primed by TNF-α, GM- and G-CSF[165], and primes neutrophils for enhanced release of O_2^- when stimulated by other factors (f-MLP, PMA)[166]. Other functions include stimulation of the enzymes phosphatidylinositol-4-phosphate kinase[167] and arachidonate-5-lipoxygenase[168] which have roles in the synthesis of lipid second messengers and bioactive lipid mediators, and release of LTB_4 and PAF from GM-CSF primed neutrophils[169]. The functional significance of IL-8 in antimicrobial defence was demonstrated by its enhancement of neutrophil anti-*Candida* activity[170].

***In vivo* effects of IL-8**

In an early study, subcutaneous injection into rabbits was one of the assays used as the basis for purification of IL-8[139]. Swelling and redness were seen within 3h, which was related to neutrophil infiltration, on histopathological examination. Later more systematic studies[171,172,173,174] showed that injection of IL-8, particularly when co-injected with the vasodilator prostaglandin E_2 (PGE_2) induced the infiltration within 30min of large numbers of neutrophils, accompanied by neutrophil-dependent plasma leakage. IL-8 was more potent than f-MLP, and the effect was not due to a secondary protein mediator, since it was not blocked by the protein synthesis inhibitor actinomycin D. No monocytes, eosinophils, basophils or lymphocytes were observed in the lesions. IL-8 has been administered intravenously to primates[175]. It caused an initial rapid but transient neutropenia, which was followed by a granulocytosis. Although some margination of neutrophils was observed, no neutrophil mediated damage took place. There was no secondary rise in plasma TNF-α, IL-1 or IL-6, and the animals remained healthy.

Effects of IL-8 on cells other than neutrophils

Whilst the most important effects of IL-8 are on neutrophils, it was reported to have chemotactic activity for T lymphocytes[176] (which led to its being deemed an interleukin). These effects have since been shown to be small in magnitude, relative to other more specific lymphocyte chemokines[177]. The effect of IL-8 on lymphocytes was surprising, since an early study detected no calcium flux after IL-8 stimulation[163], and very few if any

receptors for IL-8 were detected[178]. A more recent study detected small calcium flux, but a substantial increase in generation of IP_3 by lymphocytes in response to IL-8[179], indicating that signalling was indeed occurring. Receptors for IL-8 on lymphocytes have still not been fully characterised, but concentration/response curves indicate that lymphocytes are more sensitive to IL-8 than neutrophils[176], suggesting that the effects are mediated by a small number of very high affinity receptors.

Eosinophils from normal individuals are not usually responsive to IL-8. However, eosinophils from patients with atopic dermatitis and asthma, and those from normals primed by culture in GM-CSF, IL-3 or IL-5 respond to IL-8 in chemotactic assays[180,181,182], and presumably express IL-8 receptors. IL-8 has also been reported to inhibit histamine release from stimulated human basophils, and IgE production by IL-4 stimulated B lymphocytes. It has been reported to be chemokinetic for IL-2 activated natural killer cells. The biological significance of these latter effects has not been well established. IL-8 was reported to induce angiogenesis in an *in vivo* model in rabbits[183], suggesting an effect on EC, although this could have been secondary to other factors. However, moderate chemotactic and proliferative effects on EC were also reported[184]. EC have not formally been demonstrated to have receptors for IL-8, although they were shown to respond to MGSA[185], which shares the IL-8 receptor[186]. Monocytes have so far been reported to be entirely unresponsive to IL-8.

Length heterogeneity of IL-8

IL-8 was initially purified as a 72 amino acid (aa) peptide, but was subsequently found to be heterogeneous in the number of N-terminal aa[187]. IL-8 is generated as a 99 aa precursor with a leader sequence of 22 aa[143,188], and naturally occurring species of 79, 77, 72, 71, 70 and 69 aa have been detected[187]. IL-8 purified from leukocytes is predominantly of the 72 aa form, whereas a 77 aa form predominates in EC supernatants[189]. 77 aa IL-8 is readily converted to the 72 aa form by thrombin[189]. Comparisons of pure recombinant[189,190] or synthetic[160] forms of each of these indicates that 77 aa IL-8 is 2-10 fold less potent in receptor binding and functional assays. In *in vivo* inflammation assays however, 77 aa IL-8 was equipotent with the 72 aa form, suggesting that it may have been rapidly converted by endogenous enzymes[190]. Interestingly, N-terminal truncations of 2 or

3 aa (to produce 70 or 69 aa peptides) resulted in increased potency *in vitro*, whilst truncations of 4 aa reduced potency considerably, and truncations of 6 aa resulted in a peptide that was completely inactive but had receptor antagonist properties(160).

IL-8 family- the chemokines

IL-8 is now known to be a part of a large homologous family of cytokines, which have been named the chemokines, because most (but not all) have chemotactic activity. Structurally they are characterised by 4 cysteine residues forming 2 intra-chain disulphide bonds; the family are further subdivided by the presence or absence of an intervening amino acid between the first two amino-terminal cysteines, and are therefore referred to as C-X-C or C-C peptides. This division also has functional significance, since the C-X-C peptides include IL-8 and are active mainly on neutrophils, whereas the C-C peptides act mainly on monocytes, lymphocytes and eosinophils. MGSA is a C-X-C peptide with 43% homology to IL-8 at the amino acid level, which was originally isolated on the basis of its ability to stimulate proliferation of melanoma cell lines(191). This factor activates neutrophils similarly to IL-8(192), and interacts with the IL-8 receptor on neutrophils(186). There is also evidence that a novel receptor for MGSA exists on melanoma cells which is not competed by IL-8(193), however, a recent report indicates that IL-8 can also have melanoma growth stimulatory activity(194)!

A series of peptides in the C-X-C family are of identical sequence but vary in their NH_2 terminal lengths. Platelet basic protein (PBP), connective tissue activating peptide-III (CTAP-III) and β-thromboglobulin (βTG) are all released from activated platelets, the second two being completely homologous with the first but truncated at their NH_2 termini. They have a 44% homology to IL-8, but none activates neutrophils(195,196). However, cleavage of these factors by monocyte proteases produces a 70 amino-acid peptide with strong neutrophil activating properties. Mixed cultures of monocytes and platelets produce this factor, called NAP-2(197), which also binds to the same receptors on neutrophils as IL-8(186). Platelet factor 4 (PF4) is another platelet-derived C-X-C chemokine with 33% homology to IL-8, but it has no neutrophil stimulating properties.

IL-8 receptors

Neutrophils have two separate receptors for IL-8, cloned in 1991[111,112], and since named the A and B type IL-8 receptors. They are 77% homologous to each other, and also have homology to receptors for f-MLP and C5a. All are part of a G protein linked receptor superfamily, and IL-8 signalling has been shown to be mediated through specific G protein subunits[198]. Both IL-8 receptors (IL-8R) bind IL-8 with high affinity (K_d ~2nM), but the IL-8R-B also binds MGSA with high affinity, while IL-8R-A binds MGSA with low affinity (K_d ~450nM) and does not signal in response to MGSA[199]. Therefore, the IL-8R-A is a specific receptor for IL-8 alone, while the IL-8R-B is a more promiscuous receptor for C-X-C chemokines. This is consistent with binding data, *ie.* MGSA competes only partially for binding of IL-8 to neutrophils, whereas IL-8 competes fully for the binding of MGSA[199]. Desensitisation experiments can also provide useful data about receptor usage by different factors. After incubation of neutrophils in IL-8, they no longer responded to stimulation with MGSA and NAP-2, suggesting that all receptors for MGSA and NAP-2 were internalised by, and therefore bound to, IL-8. However, preincubation of neutrophils with MGSA or NAP-2 only partially desensitised to IL-8[186]. This supports the data above, and indicates that the IL-8R-B is also a receptor for NAP-2. Down-regulation of IL-8 receptors by internalisation after binding of IL-8 has been formally demonstrated[200], and receptors were recycled. Neutrophils appear to have approximately equal proportions of A and B type receptors, although some variability has been reported[199].

C-C chemokines

The other branch of the chemokine family, the C-C chemokines, have a range of effects on other leukocytes, but none act on neutrophils so they are unlikely to have any role in neutrophil transendothelial migration. RANTES (*r*egulated on *a*ctivation, *n*ormal *T* cell *e*xpressed and *s*ecreted) (also released by activated platelets[201]) has chemotactic activity for monocytes and memory T lymphocytes[202], and chemotactic and degranulation activity for eosinophils and basophils[203,204]. Other C-C chemokines include monocyte chemotactic peptide-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and MIP-2. MIP-1α and β have the interesting properties of stimulating migration of predom-

inantly CD8+ and CD4+ T lymphocytes respectively[177,205]. A receptor for C-C chemokines has also been isolated[206], which binds RANTES, MCP-1, MIP-1α and MIP-1β with varying affinities. The chemokine IP-10 (interferon-inducible protein-10) is an exception to the chemokine subfamily dichotomy, since it has a C-X-C structure, yet acts on monocytes and lymphocytes and not neutrophils[207]. It is likely that combinations of these leukocyte specific chemokines and specific adhesion molecules control the particular types of leukocytes which are attracted to an inflammatory site. These factors are under continuing investigation in our laboratory, but will not be further dealt with in this thesis.

The erythrocyte IL-8 receptor

It has been reported that erythrocytes bind, adsorb and neutralise IL-8[208]. A novel chemokine receptor was isolated, which was shown to bind to IL-8, MGSA, RANTES and MCP-1. The function of this receptor is unknown, except that it may serve to neutralise chemokines released into the bloodstream. Interestingly, it was found that this receptor is also the Duffy blood group antigen, which was known to be the receptor for the malarial parasite *Plasmodium vivax*. MGSA and IL-8 blocked the binding of the parasite to erythrocytes[209].

IL-8 in disease

IL-8 has been implicated in human inflammatory pathology. Inflammatory suction-induced skin blisters, which are known to induce neutrophilic infiltrates, were found to contain IL-8 in the blister fluid[210]. IL-8 was also detected in the blood of normal volunteers injected with endotoxin[211]. These show the association of IL-8 with both localised and systemic experimental inflammation *in vivo*. IL-8 was detected in the skin in psoriasis, a skin disease characterised by neutrophilic inflammation[212]. It was shown to be produced by mesothelial cells after intrapleural instillation of asbestos in rabbits[213]. Development of adult respiratory distress syndrome, in which neutrophils probably play a pathogenic role, was predicted by the concentration of IL-8 in bronchoalveolar lavage fluid[214]. In rheumatoid arthritis, a chronic inflammatory disease characterised by the infiltration of neutrophils into the joint space, IL-8 was detected in synovial fluid[215,216], and its production was shown to be increased in synovial macrophages *ex vivo*[217]. IL-8, normally

resistant to proteases, was found to be inactivated by a protease in normal serosal fluids, which is absent in serosal fluids from patients with familial mediterranean fever, potentially explaining the pathogenesis of this disease(218). Finally, antibodies against IL-8 were found in the blood of rheumatoid arthritis patients(219), and moreover, normal subjects(220), suggesting that IL-8 can act as an autoantigen, and that endogenous antibodies may act to limit the effects of IL-8 in the circulation.

In summary, IL-8 is a specific neutrophil chemoattractant, produced by a wide variety of cells. These features suggest that it is likely to have a significant role in neutrophil transendothelial migration. The exploration of this hypothesis is a central theme of this thesis, and will be expanded upon in subsequent chapters.

1.6 Activation of endothelium

Endothelium has been considered in the past as simply a barrier between the blood and tissues, allowing the passive diffusion of fluids and macromolecules, allowing leukocytes to enter the tissues in response to exogenous chemotactic gradients, and maintaining a non-coagulogenic surface unless disturbed. It has now become evident that endothelial cells function actively in inflammation and coagulation, altering their surface properties and barrier function in response to information conveyed by cytokine messengers. In addition, EC secrete mediators of inflammation, such as cytokines, and produce regulators of vasomotor control. With regard to the latter, the production of endothelin-1, a vasoconstrictor, and endothelium derived relaxing factor (EDRF) or nitric oxide (NO) will not be further discussed. The endothelial properties which can be modulated include- expression of adhesion molecules, antigen presenting molecules (class I and II MHC) and regulators of the complement and coagulation pathways; permeability to fluids, macromolecules and cells; production of cytokines and other mediators; and endothelial proliferation and angiogenesis, the formation of new vessels.

Variation in endothelial cell phenotype

Before discussing activation, it should be mentioned that the normal "resting" state of endothelium may vary. Endothelia of different sized vessels, in different organs, are not uniform in their properties. Well known examples are the specialised endothelium of the

brain, which have tight junctions and form the relatively impermeable blood-brain barrier, and the fenestrated or discontinuous endothelium of the liver sinusoids and bone marrow. It has been known for some time that the principal sites of leukocyte extravasation in inflamed tissues are the post capillary venules, and also that the high endothelial venules (HEV) of the lymphatic tissues are specialised to direct recirculating lymphocyte traffic.

The expression of adhesion molecules in normal vessels from different tissues has been investigated(221,222). All EC uniformly express factor VIII related antigen (von Willebrand factor), and CD31 (PECAM), which appears to be necessary for junction formation. CD34, a haematopoietic progenitor cell antigen that has been described on EC, was present mainly on capillaries but not larger vessels(except umbilical artery and vein), in a range of tissues(102). ICAM-1, considered to be constitutively present but upregulated on EC activation, was present in all tissues except thymus and colon, more intensely on capillaries and smaller vessels than large, but with some variability. VCAM-1 and E-selectin, usually thought to be absent on non-inflamed endothelium, were in one study(222) found on normal thyroid tissue vessels, on normal lymph node, tonsil (E-selectin) and thymus HEV (VCAM-1), and in another study(221), E-selectin was described on normal coronary and pulmonary arteries and umbilical artery and vein, while VCAM-1 was on coronary and umbilical arteries. P-selectin was expressed predominantly on medium sized vessels (venules and arterioles) and not on capillaries, in nearly all tissues, with the exception of kidney and heart. The significance of these findings are that P-selectin mediated rolling would be expected to occur in normal small vessels, and also that assumptions about regulation of VCAM-1 and E-selectin are not applicable in all tissues. There is also recent evidence of unique surface glycoprotein expression on EC which are migrating(223), and those which form the vascular bed of tumours(224). Many *in vitro* studies use human umbilical vein endothelial cells (HUVEC), which appear to respond to stimuli to produce a similar phenotype to post capillary venular cells in inflammation, but whilst they are very useful as a model, they cannot be presumed to replicate endothelial function in all vessels and tissues.

Regulation of endothelial permeability

The barrier function of endothelium is regulatable, and increased permeability can be controlled by EC actively, rather than simply resulting from EC damage. Increased permeability of the endothelium to serum is a characteristic feature of inflammation, and results in exudation and tissue swelling. Transendothelial permeation of macromolecules can occur through cell junctions (paracellular), through pores through cells (transcellular) or by vesicular transport. The junction between adjacent endothelial cells is maintained by adhesion molecules including PECAM[47], endothelial cadherin (cadherin V)[225] as well as other cadherins, and integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$)[226] The junctional complexes, seen by electron microscopy (EM), form a tight junction that is less well developed than that seen in epithelia, but has similar characteristics[227]. The cytoskeleton plays a role in maintenance of the junction, particularly the dense peripheral band of actin[228] which links to the junctional complexes through proteins such as vinculin. Treatment of EC monolayers with cytochalasin B, which disrupts the peripheral actin band, increased monolayer permeability[227,228], as did antibodies to β_1 integrins[226] and cadherins[225]. Physiological stimuli of increased endothelial permeability include histamine and thrombin, which induce retraction of EC and opening of intercellular junctions, which is reversible and not caused by EC damage. Increased permeability is also seen after treatment of EC with cytokines. TNF-α treatment resulted in increased permeability of EC monolayers which was also reversible (*ie.* was presumably not due to cell death or damage), yet this was shown at massive TNF-α concentrations (the equivalent of 1000-10,000U/ml)[229]. LPS increased permeability, but this may have been due to cell death or damage[230]. When cytokines and the "acute agonists" histamine and thrombin were combined, then increases in EC permeability were seen at more modest concentrations[230], and this may reflect a more physiological situation at the inflammatory site.

Modulation of permeability appears to be under the control of protein kinase C, since PKC activators (PMA) also increased permeability[231,232], and the PKC inhibitor, H7, abrogated the effect of thrombin[230,232]. A rise in intracellular Ca^{2+}, seen after stimulation of EC with thrombin, was necessary but not sufficient for an increase in permeability[233], since the calcium elevating agent ionomycin failed to alter permeability[231]. G proteins are required,

since PT inhibited increases of permeability caused by both cytokines[229] and thrombin[230]. Interestingly, cyclic adenosine monophosphate (cAMP) reduced the permeability of EC monolayers[231], and may have a role in the relative impermeability of brain endothelium[234]. These pathways all indicate that EC have active control of their permeability, which is modulated in response to various mediators involved in inflammation.

Regulation of coagulation

Coagulation is another endothelial function which is regulated. EC actively maintain an anticoagulant surface, by synthesising prostacyclin (a potent platelet inhibitor), and displaying natural heparin-like molecules and thrombomodulin (which is a promoter of the protein S-protein C anticoagulant pathway) on their surface[235]. EC activated by cytokines reduce these anticoagulant properties and become actively procoagulant, by synthesising tissue factor, producing coagulation components, and plasminogen activator inhibitors[235,236]. The endothelial role in coagulation will not be further discussed in this thesis, however it should be borne in mind that this is a significant feature of the inflammatory response.

Cytokine activation of endothelial cells

The major EC functions of interest from the point of view of cell migration in inflammation are the expression of adhesion molecules and the production of inflammatory mediators by EC. IL-1, TNF-α and LPS seem to induce a common distinct pattern of activation, and will be considered first. IL-1 activates EC through the type I IL-1R[237], and its effect is blocked by the IL-1 receptor antagonist[238]. TNF-α has two separate receptors, both of which are present on EC, and the usage of these receptors is investigated in chapter 7. Although EC lack CD14, the receptor for LPS, soluble CD14 in serum can bind LPS-LBP (LPS binding protein) complexes and then interact with EC to produce a strong activation[239,240]. The signalling pathways of all of these factors are unknown, but all cause PKC independent activation of NF-κB[241]. NF-κB and its inhibitor, IκB-α, are inversely regulated by TNF-α, and IκB-α has been shown to inhibit the TNF-α induced up-regulation of E-selectin[242]. TNF-α as well as IL-1 and LPS cause up-regulation of ICAM-1, induction of E-selectin, and production of IL-8 by EC. A dissociation of the

effects of these stimuli was seen in the ability of TNF-α but not IL-1 to increase total mRNA and protein synthesis, and cell size (*ie.* induce hypertrophy); in fact IL-1 antagonised this[243]. Also, TNF-α but not IL-1 also increased MHC class II expression[60].

IL-4 acts on EC by a different pathway, since its signal is cAMP dependent, whereas TNF-α is not[244]. It induces a different (although overlapping- *ie.* both induce VCAM-1) set of pro-inflammatory EC functions and antagonises some TNF-α and IL-1 effects. TGF-β inhibits the induction of EC inflammatory functions by other cytokines, while activating functions involved in wound healing and repair (IL-4 and TGF-β are reviewed in chapter 9). IFN-γ induces yet another pattern of activation, by increasing ICAM-1 and class I MHC[60], and inducing MHC class II[245], which could be said to favour immunological events, by enhancing T cell-endothelial interaction. IFN-γ interacts with TNF-α but not IL-1 to increase and prolong E-selectin induction[60].

The effects of haematopoietic growth factors on EC

Other factors implicated in EC activation are the haematopoietic growth factors. The effect of G- and GM-CSF on EC has been controversial. These factors were initially reported to induce EC to migrate and proliferate, and specific binding to EC was demonstrated[246]. Other groups[247] and our own laboratory (unpublished results) were unable to replicate these findings, although a slight increase in proliferation in response to GM-CSF was noted in one study[245]. No inflammatory effects were found for either of these factors on EC[247,248]. Sensitive RNAse protection assays failed to detect mRNA for the α-chain of the GM-CSF receptor in resting or activated EC[249]. The same assays did detect mRNA for the α-chain of the IL-3 receptor; previously, IL-3 had been shown to be inactive on EC[248], although one recent study showed some pro-inflammatory activation[250]. Activation of EC by IL-3 is investigated in chapter 8. Interestingly, erythropoietin was also shown to bind specifically to EC and activate proliferation and migration, but inflammatory functions were not investigated[251].

Variability of activation patterns

Finally, it should be noted that endothelial activation, like resting endothelium, may not be uniform in different vessels in different tissues. Investigations of this have been limited, but it was noted that human dermal microvascular endothelial cells (HDMEC) respond

differently to cytokines than HUVEC. *In vitro*, although TNF-α induced VCAM-1 expression in both cell types, HDMEC did not up-regulate VCAM-1 in response to IL-1 or IL-4 as did HUVEC[(252)]. In a skin organ culture model, immunohistological studies showed that the expression and inducibility of VCAM-1 and E-selectin by TNF-α and IL-1 varied according to vessel type, vessel location in the superficial or deep dermal venous plexus, and whether examined in-vivo, in organ culture or in cell culture, and again, these responses differed from HUVEC[(253)]. EC derived from synovium of patients with rheumatoid arthritis differed from HUVEC in its regulation of ICAM-1 expression in response to cytokines[(254)]. IL-4 acted as a mitogen for capillary endothelium but not HUVEC[(255)], and growing or motile EC were more sensitive to TNF-α than post-confluent endothelium[(256)]. These findings indicate that the standard experimental model employing HUVEC may need to be interpreted with caution when extrapolating to *in vivo* pathology.

In summary, the vascular endothelium is an active participant in the inflammatory reaction, and is able to regulate a number of functions which influence fluid exudation and cell migration. Investigations of factors which activate endothelium, and the role of endothelial activation in neutrophil transmigration will be presented and discussed in subsequent chapters.

1.7 Neutrophil transendothelial migration

Although neutrophil emigration from vessels was initially observed more than 100 years ago, the process has only recently been broken down into its component steps of rolling, adhesion and transmigration, and the molecular mechanisms of each examined. Previous sections have reviewed the adhesion steps. Detailed investigation of transmigration required the ability to isolate and culture endothelium *in vitro*, and several different model systems have been developed to replicate the vessel wall. Transmigration studies have also been facilitated more recently by a knowledge of neutrophil and EC surface molecules and secreted mediators. Subsequent *in vivo* studies using newer reagents have provided further information, but several aspects of the basic mechanism remain unclear.

Transmigration- *in vitro* observations on slides

Early observations of neutrophil-endothelial interactions, including transmigration, were made by co-incubating leukocyte suspensions with EC cultured on glass coverslips[257,258]. Experiments were carried out under conditions of flow, by rotating the monolayers at an angle. Neutrophils were observed to adhere to the endothelial surface, and using phase contrast and EM, these cells remained spherical and phase bright. Some neutrophils were observed which were flattened and enlarged. Nomarski differential interference microscopy revealed that these cells were below the plane of focus of the rounded cells, and indeed appeared below the plane of the endothelium. Transmigration was confirmed by EM transverse sections, which showed flattened neutrophils against the glass slide beneath the endothelium, and in some cases revealed neutrophils in the process of migrating between endothelial cell junctions. Only limited manipulation of the system was performed, which did not reveal any significant regulators of transmigration. This experimental system does not allow the creation of a chemotactic gradient across the endothelium, but has subsequently proven valuable particularly for assessing transmigration in response to activated endothelium ((259), and see experiments reported in chapters 4, 9).

Transmigration- polycarbonate filter model

Another experimental system for the examination of transendothelial migration of leukocytes was first reported in 1981. Endothelial monolayers were cultured on gelatin-coated polycarbonate filters, which had 5μm pores. Confluence of the monolayers was confirmed by EM, which demonstrated that the EC were continuous and formed intercellular junctions similar to those seen *in vivo*. The filter with monolayer was placed horizontally between an upper and lower compartment containing medium. Functional indication of confluence was provided by resistance of the monolayers to the passage of radiolabelled (^{125}I) albumin. In this system, a nitrocellulose filter was placed below the polycarbonate one to collect the neutrophils which migrated. Radiolabelled (^{51}Cr) neutrophils were used, and counts were taken of the nitrocellulose filter. Minimal counts were measured in the absence of a stimulus, but if f-MLP were placed in the medium in the lower compartment, creating a concentration gradient across the monolayer, then

significant numbers of neutrophils migrated into the lower filter. This demonstrated that a factor which had previously been demonstrated to have chemotactic activity in a Boyden chamber assay using a filter alone[105] was also able to induce neutrophils to migrate across a confluent endothelial monolayer.

Permeability of endothelial monolayers *in vitro*

Several groups developed cultures of endothelial monolayers on filters, for the purpose of studying endothelial permeability in response to various factors. Regulation of endothelial permeability was discussed in the previous section, but these systems will be reviewed since they have relevance to measuring leukocyte transmigration, and permeability is also of interest in this context. An interesting approach was to culture EC on the stromal surface of human amniotic membrane[260]. EC cultured for 8 days formed a confluent monolayer, with intercellular and cell-substrate adhesion structures similar to those seen *in vivo*, including a well-developed basement membrane structure. The borders of the EC could be stained with silver nitrate ($AgNO_3$) and produced a pattern similar to EC *in vivo*. The cell junctional regions were able to exclude the passage of a macromolecular probe, horseradish peroxidase (HRP) as assessed by EM. Another measure of permeability was also used, that of transendothelial electrical resistance, which reflects permeability to ions. In another study[261], EC cultured on polycarbonate filters in transwells (see materials and methods, Fig. 2.1) formed a tight monolayer within 3 days, which had an electrical resistance of $17 \pm 4\Omega{\cdot}cm^2$. The resistance of the filter alone was $3{\cdot}2\Omega{\cdot}cm^2$, while the resistance of capillary endothelium *in vivo* had previously been measured at $13\text{-}40\Omega{\cdot}cm^2$ (except brain endothelium, which has a much higher resistance). Electrical resistance could be measured continuously, and was shown to be reduced by incubating EC in serum concentrations below 10% (optimal HUVEC culture requires 20% serum), and by the removal of calcium with EGTA, a calcium chelator (endothelial intercellular adhesion molecules cadherin-V, PECAM and the $\beta 1$ integrins are known to be calcium dependent). This culture system was used as a model on which my own permeability (Ch. 2) and transmigration experiments were based.

Effects of transmigration on monolayer permeability

Human amnion, as a substrate to endothelial monolayers, was also used for studies of neutrophil transendothelial migration and permeability in parallel(262). Migration between EC into the amnion collagenous matrix, in response to an f-MLP gradient, occurred rapidly, within 5-10min, and was quantitated by microscopic counting. Neutrophil transit time through the EC intercellular junction was calculated to be approximately 1min. Electrical resistance and albumin permeability were measured simultaneously with neutrophil migration(263), and were found not to change when the ratio of migrating cells to EC was up to 5:1, suggesting that transmigration was not accompanied by generalised endothelial leakiness. A common feature of EM of neutrophils in the process of transmigrating in this and other studies was the close apposition of the neutrophil and EC membranes, effectively sealing the intercellular junction during migration. The membrane gap was only ≤150Å, although no junctional structures were observed between EC and neutrophils. A calculation of area based on EM photographs suggested that the increase in paracellular pathway that results from neutrophil transmigration was only ≤0·22%, which would not be expected to result in measurable increases in permeability. However, another study using EC on polycarbonate filters(264) showed that the migration of monocytes was associated with a unidirectional transport of low density lipoprotein (and bi-directional increase in albumin permeability) across the endothelium, which the authors concluded was due to disruption of endothelial junctions. Whether monocyte migration is different to neutrophil migration in this respect, or whether the differences were due to different culture systems, species differences (bovine EC, human monocytes) or lower FCS (8%) in the latter paper is not clear.

Cytokine-activated endothelium induces neutrophil transmigration

A significant finding reported by several groups in 1988-89, using each of the different assay systems described above, was that preincubation of endothelium with the inflammatory cytokines TNF-α or IL-1, or bacterial LPS, enhanced transmigration of subsequently added neutrophils(259,265,266). TNF-α and IL-1 were equipotent at optimal concentrations, and no additional effect was seen when both factors were combined. The effect was on the EC, since the cytokine was washed away prior to addition of the

neutrophils, and incubating neutrophils with EC in the presence of TNF-α or IL-1 without prior incubation of the EC did not cause transmigration (IL-1 does not affect neutrophil motility in any case). Preincubation of EC for 60min was sufficient to lead to significant migration, but 4-6h was optimal, and the effect was sustained for 24h after addition of the cytokines to the EC (more so for TNF-α than IL-1). New protein synthesis by EC was required, since co-incubation with actinomycin D during the EC activation phase blocked the induction of transmigration. Increased migration was not due to a failure in the barrier function of EC, since monolayer permeability to albumin[265] or electrical resistance[266] was not altered by cytokine preincubation of EC. An EC-produced neutrophil chemotaxin was not detected[265]. No consistent additive effect was seen when a transendothelial chemotactic gradient was combined with endothelial activation. One study suggested that EC stimulated from the lower (abluminal) side were more effective at induction of neutrophil transmigration than when stimulated from the upper compartment[267]. These findings suggested an active role for EC in the promotion of neutrophil diapedesis in inflammation (as well as adhesion- see previous section), where TNF-α and IL-1 are likely to be present.

Integrin-ICAM interactions in transmigration

The requirement for adhesive interactions in transmigration has been investigated. Antibodies to endothelial ICAM-1 blocked transmigration in response to IL-1 activated endothelium on a glass slide (slide assay) almost completely. ICAM-1 is a ligand for the leukocyte integrins CD11a/CD18 (LFA-1) and/or CD11b/CD18 (Mac-1) on activated neutrophils. Antibodies to CD18 also blocked completely; interestingly, while CD11b antibodies did not block, CD11a antibodies blocked partially, and a combination of CD11a and CD11b antibodies blocked completely. This suggests that LFA-1 can mediate transmigration alone when Mac-1 is unavailable, but that Mac-1 is less effective, and mediates transmigration only partially when LFA-1 is unavailable. When neither is available, transmigration does not occur. This was further substantiated by the use of neutrophils from a LAD-I patient, which lack β_2 (leukocyte) integrins altogether. Although these neutrophils were able to adhere to IL-1 activated endothelium (E-selectin mediated adhesion), they did not transmigrate. In experiments of chemotactic transmigration of

neutrophils across (unactivated) endothelial monolayers on amnion, blocking antibodies also demonstrated an essential role for neutrophil β_2 integrins and EC ICAM-1, even though on unactivated endothelium, ICAM-1 is expressed at lower levels[268].

These studies, and others conducted *in vivo* with antibodies, as well as the experience with LAD-I patients, prove that the leukocyte integrins are essential for neutrophil transendothelial migration, and that they function by interacting at least with endothelial ICAM-1 (the potential role of ICAM-2 as a ligand for β_2 integrins in transmigration has not been explored). Yet the way in which this interaction facilitates transmigration is not well understood. Questions remaining include the role of conformational change of the integrins, the possible role of intracellular signalling of either of these adhesion molecules to alter neutrophil motility or endothelial junction opening, or other potential functional mechanisms. An interesting exception to the absolute requirement for the integrin/ICAM interaction was provided by *in vivo* studies in rabbits[269]. MAb to CD18 effectively inhibited neutrophil emigration to inflammatory reactions in the abdominal wall skin, and to peritoneal inflammation induced by the bacteria *streptococcus pneumoniae*. The MAb also inhibited neutrophil emigration into PMA induced lung inflammation (pneumonia) but not into pneumonia induced by *s.pneumoniae* or HCl, and inhibited only partially when pneumonia was induced by LPS. The alternative adhesion pathway available in the lung that replaces CD18 integrins and ICAM has not been reproduced *in vitro* and has not been characterised at a molecular level.

E-selectin in transmigration

E-selectin, however, is not absolutely required for transmigration. Although an early study showed inhibition of transmigration with antibodies to E-selectin[270], subsequent studies indicated that this may only be the case at low concentrations of activating cytokines, while at optimal concentrations, blocking E-selectin antibodies had no inhibitory effect[271]. Therefore E-selectin may play an accessory role in promoting transmigration through endothelium which is only partially activated (possibly by its ability to activate leukocyte β_2 integrins[272]), but other more important mechanisms render this unnecessary when activation is optimal. Transmigration was shown to be induced after activation of EC for 24h with TNF-α, at a rate of about 80% of the optimal at the time point of 4-6h[265]. At this

time, however, expression of E-selectin has diminished to virtually the same level as is expressed on unactivated EC, again suggesting that it is not necessary for transmigration. In a system of flow, however, and indeed *in vivo*, inhibition of selectin function would ultimately inhibit migration, since the initial rolling step is necessary for subsequent firm integrin mediated adhesion and then transmigration.

CD31 (PECAM)

Although this interendothelial cell adhesion molecule has not been demonstrated to have a direct role in leukocyte-endothelial adhesion, it was recently found to play an important role in transmigration. Soluble PECAM or a PECAM antibody blocked spontaneous transendothelial migration of monocytes, and transmigration of both neutrophils and monocytes induced by activation of the EC[56]. The leukocytes remained tightly bound to the EC surface, suggesting that the transmigration step and not adhesion was being inhibited. Inhibition resulted if either the EC or the leukocyte was pretreated with antibody, and these were not additive, suggesting the homophilic interaction of PECAM on both cell types. The mechanism of action of PECAM has not yet been established. It may act indirectly by activating β_2 integrins on the leukocyte, or directly as an adhesion molecule at the EC junction, or as a "zipper" to undo endothelial cell junctions to allow the leukocyte to pass, while maintaining a seal between the leukocyte and the EC during migration. *In vivo* studies lend support to the suggestion of PECAM as a transmigration molecule since it was detected at high levels on transmigrating lymphocytes in mice[273]. However, some puzzles remain, since in lymphocytes, PECAM is a marker more closely associated with a naive phenotype[274], whereas memory cells are more transmigration-competent[275], and recently, PECAM expression was found to correlate negatively with transmigration in mixed lymphocyte populations[276].

Mechanism of induction of transmigration by activated endothelium

Although transmigration had been observed, and adhesion molecule requirements determined, the mechanisms by which activated EC stimulate neutrophil motility and migration across the endothelial junction remained unclear. It is important to note that adhesion of neutrophils to EC does not in itself lead inevitably to transmigration, and that

the two steps are clearly separate. When neutrophils were tethered to EC monolayers via their Fc receptors, adhesion was increased but transmigration did not occur[265]. Likewise, pre-incubation of neutrophils with f-MLP or TNF-α, which increased their adhesiveness for endothelium by activating β_2 integrins, did not result in transmigration[265]. Transmigration requires either the presence of a gradient of chemoattractant, or the activation of endothelium by cytokines. The finding, reported in 1989, that EC activated by IL-1, TNF-α and LPS produce the neutrophil chemotactic peptide IL-8 offered a possible mechanism for the latter. Certainly the range of factors which induce IL-8 production by EC and those which activate EC to induce neutrophil transmigration are the same. Formal proof awaited studies in which IL-8 production by activated EC was neutralised (Ch 5, (277)). Evidence indicated that IL-8 was not the sole mediator of EC-induced neutrophil transmigration (Ch. 3, 4, 5, (277)), and candidates for co-mediators included G- and GM-CSF (Ch. 6), PAF (Ch. 5, (278)) and eicosanoids such as thromboxane B_2 (TxB_2)[279]. These and other factors will be further discussed in subsequent chapters and in the Discussion section.

Neutrophil intracellular signalling in transmigration

Few studies have addressed intracellular events occurring in either the neutrophil or the EC during transmigration. It is likely that communication between the cell types occurs, since the EC must release intercellular connections at the point where the neutrophil migrates between cells, and reseal the junction afterwards. On the part of the neutrophil, a Ca^{2+} flux has been observed on its contact with activated EC, which was attributed to endothelial expression of membrane PAF[280]. As previously reviewed, Ca^{2+} fluxes are intimately related to, but not essential for, neutrophil chemotactic mobility. Loading of neutrophils with an intracellular calcium chelator prevented the Ca^{2+} rise, but did not prevent actin polymerisation responses and did not alter adhesion or transendothelial migration, either in response to cytokine activated endothelium or to a transendothelial gradient of f-MLP[281]. Neutrophils which have migrated across cytokine activated endothelium show an up-regulation of β_2 integrins, and a loss of surface L-selectin. The integrin up-regulation was prevented by calcium buffering, but not the loss of L-selectin. The involvement of neutrophil G proteins in the transmigration response was demonstrated by PT pretreatment

of neutrophils, which inhibited transmigration[265]. Whether PT inhibited the transmigration step specifically, or inhibits adhesion primarily and migration secondarily is not certain, but adhesion of PT pretreated neutrophils to IL-1 activated EC, whilst not quantitatively reduced, became susceptible to shear stress, so is clearly qualitatively altered. IL-8 and PAF, as well as other potential mediators, depend on G protein linked neutrophil receptors for activity. PT treatment of neutrophils also inhibited their accumulation at inflammatory sites *in vivo*, whether the inflammation was induced by the direct neutrophil chemoattractants f-MLP, C5a or IL-8, or the endothelial activator (with no neutrophil chemotactic activity) IL-1[282].

Endothelial calcium flux and the opening of junctions

Transmigration-associated calcium changes in EC have also been examined. Resting or IL-1 activated EC underwent a transient increase in intracellular Ca^{2+} in response to transmigrating neutrophils (transmigration across resting EC was in response to an f-MLP gradient)[283]. Ca^{2+} transients are seen after stimulation of EC with histamine or thrombin, and are necessary for the increase in permeability of monolayers which occurs in response to these agents. It therefore seems likely that the increase in intracellular Ca^{2+} which occurs when neutrophils transmigrate has a role in the opening of EC junctions to allow neutrophil passage. Indeed, clamping of EC Ca^{2+} with an intracellular buffer, which was effective at preventing Ca^{2+} flux in response to histamine as well as to neutrophil contact, inhibited neutrophil transmigration (but not adhesion) in response to both IL-1 activated endothelium, and an f-MLP chemotactic gradient. The pathway by which neutrophils, in the process of transmigration, communicate with EC to induce a calcium flux is unknown. Resting neutrophils contacting EC, which did not transmigrate, did not induce a Ca^{2+} flux. Interestingly, f-MLP activated neutrophils contacting EC, which adhere via β_2 integrins to endothelial ICAM-1, did induce a rise in Ca^{2+}, despite the fact that they did not transmigrate. This rise was accompanied by an increase in endothelial permeability. This suggests the possibility that ligation of ICAM-1 may transmit an intracellular signal in EC which causes the elevation of Ca^{2+}. Alternatively, the message may be transmitted from neutrophils to EC via a soluble mediator. The former possibility seems more attractive, for it allows the hypothesis that neutrophils can induce a localised signal, over only a part of

the endothelial cell, which allows a localised separation of the junction to allow passage of the neutrophil, as has been observed in EM studies[263]. This may be the basis of the absolute requirement for β_2 integrins in transmigration, referred to earlier.

Neutrophil penetration of the endothelial basement membrane

Subsequent to its migration through the interendothelial cell junction, the neutrophil flattens between the EC and the subendothelial basement membrane (BM). Many studies, using EM or light microscopic visualisation, describe a delay at this point, as though further time is required before the neutrophil can penetrate the BM and enter the tissues, and thereby suggest a further or fourth step in the process of diapedesis. This was studied using EC cultured on a collagen matrix for 21d, after which time a well-developed basement membrane was evident[284]. Neutrophils penetrated this barrier in response to chemotactic gradients, and associated with this was a disruption of the BM, which was rapidly repaired by the overlying endothelium. The mechanism of BM penetration was investigated using a range of protease inhibitors. Cathepsin G and elastase could be irreversibly inhibited in neutrophils before adding them to the assay, but this had no effect on BM penetration. A wide range of protease inhibitors also did not affect penetration, however, this is probably due to the ability of neutrophils to create a protected environment for proteolytic degradation, from which most soluble inhibitors are excluded[285]. Components of the subendothelial matrix have been noted to reduce neutrophil activation, which may act to prevent damage to EC during transmigration[286].

Transmigration of other leukocyte types

Transendothelial migration of other leukocyte types has also been studied. Monocytes have also been shown to migrate through endothelial monolayers in response to chemotactic gradients (f-MLP, LTB4[264,287]) or preactivation of the monolayers by IL-1, TNF-α and LPS[288]. Interestingly, monocytes are able to use the β_1 integrin VLA-4 for transmigration when the β_2 integrins are blocked, so their transmigration is not absolutely dependent on β_2 integrins as it is in neutrophils. This correlates with the finding that in patients with LAD-I, some monocytes are observed to migrate into inflammatory sites[288]. Eosinophils also migrated through IL-1 or TNF-α activated endothelium, but interestingly, only after priming of the eosinophils with GM-CSF, IL-3 or IL-5[289]. Activation of

endothelium with IL-4 resulted in an even stronger stimulus for eosinophil transmigration, and this induction was specific, since neutrophils did not respond[290]. Eosinophil transmigration through EC activated with IL-1 or TNF-α appeared to be dependent on β_2 integrins only, with no inhibition from antibodies to VLA-4, but migration through IL-4 activated EC used both β_2-ICAM and β_1-VCAM adhesion mechanisms. Lymphocyte transmigration is regulated by the activation of EC by IFN-γ, in addition to the other factors[291], and is complicated by the differential ability of T cell subsets to migrate[275]. There have also been some interesting studies of the transepithelial migration of neutrophils, such as might occur in intestinal inflammation, which indicate that IFN-γ may be the most important mediator[292].

In this chapter, I have reviewed neutrophil-endothelial adhesion and the adhesion molecules involved, neutrophil chemotactic motility and the important neutrophil chemotactic factor IL-8, the different activation states of endothelial cells, and finally, what is known about neutrophil transendothelial migration itself. This provides a framework in which to present the results of my own research. While much of the information in this chapter was available at the time of commencing this project, a great many studies referred to were published within the past 5 years, indicating the rapid progress of research in this area. In chapter 10, I will discuss the significance of my results in the context of current concepts of neutrophil-endothelial interactions, how they have contributed to the field, and the future directions of research in this area.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media

2.1.1 HUVEC medium

Medium for HUVEC culture was made up from M199 with Earles salts (Cytosystems, NSW), with 20% fetal calf serum (FCS) (Flow, Australia), 20mM HEPES, 1% glutamine (Cytosystems), 1% non-essential amino acids (Cytosystems), 1% sodium pyruvate (Cytosystems), penicillin, gentamicin and fungizone, and 3% sodium bicarbonate.

2.1.2 HUVEC wash/assay medium

Medium for washing HUVEC during passaging procedures and after trypsinisation, as well as for conducting neutrophil chemotaxis, and neutrophil/endothelial adhesion assays, consisted of M199 with Earles salts, 2½% FCS and 10mM HEPES.

2.1.3 Medium for immunofluorescence/flow cytometry staining

Flow cytometry wash (FC wash), used to dilute antibodies for cell staining and to wash unbound antibody from cells, consisted of phosphate buffered saline (PBS) with 5% newborn calf serum (NCS) and 0·2% sodium azide. Medium to fix stained cells for flow cytometry (FC fix) was PBS with 0·4% formaldehyde, 2% glucose and 0·02% sodium azide

2.2 Reagents

2.2.1 Interleukin-8

Chemically synthesised IL-8 was a gift of Dr. Ian Clark-Lewis, Centre for Biomedical Research, Vancouver, Canada. It was produced as the 72 and 77 amino acid IL-8 forms using automated solid-phase methods[(293)]. The IL-8 was folded and purified by reverse phase HPLC and its chemical properties and bioactivities examined. The synthetic IL-8 had the correct sequence as determined by Edman protein sequencing methods. It was homogeneous by criteria of reverse phase HPLC, isoelectric focusing and mass spectrometry. The 72 amino acid form had equivalent potency to recombinant 72-IL-8 in assays for induction of neutrophil chemotaxis, elastase release, cytosolic free calcium and

superoxide production[293]. Endotoxin levels in the stock preparation were <10 pg/ml, as determined by limulus amoebocyte assay.

2.2.2 Cytokines

Recombinant human TNF-α (batch no. 3056-55, at 0·5 mg/ml, equivalent to 2×10^7U/ml) and TGF-β (lots 8987-53 and G098D) were kindly supplied by Genentech (South San Francisco, CA, USA). Endotoxin levels were <12·5 pg/ml by limulus amoebocyte assay. IL-1 was kindly supplied by Immunex (Seattle, WA, USA)(10^8 thymocyte mitogenic units/mg). Endotoxin levels were <40pg/ml. N-formylmethionylleucylphenylalanine (f-MLP) was obtained from Sigma. G- and GM-CSF were obtained from Amgen, Thousand Oaks, CA, USA (recombinant human, expressed in *e.coli*). CHO cell (glycosylated) recombinant human GM-CSF was a gift of Dr. Angel Lopez, I.M.V.S. IL-3 was obtained from Genetics Institute, Cambridge, MA, USA. TNF-α wild type and mutants used in chapter 7 were kindly provided by Dr. X. van Ostade and Dr. W. Fiers, University of Ghent, Belgium. PAF was obtained from Boehringer Mannheim, Mannheim, FRG, and WEB2086 was from Boehringer Ingelheim, Ingelheim, FRG.

2.2.3 Cell culture reagents

Human purified fibronectin (Boerhinger Mannheim, Mannheim, FRG) was diluted in phosphate buffered saline pH 7·3 (PBS) to 50μg/ml for use in coating plastic surfaces. Heparin was obtained from Sigma. Endothelial cell growth supplement (ECGS) was obtained from Collaborative Research Bedford, MA, USA.

2.2.4 Antibodies

Monoclonal antibodies 60·1(IgG1,κ), 60·3(IgG2a,κ), 60·5(IgG2a,κ) (Ch. 3) were kindly provided by Dr. P. Beatty, Fred Hutchinson Cancer Centre, Seattle WA. 60·1 is directed against the α chain of the leukocyte integrin Mac-1 (CD11b/CD18) and 60·3 recognises the common β chain of the LFA-1, Mac-1 and P150,95 complex (CD18). IgG purified from ascites was diluted 1:200 for use (Ch. 3). HB203, anti CD18, IgG1, was obtained through the American Type Culture Collection (ATCC), Rockville, MD, USA (Ch. 5). Anti GM-CSF antibody HGM2/3.1 (Ch. 6) was obtained from Genetics Institute, Cambridge, MA,

USA. Neutralising antiserum to IL-8 was a gift from S.L. Kunkel, University of Michigan, Ann Arbor, MI, USA. TNF receptor antibodies were HTR-9, anti p55, IgG1, and HTR-1, IgM (Ch. 7). Other MAb were raised at the IMVS by Joe Wrin and Sun Qiyu.

2.2.5 Radiolabelled albumin

Bovine serum albumin (BSA)(Commonwealth Serum Laboratories, Melbourne, Vic) in PBS was labelled with ^{125}I (Amersham) by the Chloramine-T method[294]. Activity of each preparation was approximately 5×10^5 cpm/ml, and the trichloroacetic acid precipitatable activity was >97%.

2.3 Neutrophil preparation

Neutrophils were prepared from fresh blood from healthy volunteers. Citrated blood was dextran sedimented, and the buffy coat separated by Ficoll/Hypaque (Nycomed, Oslo, Norway) gradient centrifugation followed by hypotonic lysis of remaining red cells. Purity of preparations was >93% neutrophils as judged by morphological examination of Wright's stained cytocentrifuge preparations. Contaminating cells were mostly eosinophils.

2.4 Endothelial cell culture

HUVEC were isolated by collagenase treatment of umbilical veins[295]. Umbilical cords were obtained within 24h of delivery. The vein was cannulated and flushed with M199 + antibiotics, then filled with collagenase and incubated in a 37°C water bath for 10min. It was then washed, the washings retained and centrifuged, and the cells resuspended in HUVEC medium. EC were cultured in 25cm² or 75cm² gelatin coated plastic flasks, and when they reached confluence, harvested by detachment with trypsin/EDTA, washed, and split 1 in 2 (passaging). Cells were used between passages 2 and 6, except for experiments described in chapter 9 where primary cells (first subculture) were used. Endotoxin levels in media used were <50pg/ml by limulus amoebocyte assay.

2.5 Neutrophil adhesion assays

U-bottomed 96 well microtitre trays (Nunc, Denmark) were used for experiments of neutrophil adhesion to plastic. For assays of neutrophil adhesion to EC, HUVEC monolayers were prepared by harvesting EC with trypsin/EDTA and plating them onto gelatin coated flat- bottomed microtitre trays (Nunc, Denmark) at 2×10^4 cells per well in 200µl HUVEC medium. Monolayers were grown to confluence overnight, and washed in M199 with 2·5% FCS (assay medium) before use. For some experiments monolayers were incubated with TNF-α 100U/ml for 4 hours prior to the assay.

Adhesion of neutrophils was quantified by a method previously described[(296)]. 5×10^5 neutrophils with or without stimulant in assay medium were added to each well and incubated for 30min at 37°C, 5%CO_2. After removal of supernatant, 200 µl of the vital dye Rose Bengal (0·25% in PBS) was added to each well, and incubated for 5 minutes at room temperature (RT). Wells were then washed twice gently to remove non adherent cells, and 200µl of ethanol/PBS (1:1) added to fix the cells. After at least 60min the optical density reading at 570nm (OD_{570}) of each well was determined using an ELISA reader. For measurement of the level of adherence to endothelial monolayers the OD_{570} of wells containing EC alone was subtracted from the OD_{570} of wells containing EC and attached neutrophils. There is a linear relationship between the OD_{570} and the number of adhering cells. The proportion of cells adhering is calculated by expressing the OD_{570} of the adherent cells as a percentage of the OD_{570} of 5×10^5 stained and ethanol:PBS treated neutrophils.

In some cases, neutrophils were preincubated with stimuli prior to the adhesion assay. 0·5ml of neutrophil suspension at 10^7 cells/ml in assay medium was incubated with IL-8, TNF-α, or f-MLP for 15min at RT. 1ml of ice cold assay medium was added, and the tubes spun for 15 seconds in a microcentrifuge at 6500rpm. The cells were then washed again and resuspended in warm assay medium prior to addition to the EC monolayers. The whole washing procedure was completed in 4-5min. To further shorten the time between stimulation and adhesion, following the addition of neutrophils the trays were spun at 10g

for 1 minute to settle the neutrophils. The plates were then incubated for 10min at 37°C, and adhesion quantified as described above.

2.6 Neutrophil chemotaxis assays

10^6 neutrophils in 100μl of assay medium were placed into transwells (Costar, Cambridge, MA, USA) (6·5mm diameter), which have as their base a polycarbonate filter membrane with 3μm pores. These upper compartments were then placed into a 24 well culture dish, which had been precoated with gelatin (to prevent neutrophil adhesion). The lower compartment contained 600μl of assay medium. The lower and/or upper compartments could also contain IL-8 or G- or GM-CSF at varying concentrations. Neutrophils which migrated through the filters after 45 minutes incubation (37°C, 5%CO_2) were retrieved from the lower compartment and counted using a Coulter Counter (model ZF, Herts, UK). Counts are expressed as a percentage of the cells added.

2.7 Neutrophil transmigration assays

2.7.1 Transwells

Transwells (6·5mm diameter, 3μm pores) were coated with fibronectin (50μg/ml for 30min at RT) and seeded with EC at 5×10^4 cells per well in 150μl of HUVEC medium. These were placed in 24 well cluster trays (Costar),the lower well containing 700μl of HUVEC medium, and cultured for 3 or 4 days at 37°C, 5%CO_2 (Adaptation of methods of (261,265,297)) (Fig. 2.1). Confluence was determined both prior to and following treatment with cytokines by measuring permeability to radiolabelled albumin[297]. Briefly, endothelial monolayers were washed and 150μl of HUVEC medium containing ^{125}I-albumin (1:20 dilution of stock solution) was pipetted into the well. The transwells were then placed into 24 well trays, the lower compartment containing 700μl of HUVEC medium. After 60min incubation at 37°C, 5%CO_2, the transwells were removed and 200μl was sampled from the lower well and the radioactivity counted. Transwells without endothelium (filter alone) were used for comparison. Diffusion of albumin was expressed as a percentage of equilibrium, which was simulated by mixing 150μl of medium

containing ^{125}I-albumin with 700μl of medium. Transwells were used after 3 or 4 days of culture, at which time albumin diffusion was generally <10% of equilibrium at 60min (Fig. 2.2)[261]. HUVEC monolayers with permeability >10% were found to give poor results in transmigration assays and were discarded.

In some experiments, monolayers cultured in transwells were preincubated with IL-1 or TNF-α by adding this in equal concentrations to the upper and lower wells 4 hours prior to the assay. IL-1 or TNF-α treatment did not increase the permeability of the monolayers (BSA diffusion- unstimulated HUVEC monolayer 7·0 ± 1·0%, IL-1 treated HUVEC monolayer 5·5 ± 0·8%, p=0·257 by independent t-test, mean of 5 experiments performed in duplicate).

Monolayers were washed with assay medium prior to each assay, by exchanging medium in the upper and lower compartments. The assay medium in the upper compartment was aspirated and 10^6 neutrophils were added to each transwell, in a final volume of 100 or 150μl of HUVEC medium. The medium in the upper compartment in some experiments also contained antibodies or cytokines *ie.* IL-8, calculated to an appropriate final concentration. The transwells were then placed in 24 well culture trays which had been precoated with gelatin. The lower well contained 600 or 700μl of HUVEC medium, with or without IL-8 or other factors. This assembly was incubated for 45-60 mins, and at the end of this time the wells were shaken to dislodge neutrophils from the lower surface of transwells, and the transwells removed. The medium in the lower wells was thoroughly mixed, and aliquots taken for counting of migrated neutrophils in a Coulter Counter (model ZF, Herts, UK) Counts are expressed as a percentage of the total cells added. Modifications and variations of this basic assay will be discussed in the relevant chapters.

As a test of the validity of this method of quantifying transmigration, I determined whether a significant number of cells remained adherent to the underside of the filter after the assay, using ^{51}Cr labelled neutrophils[9]. Transmigration experiments were carried out as described above, using an IL-8 chemotactic stimulus or TNF-α activation of endothelium. At the end of the assay, cells were retrieved from the lower well, and also scraped from the

lower surface of the filter, for counting. Using this method the number of cells adherent to the filter was <4% of the total, and proportional to the total, for either stimulus.

In some experiments (Fig. 3.8) albumin diffusion was tested during neutrophil transmigration, by adding 50μl of ^{125}I-albumin in HUVEC medium to 100μl of neutrophil suspension in the upper chamber, and measuring activity of aliquots from the lower chamber after the assay. To exclude transport of albumin in or bound to neutrophils, aliquots from the lower chamber were centrifuged (5 mins, 1500rpm) and the supernatants counted in parallel with the pre-centrifuged samples.

2.7.2 Slide assay

Methods were adapted from those previously described(258,259). Endothelial cells (10^5 cells per well, in 300μl HUVEC medium) were plated into wells on 8 well Labtek chamber slides (Nunc, Naperville, IL, USA) precoated with fibronectin, and allowed to reach confluence over 3-4d. 4h prior to experiments, 100U/ml IL-1 or TNF-α was added to some wells. All wells were washed twice with assay medium immediately prior to the addition of neutrophils. Neutrophil suspensions, in some experiments preincubated (desensitised) in chemotactic factors or medium, were diluted to 10^6 cells/ml and 200μl was added to the endothelial monolayers. The slides were then incubated at 37°C, 5%CO_2 for 20 min. At the end of this time, the medium and non adherent cells were tipped off, the upper chamber structures were removed, and non- or loosely adherent cells removed by dipping the slides into pre-warmed assay medium. The slides were then viewed under phase contrast microscopy within the next 5 minutes, and 5 high power fields (40× objective) were randomly selected from each well and photographed. Adherent cells were clearly recognised by their rounded or polarised phase bright appearance, while transmigrated neutrophils were easily distinguished since they were spread out on the glass, phase dark but with a bright halo (Fig. 4.7)(258). Counts are given as cells per high power field (photograph) and are means of the 5-6 fields. Pooled figures refer to pooling of the means from different wells.

Sample HUVEC monolayers cultured on fibronectin-coated glass slides were stained with silver nitrate ($AgNO_3$) to demonstrate cell junction formation and confirm confluence (Fig. 2.5). For $AgNO_3$ staining, slides were washed in M199 to remove protein, and flooded briefly (10s) with a solution of $AgNO_3$ 0·1% in H_2O[298], then rinsed again in M199 and fixed in M199/1% formaldehyde for 30min before mounting. In transmigration slide assays, confocal microscopy confirmed that the flattened cells visible by phase contrast microscopy had indeed migrated beneath the monolayer and were not merely spread on top of the EC (see next section, Figs. 2.6, 2.7).

The two assays of transmigration outlined here provided complementary information. The transwell assay offers the opportunity to create a chemotactic gradient across the endothelial monolayer, as well as to pre-incubate the endothelium in various cytokines which may activate the EC to induce transmigration. It is a quantitative assay which is generally quite reproducible, although some variation occurs with different lines of endothelial cells and neutrophil donors. However, it is not possible to visualise the endothelium directly, and gives no information about neutrophil-endothelial adhesion. It relies on the ability of neutrophils, having transmigrated through the endothelium, to also migrate through the polycarbonate filter and drop off into the medium of the lower compartment. This appears not to be a great problem, since radio-labelling experiments indicate that few neutrophils remain associated with the filter. Some preliminary experiments with monocytes, however, indicated that most cells remained adherent to the undersurface of the filter, suggesting that this may not be a suitable assay for monocyte transmigration. The slide assay has the advantages of direct visualisation and counting of both adhesion and transmigration, as well as the ability to see the endothelial monolayer. It may be adaptable to monocytes and other leukocyte types (work is continuing in our laboratory) and has the advantage, more important in other leukocyte types, of requiring far less cells. However, quantitation is less accurate, due to variability in adhesion and transmigration rates in different parts of the same slide, in addition to potential observer variation in interpretation of cellular appearance. In some cases, where possible, both assays were used (Ch. 4) to examine the same or related questions.

2.7.3 Desensitisation of neutrophils

In some experiments (Ch. 4) neutrophils were desensitised to chemotactic factors prior to transmigration experiments. Neutrophils (10^7/ml) were preincubated in assay medium at 37°C for 30min with frequent manual agitation, with chemotactic factors, at the concentrations indicated in the text. At the end of the incubation the cell suspension was placed on ice, and washed twice in PBS at 4°C before resuspending in assay medium for transmigration experiments. These cells remained viable by criteria of trypan blue exclusion (<99%) and showed no increase in release of lactate dehydrogenase at IL-8 concentrations of up to 1µM. Control cells were subjected to the same treatment except the initial incubation was in medium alone. The purpose of cooling to 4°C was to preserve the desensitised state, since washing at RT resulted in less effective specific desensitisation. The transmigration of neutrophils was not affected by washing at 4°C, since transmigration of control cells was comparable with unwashed cells to all stimuli tested, as was the response of desensitised cells to the heterologous chemotactic stimulus.

2.8 Confocal microscopy

Confocal laser scanning microscopy was used to examine transmigration of neutrophils through endothelium cultured on glass slides (section 2.7.2), for the purpose of verifying that neutrophils migrated beneath the endothelium, and to image the immunofluorescent staining of HUVEC for IL-3Rα chains (Fig. 8.3).

For studying slide transmigration assays, HUVEC monolayers were cultured on fibronectin coated glass slides as described above, preincubated with TNF-α for 4h, and incubated with neutrophils for 20min, to allow transmigration to occur. Slides were washed of non-adherent neutrophils, stained with $AgNO_3$ to mark cell borders, and fixed in M199/1% formaldehyde for 30min at RT. The slides were visualised by fluorescence microscopy using a Bio-Rad MRC-600 confocal microscope equipped with an argon-ion laser (excitation wavelength 488nm) and bandpass filters set to detect an emission wavelength of 510-515nm. The samples were unstained, and images were detected using cellular autofluorescence, which is known to be high for neutrophils, and was lower for endothelial

cells. Fluorescence was reasonably bright, which enabled a narrow confocal aperture, which maximises the confocal effect of optical sectioning. This means that light from the plane of focus only was displayed, with light from above and below that plane being excluded, resulting in an image that represents a section of the cells parallel to the slide. Several different planes at different levels above the surface of the slide were then imaged, resulting in a series of sections (Fig. 2.3). Phase contrast micrographs were also acquired in parallel using the laser scanning microscope's transmitted light capture unit. A vertical section, perpendicular to the plane of the slide (*xz*-section, as opposed to *xy*-section) was acquired by scanning a single line across the slide and moving the microscope stage, relative to the plane of focus, such that repeated single line scans built up an image in the vertical plane. Serial sections (Fig. 2.3) and vertical sections (Fig. 2.4) of the slide assay demonstrates that neutrophils can be detected both above and below the EC monolayer.

For immunofluorescent studies, HUVEC were cultured on fibronectin coated glass slides, and treated with TNF-α 100U/ml for 24h. The monolayers were washed, incubated with saturating amounts of MAb for 30min at 4°C, washed again, and then fixed/permeabilised by dipping the slides in methanol/acetone (1:1) for 30s. Cells were stained with primary MAb before fixation/permeabilisation to ensure that surface structures only were stained. The slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Silenus, Hawthorn, Victoria) for 30min at 4°C, washed, and mounted under coverslips. IL-3Rα stained slides showed very dim fluorescence, which required a fully opened confocal aperture, resulting in maximum light acquisition but no optical sectioning, *ie*. equivalent to normal immunofluorescent microscopy. The advantage of using the laser scanning microscope in this case was improved sensitivity, as well as the digital acquisition of images which allowed brightness and contrast enhancement in a highly controlled fashion. Negative control as well as IL-3Rα stained samples were enhanced to the same degree.

2.9 Flow cytometry

Flow cytometry was used to examine HUVEC expression of E-selectin (Ch. 7) and IL-3Rα (Ch. 8). HUVEC were cultured in 24 well cluster dishes, and treated with TNF-α or TNF-α mutants, for 4 or 24 hours, as indicated. The wells were washed twice with assay wash, and incubated with MAb 10μg/ml in assay wash for 30min at 4°C. After a further wash, the wells were incubated with FITC-labelled rabbit anti-mouse IgG, 1:80 in assay wash, for 30min at 4°C, and then washed with FC wash. The cells were then removed from the wells with trypsin/EDTA, centrifuged and resuspended in FC fix. The fluorescence intensity of the cells was determined by a Coulter Epics Profile II flow cytometer, counting 5,000 or 10,000 cells for each experimental point. Frequency histograms of fluorescence intensity for each cell population were constructed by the Profile software, and the mean fluorescence intensity (MFI) of each population was calculated. In some figures, MFI values from multiple experiments were pooled for statistical analysis (Fig. 8.2). Histograms were mathematically smoothed, without changing the shape of the curve, using a smoothing program written by Mr. Alan Bishop, Flow Cytometry supervisor, of the Hanson Centre, IMVS.

2.10 Enzyme linked immunosorbent assay for IL-8

IL-8 in the supernatants of EC was quantitated by enzyme linked immunosorbent assay (ELISA). The ELISA was a sandwich-type, using polyclonal anti-IL-8 as a capture layer, and biotinylated polyclonal anti-IL-8 (the same antibody) as a detection layer. The method was developed from the technique described[299].

A high-titre anti-IL-8 antiserum, raised in rabbits by immunisation with human IL-8, was obtained from Dr. S. L. Kunkel, University of Michigan, Ann Arbor, MI, USA. Immunoglobulin was purified from the serum by sequential ammonium sulphate precipitation and protein A column chromatography[300]. This purified antibody was used to coat polyvinylchloride (PVC) 96 well assay plates (Costar), which had previously been found by experience in our laboratory to provide the best substrate for protein coating. The antibody was diluted to approximately 1μg/ml in bicarbonate buffer, pH9·6, and pipetted onto the

plate at 50µl/well, then incubated overnight at 4°C. A blocking step was tried but found not to be necessary, since samples were invariably measured in supernatants containing 20%FCS, which blocked nonspecific binding. Standards were prepared (in a large batch) by dilution of synthetic human IL-8 (section 2.2.1) in HUVEC medium. Samples were the undiluted supernatants of HUVEC and therefore also in HUVEC medium.

After washing the wells 3 times with PBS/ 0·05%Tween, samples and standards, 100µl/well, were incubated for 1h, 37°C, 5%CO_2, and the wells washed again 4 times. Biotinylated anti-IL-8 was added next. Purified anti-IL-8 was biotinylated using either an Amersham biotinylation kit (Amersham, Bucks., England) or sulfo-NHS-biotin (Pierce, Rockford, IL, USA), according to the manufacturers protocol. Biotinylated antibody was diluted 1:250 in PBS/ 0·05%Tween/ 1%FCS, and 50µl was incubated in each well for 30min, 37°C, 5%CO_2, and then washed. A complex of streptavidin/ biotinylated HRP (Amersham, Bucks., England) was diluted 1:1000 in PBS/ 0·05%Tween/ 1%FCS, and 100µl was incubated in each well for 30min, 37°C, 5%CO_2, and washed. The final step was the addition to each well of HRP substrate, OPD (o-phenylenediamine) (Sigma) in citrate buffer with 0·03% H_2O_2, which was incubated at RT until colour development was adequate (generally 5-30min) and then stopped by the addition of 50µl of 1M H_2SO_4 to each well. The procedure is shown diagrammatically in Fig. 2.8. Optimal dilutions of primary coating antibody, biotinylated antibody, and streptavidin/biotinylated HRP were determined by checkerboard analysis of negative and positive standard samples. The assay was shown to be specific for IL-8, both 72 and 77 amino acid forms, and no reactivity was seen with standard preparations of TNF-α, IL-1, GM-CSF, heparin/ ECGS (used in HUVEC culture), and MGSA which is a member of the IL-8 family.

Optical density at 490nm (OD_{490}) was determined on a multi-well spectrophotometer. The standard curve, expressed as a semi-log graph, was approximately linear over the range 0·1-10ng/ml, but the values were best fit by an asymmetric sigmoidal curve fit (Fig. 2.9). The sample values were calculated, using this equation, in ng/ml of IL-8. Samples from each separate supernatant were assayed in duplicate, and the mean value (ELISA mean) was then pooled with replicate supernatants to give a mean ± SD or SEM (experimental

mean). The coefficient of variation for the same sample assayed on the same plate (intra-assay CV) was generally <5%. The inter-assay CV was large at 10-50%, so samples tested on different days were not generally directly compared or pooled.

2.11 Northern blotting

Northern blotting was used to compare levels of mRNA for IL-8 in HUVEC activated with TNF-α alone or in combination with IL-3 (Ch. 8) or TGF-β (Ch. 9). Standard molecular biology solutions and techniques were as in "Current protocols in molecular biology"[301].

2.11.1 Total RNA preparation

Total cellular RNA was prepared according to the method of Chomczynski and Sacchi[302]. Briefly, HUVEC cultured in $75cm^2$ flasks or 6 well dishes were washed with PBS and then extracted into a solution of 25mM sodium citrate with 4M guanidine thiocyanate, 0·5% Sarkosyl, and 0·1M 2-mercaptoethanol. This was then combined with 0·1vol 2MNaOAc, 1vol water saturated phenol, and 0·2vol chloroform/isoamyl alcohol (49:1), cooled and centrifuged. The aqueous phase, containing the RNA, was then precipitated with 1vol isopropanol, resuspended in the guanidine thiocyanate solution described above, and re-precipitated with isopropanol. The pellet was washed in cold 70%ethanol/H_2O to dissolve salts and dried. The RNA was then dissolved in diethylpyrocarbonate (DEPC) treated H_2O and quantitated on a spectrophotometer using the absorption at 260nm.

2.11.2 Electrophoresis and transfer

The RNA was separated by electrophoresis on a formaldehyde/ 1% agarose gel using 10μg of total RNA per lane. The gel was stained with ethidium bromide and inspected and photographed under UV light to check for adequate separation and equal lane loading. The RNA was transferred to a nitrocellulose filter by capillary transfer, and cross-linked to the filter with UV light.

2.11.3 Preparation and labelling of DNA probes

IL-8 cDNA was obtained from Genentech, USA, and was in the pUC19 plasmid vector. The cDNA for glyceraldehyde phosphate dehydrogenase (GAP-DH) (ATCC no. 57090)

was in the vector pBR322. The plasmids were transformed into competent *e.coli*, colonies selected on ampicillin or tetracycline respectively, and grown up in 400ml cultures. Plasmid DNA was isolated from the bacterial pellets by chromatography using the Qiagen-tip and kit, according to the manufacturer's protocol. The plasmids were checked by running on a 1% agarose/TAE gel. The IL-8 plasmid was then digested with EcoR-I, and the GAP-DH plasmid with both Xba-I and Pst-I, yielding 478 and 780 base pair fragments respectively. The fragments were purified by cutting the bands from a low-melting point agarose gel, and the DNA extracted using the Geneclean kit (BIO 101, La Jolla, CA, USA). After quantitation of the DNA, 50ng aliquots were random prime labelled with ^{32}P-dATP using the Gigaprime kit (Bresatec, Adelaide, SA). Incorporation was checked by spotting a sample on a polyethyleneimine (PEI) thin layer chromatography strip, and separating incorporated from unincorporated label in a 0·8M NaH_2PO_4 buffer, pH3·5. The label was usually >70% incorporated into the cDNA fragment.

2.11.4 Hybridisation and phosphor screen exposure

The nitrocellulose filter containing the RNA was prehybridised to block nonspecific DNA adsorption by incubating for at least 4h with a solution of 5× SSC (sodium/sodium citrate) buffer with Denhardt's solution, 1%SDS, 0·05%NaPPi, and 100µg/ml salmon-sperm DNA, at 60°C. After this, approximately 2×10^7cpm of probe was added to the solution, and hybridisation carried out by overnight incubation. The filter was washed with SSC/1%SDS at 60°C, and then exposed to a phosphor storage screen (Molecular Dynamics) for 3 days. The filter was prepared for a second hybridisation by stripping the probe DNA by washing in 1%SDS at 80°C for 1h, and complete removal was confirmed by an overnight exposure to the phosphor screen. The prehybridisation and hybridisation procedure was repeated except that the probe for GAP-DH was used instead.

2.11.5 Quantitative analysis of phosphor image scans

The phosphor screen was scanned into a computer using the Image Quant phosphorimager and software (Molecular Dynamics) printed out on a laser printer, and the digitised file was analysed. Intensity of bands was quantitated by integration of pixel intensity within a

region corresponding to each band, after subtraction of a similar sized region of background. Where possible, the intensity of the IL-8 band was expressed as a ratio of the intensity of the band for GAP-DH.

2.12 Statistical methods

The variations are expressed as the standard deviation (SD) or standard error of the mean (SEM), as stated. *P* values were calculated by Student's paired or independent *t*-test, or analysis of variance (ANOVA), as indicated.

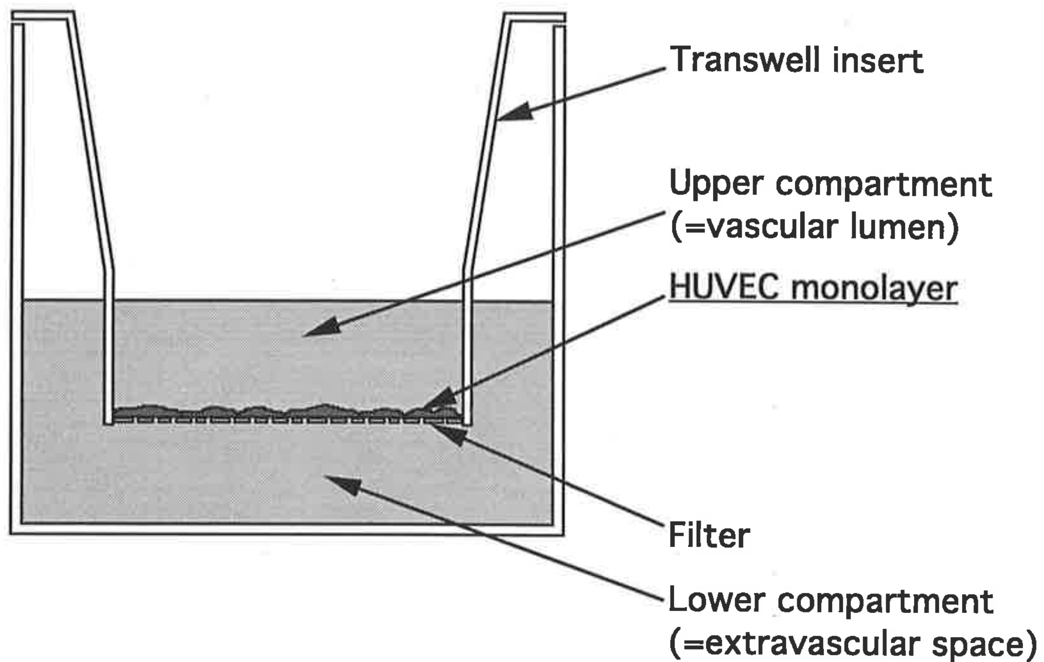


Fig. 2.1 Transwell used in transmigration assays

A diagrammatic representation of a transwell. The insert fits into a well of a 24 well cluster dish, and has as its base a polycarbonate filter 10μm thick, with pores 3μm in diameter. In order to keep the fluid levels in both the insert (upper compartment) and the well (lower compartment) approximately equal and avoid an hydrostatic gradient across the filter, the required volumes are either 100μl in the upper compartment and 600μl in the lower, or 150 and 700μl. The endothelial monolayer is cultured on the filter as shown, and forms a barrier between the upper and lower compartments. The upper compartment therefore represents the vascular space, and the lower compartment, the tissue space.

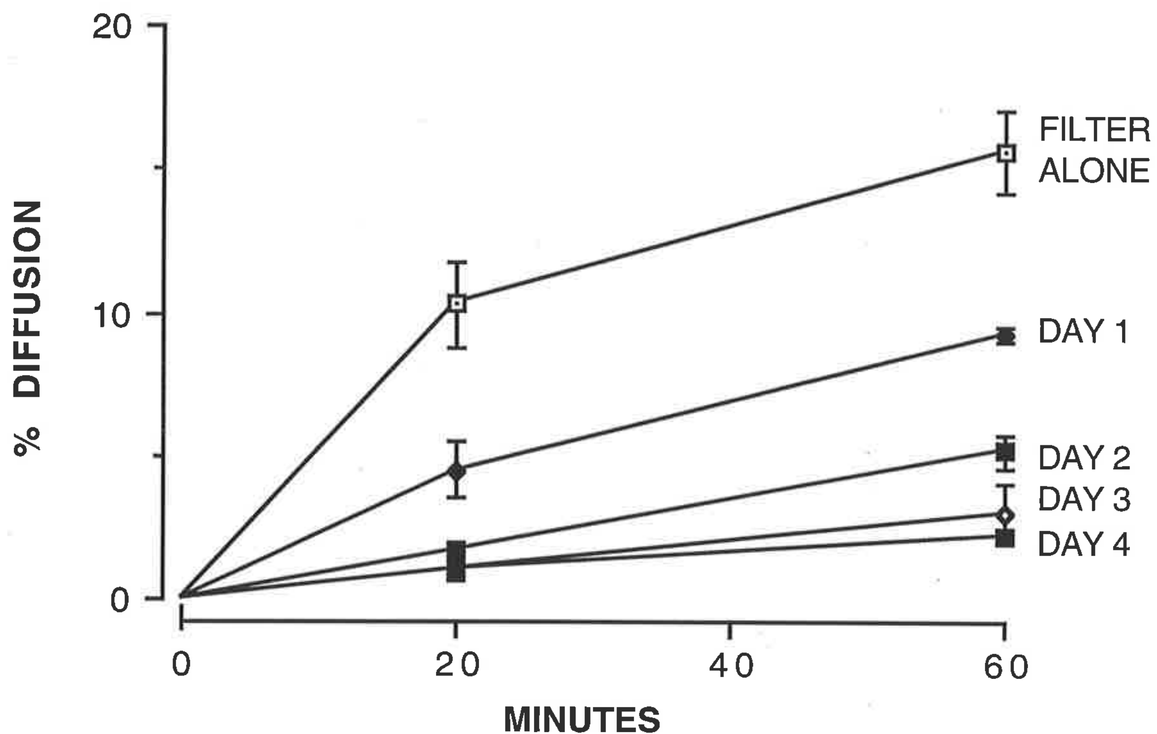


Fig. 2.2 Albumin permeability of endothelial monolayers cultured in transwells

Endothelial cells were seeded onto fibronectin-coated transwell inserts either 4, 3, 2, or 1 days prior to estimation of albumin permeability of the resulting monolayers. Filters alone, without endothelial cells, were used as a control. ^{125}I-albumin was placed in the upper compartment at time 0, and medium from the lower compartment was sampled at 20 and 60min. The diffusion of albumin through the monolayer is expressed as a percentage of equilibrium. Experiment conducted in duplicate.

Fig. 2.3 Electron micrograph of HUVEC grown on polycarbonate filters

HUVEC were cultured in fibronectin-coated transwells. The filter was cut out of the base of a transwell and prepared for transmission electron microscopy. An endothelial cell is seen overlying the dense homogeneous filter which is perforated with pores. The cell nucleus is present in the centre at the thickest part of the cell, the cytoplasm tapers off and is very thin at the cell edges. The cytoplasm is seen to lift away from the filter, which is probably fixation artifact.

Fig. 2.4 Electron micrograph of neutrophil transmigration.

The endothelial monolayer is seen overlying the filter. Two neutrophils have migrated through the endothelium, one lies between the endothelial cell cytoplasm and the filter and is entering a pore of the filter, and the other has traversed the filter and is emerging from the other side.

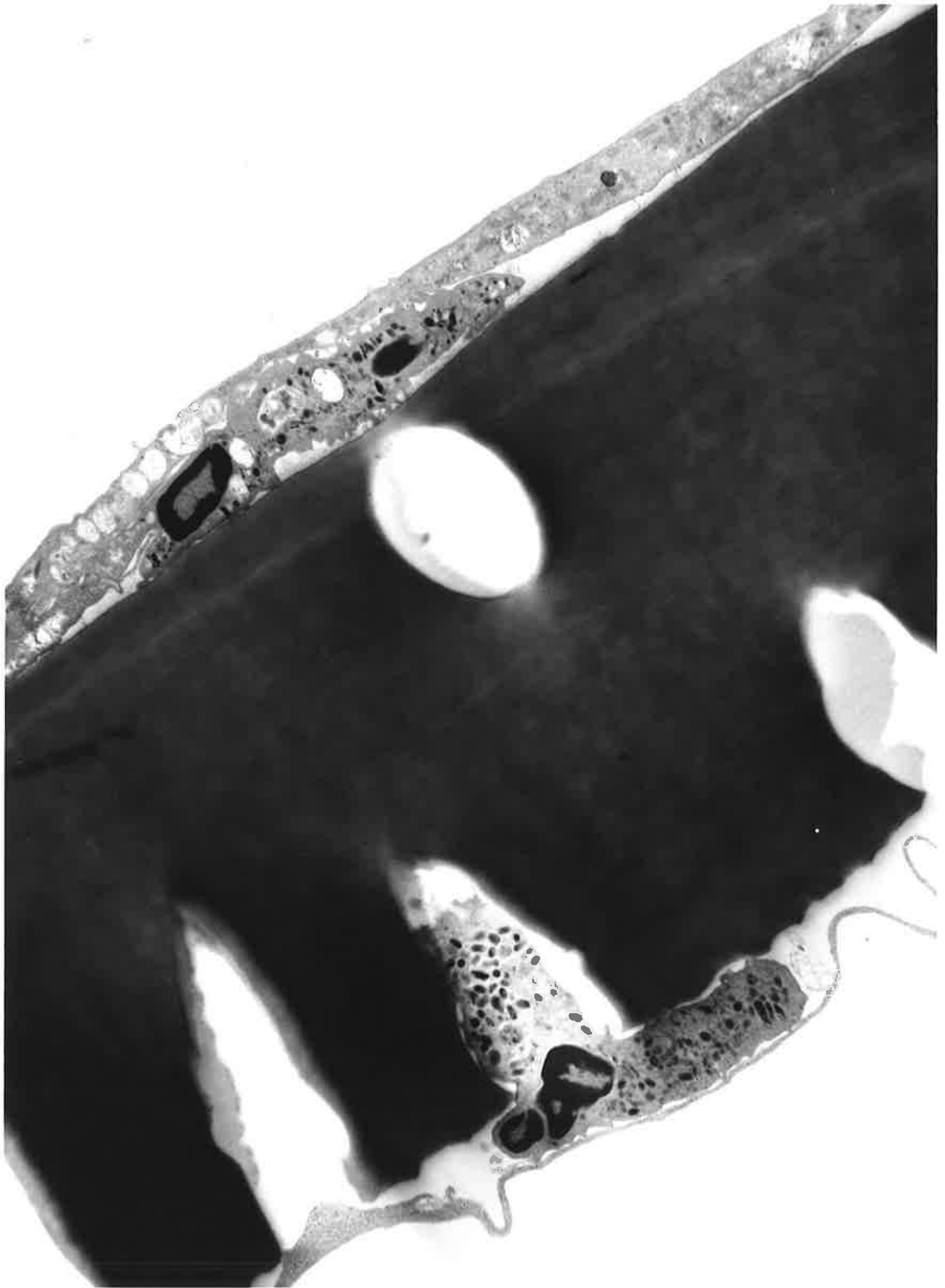


Fig. 2.5 Endothelial monolayer- silver stained

Endothelial cells were seeded into a well of a fibronectin coated multiwell Labtek chamber slide, and cultured for 4 days. Silver staining marks junctions between cells, showing that these are well developed, and indicates complete coverage of cells over the slide, and confluence of the monolayer with no gaps between cells.

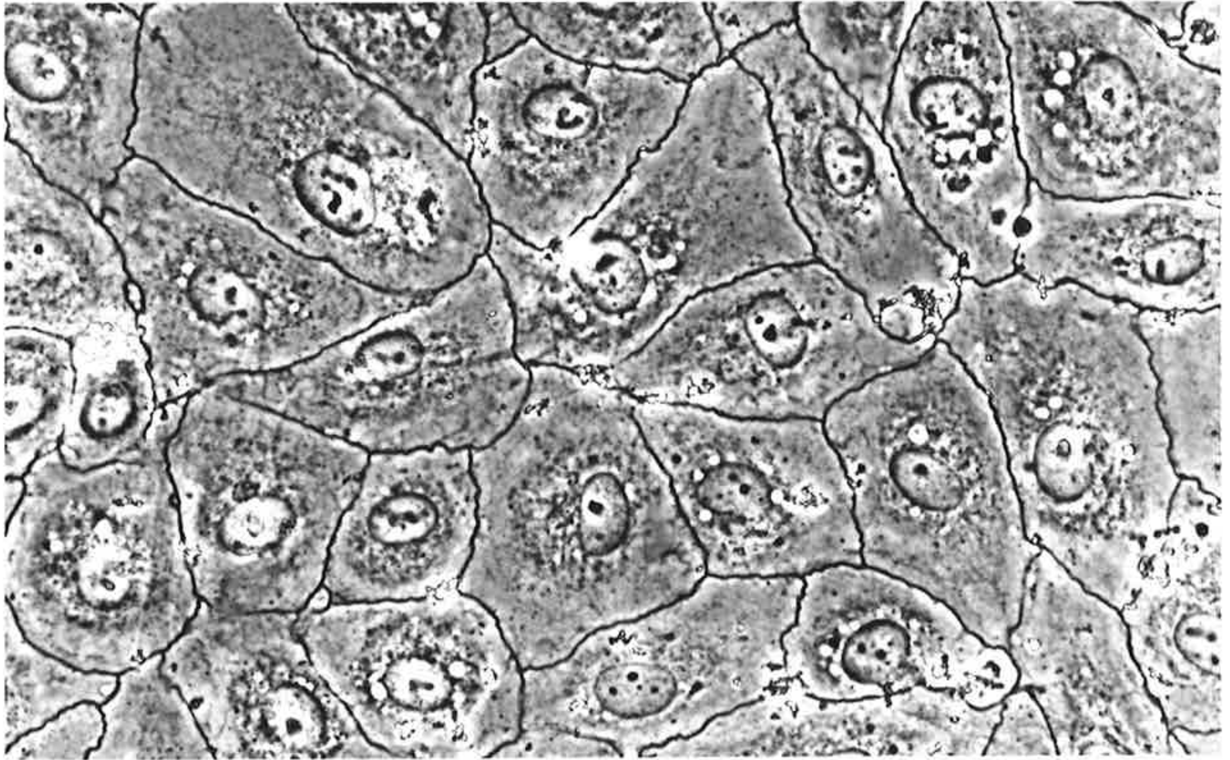


Fig. 2.6 Confocal serial section of neutrophil transmigration in the slide assay

HUVEC were cultured to confluence on a glass slide, then activated with TNF-α 100U/ml for 4h and co-incubated with neutrophils for 20min. After washing off non-adherent neutrophils, the slide was formaldehyde fixed and silver stained, and examined by fluorescence confocal microscopy using cellular autofluorescence. Panel 1 is the lowest plane of focus, immediately adjacent to the surface of the slide. A flattened neutrophil is shown clearly by fluorescence in the centre of the panel, and is in sharp focus, although faint (arrow) in the phase contrast image. As the plane of focus moves upwards, the junction between two endothelial cells becomes clear in the phase contrast image (panel 3, arrow) overlying the now out of focus neutrophil, which is not seen in the confocal fluorescent image since it is no longer in the plane of focus. This proves that the neutrophil is beneath the endothelium, having probably migrated through the overlying junction. Moving upwards further (panel 4), a rounded neutrophil is now in sharp focus, and several are shown in the confocal image. This cell is clearly above the monolayer, adherent to the surface.

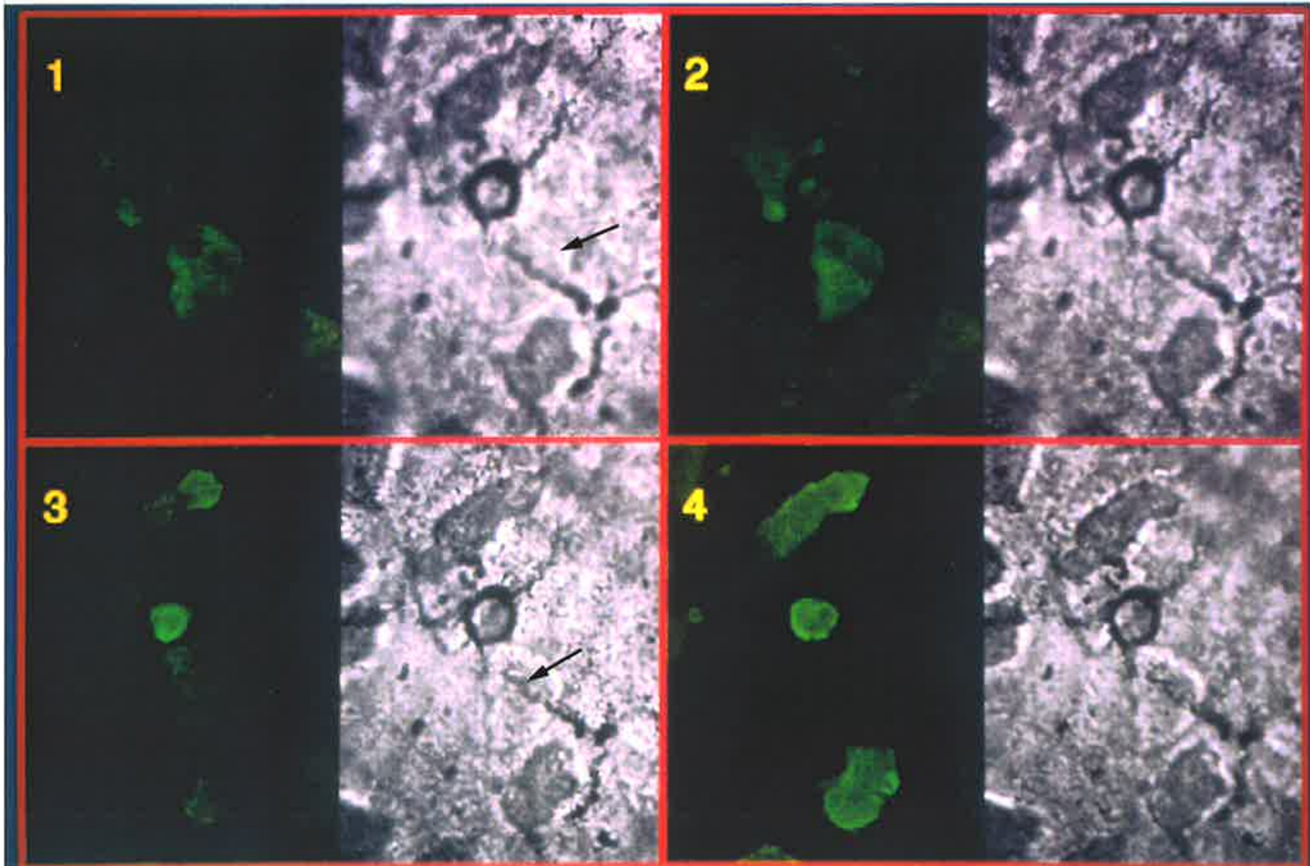

1
2
3
4

Fig. 2.7 Vertical section of neutrophil transmigration in the slide assay

The slide was the same as that used for Fig. 2.3. In this vertical section (*xz*; perpendicular to the plane of the slide), the endothelial monolayer is shown as a dim horizontal line above the slide. Neutrophils can be seen both above and below the endothelium, which lifts up over the transmigrated neutrophil, and one cell is seen in the process of migrating.

Adherent neutrophil
Transmigrating neutrophil
Endothelial Monolayer

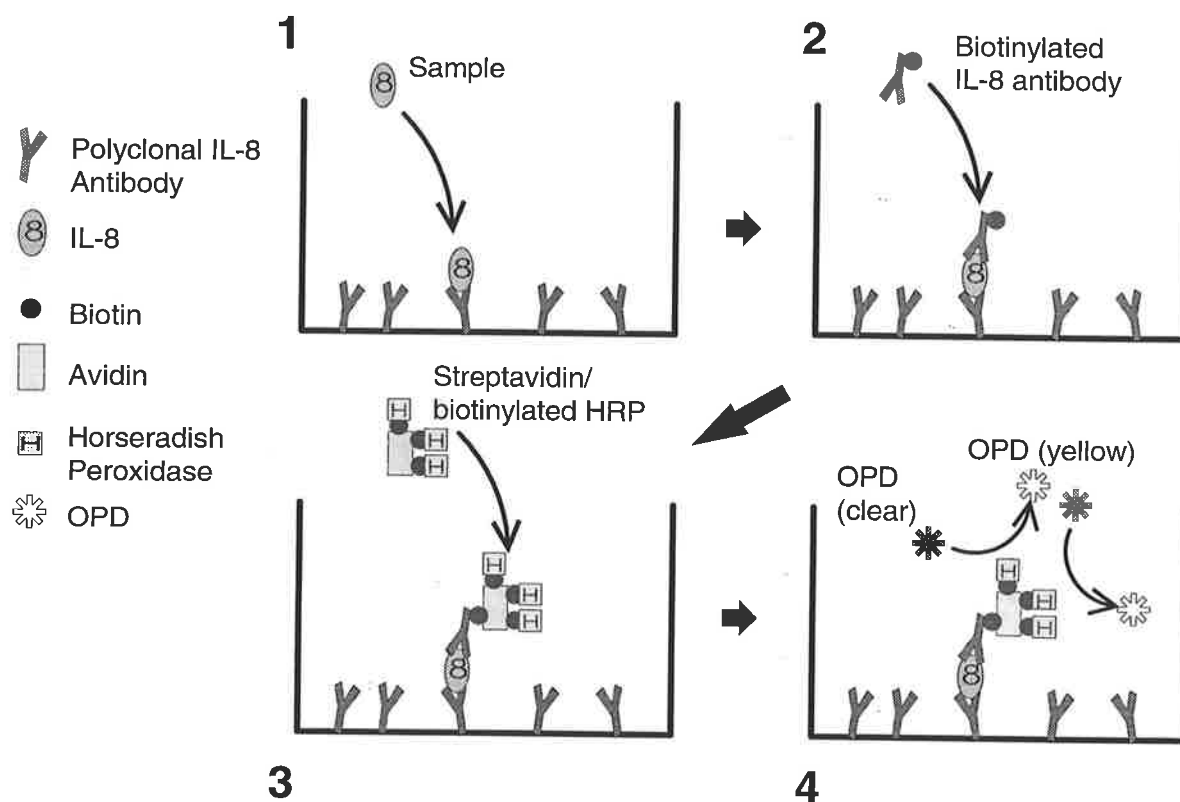
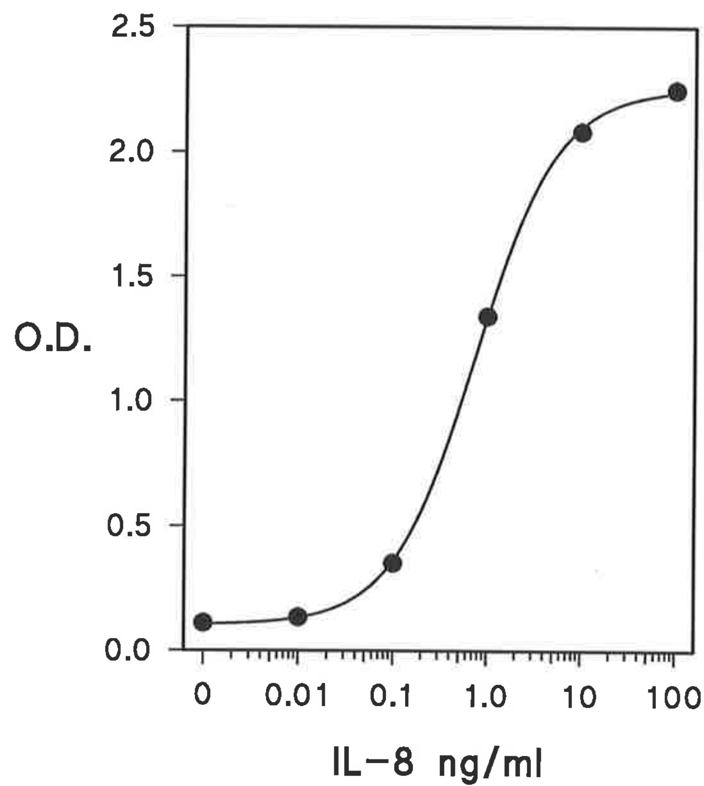


Fig. 2.8 The IL-8 ELISA

Plates were coated with polyclonal IL-8 antibody, and the samples added. IL-8 in the samples bound to the antibody on the plate (1). Biotinylated IL-8 antibody was then added, which bound to any IL-8 present (2). Excess unbound biotinylated antibody was washed away. Streptavidin, which has a very high affinity for biotin, was pre-bound to biotinylated HRP, at a stoichiometric ratio which left additional binding sites free. This was then added, and bound to any biotinylated antibody present (3). The clear OPD solution was then added, which turns a yellow colour as a result of the reaction of HRP with H_2O_2 (4). The density of this colour was proportional to the amount of HRP present, which in turn was dependent on the concentration of IL-8 in the sample. Amplification of the signal, leading to improved sensitivity, occurs at the following steps- (a) Because the detection antibody is polyclonal, more than one antibody may bind per molecule of IL-8; (b) each molecule of biotinylated antibody may be conjugated with more than one molecule of biotin; and (c) each streptavidin molecule may be bound to several molecules of biotinylated HRP.



2.9 IL-8 ELISA- representative standard curve

O.D. readings were taken at a range of IL-8 standard concentrations, in 10-fold dilutions from 100ng/ml to 10pg/ml. The standard curve is an asymmetric sigmoid function, produced using a curve-fitting program (Fig.P). Sample O.D. values were transformed using the curve-fit equation to ng/ml of IL-8.

CHAPTER 3

INTERLEUKIN-8 INDUCES NEUTROPHIL TRANSENDOTHELIAL MIGRATION

INTRODUCTION

Transmigration of leukocytes across the vascular endothelium is necessary for their tissue localisation. Adhesion of the circulating cell to the vascular endothelium is probably the first step in this process, but is not in itself sufficient to lead to transmigration. Two major mechanisms have been proposed for the induction of leukocyte transmigration: chemotactic attraction, and the activation of endothelium by inflammatory cytokines. The attraction of cells by chemotactic stimulants has become a paradigm in cell biology since its original proposal by Metchnikoff 100 years ago. Yet, the operation of this mechanism *in vivo* in attracting cells across the endothelial barrier and the agents responsible for it in inflammatory lesions have not been fully characterised. The other mechanism, only recently demonstrated, is associated with the activation of EC by the cytokines TNF-α, IL-1, and LPS. Endothelial activation was initially shown to stimulate adhesion of leukocytes[9,303] and recently to promote transendothelial neutrophil passage in the absence of exogenous neutrophil stimulation or chemotactic gradient[265,266].

IL-8, previously known as neutrophil activating peptide, induces shape change and degranulation in neutrophils, and is a powerful chemotactic agent[188]. Of particular interest is the secretion of IL-8 by EC stimulated with TNF-α, IL-1 and LPS[304], as well as by activated monocytes and fibroblasts. In this chapter, chemically synthesised IL-8 was used to investigate the role of this molecule in neutrophil adhesion and transendothelial migration. IL-8 stimulated the adhesion of neutrophils to plastic, and HUVEC monolayers *in vitro*, and this adhesion was inhibited by antibodies to the neutrophil adhesion molecule CD11b/CD18. Transmigration of neutrophils across endothelial monolayers was induced by chemotactic and chemokinetic stimulation with IL-8. Increased permeability of monolayers occurred at high rates of transmigration. In addition, I have used TNF-α preincubation of endothelium to induce transmigration, and have shown that these two stimuli can lead to additive effects.

RESULTS

3.1 IL-8 stimulates neutrophil adherence to plastic.

To determine the effects of IL-8 on neutrophil adhesiveness to plastic, neutrophils were incubated with IL-8 in uncoated polystyrene microtitre wells. Time course experiments indicated that a period of 30min incubation produced maximal and consistent adhesion (data not shown). Fig. 3.1A shows a concentration dependent stimulation of neutrophil adherence to plastic by IL-8, following 30min incubation (closed circles). This effect was significant above 10nM IL-8 and reached a maximum of 1·9× basal adherence at 100nM. Comparative concentration/response experiments indicated that even at the maximum concentration, IL-8 was a markedly less effective stimulus of neutrophil adhesion than TNF-α (Fig. 3.2)

3.2 IL-8 stimulates adhesion of neutrophils to HUVEC

The effect of IL-8 on neutrophil adhesion to endothelium was then investigated by coincubating neutrophils with endothelial monolayers in the presence of IL-8 for 30min. A concentration dependent increase in adhesion was observed, which was evident at 1nM, and increased up to 1000nM where an average of 20% of neutrophils were adherent, which was 2·7× the basal level (Fig.3.1A, open circles). To determine whether IL-8 affected endothelial cells as well as neutrophils, the endothelial monolayer was preincubated with IL-8 for varying time periods (10, 30min, 1, 4h) and washed prior to the adhesion assay. No increase in adhesion was seen (data not shown).

Confirmation that the pro-adhesive effect was solely due to neutrophil stimulation was provided by preincubation experiments. Neutrophils were preincubated with 10 or 100nM IL-8 (or with 10U/ml TNF-α or 100nM f-MLP) for 15 minutes and washed prior to addition to monolayers. After incubation on monolayers for 10min, adhesion was measured (Fig.3.1B). The adhesive effect of 100nM f-MLP and 10U/ml TNF-α were greater than that of IL-8 (p<0·05, p<0·005 respectively). With longer incubations, the effects of IL-8 preincubation were lost (data not shown), suggesting a transient effect.

Preincubation of neutrophils with MAb to CD18, the common β-chain of neutrophil integrin adhesion molecules (60·3), and CD11b, the α-chain of Mac-1 (60·1), inhibited adhesion of both unstimulated and IL-8 stimulated neutrophils. 60·5, a control antibody to HLA Class 1, had no effect (Fig. 3.3). This implies that the increase in adhesiveness of neutrophils stimulated by IL-8 is mediated at least in part by the Mac-1 molecule.

3.3 Effects of IL-8 on neutrophil adhesion to TNF-α stimulated endothelial monolayers

When monolayers were incubated with 100U/ml TNF-α for 4h prior to the assay and washed, the adhesion of unstimulated neutrophils was increased (Fig. 3.4). 10 and 100nM IL-8 led to further significant increases in adhesion.

3.4 IL-8 chemotactic transmigration

Figure 3.5 shows the effect of graded concentrations of IL-8 placed in the lower compartment on the migration of neutrophils through an endothelial monolayer. Very few neutrophils migrated randomly through the monolayer under control conditions when medium alone was placed in both compartments(2·2 ± 0·7%), confirming that the monolayer presents an effective barrier (compare filter chemotaxis). IL-8 0·01 and 0·1nM in the lower compartment also did not induce transmigration, but 40% of neutrophils migrated in response to 1nM IL-8, with maximal transmigration, of 87·4 ± 2·1% of cells added, seen at a 10nM gradient.

This can be compared with chemotaxis experiments which induce neutrophil migration across filters alone. The peak level of migrating cells is lower, perhaps indicating better preservation of the gradient in the presence of the monolayer.

3.5 IL-8 chemokinetic transmigration

Neutrophil transmigration was also induced with equal concentrations of IL-8 on both sides of the monolayer (Fig. 3.6). The concentration/response relationship was different to chemotactic experiments, with a maximum transmigration of 21 ± 3·8% reached at 1nM IL-8, which diminished and returned to baseline at higher concentrations.

3.6 TNF-α activation of endothelium

To determine the effects of TNF-α preincubation of endothelium on transmigration of unstimulated neutrophils, monolayers in transwells were incubated in medium containing TNF-α for 4h and washed prior to the addition of neutrophils. A concentration dependent increase in transmigration of neutrophils was seen (Fig. 3.7). Responses of individual HUVEC culture lines to TNF-α were variable, but 4 to 8 fold increases over baseline were generally seen, with peak levels of transmigration approaching 30-40%.

3.7 Combination of endothelial activation and IL-8 chemotaxis

TNF-α activation of endothelium led to additive increases in transmigration when combined with an IL-8 chemotactic stimulus (Fig. 3.8). This effect persisted at maximal IL-8 gradients, but was less consistent.

3.8 Monolayer permeability during transmigration

To determine whether transmigration resulted in increased permeability of the monolayer, simultaneous experiments of transmigration and albumin diffusion were carried out (table 3.1). Diffusion of ^{125}I-labelled albumin through the endothelial monolayer was increased at high rates of transmigration. The albumin was not cell bound, as centrifuged samples gave identical counts. In chemokinetic experiments where transmigration was minimal, no increase in permeability occurred despite the exposure of neutrophils to the same concentrations of IL-8, suggesting that increased permeability is not simply related to neutrophil mediated endothelial injury but is dependent on transmigration.

Table 3.1 Diffusion of albumin during transmigration

	Transmigration (% of cells added)	Albumin diffusion (% of equilibrium)	Albumin diffusion (% of filter alone)
Nil	1·6 ± 0·4	2·7 ± 0·2	13·2 ± 1·0
IL-8 10nM chemotaxis	74·3 ± 6·0	6·1 ± 1·0*	29·9 ± 4·8*
IL-8 10nM chemokinesis	10·8 ± 0·8	2·9 ± 0·2	14·4 ± 1·2

^{125}I-Albumin (10^6 cpm) was added to the transwell at the same time as the neutrophils in a chemotactic and chemokinetic transmigration assay as outlined in Methods. In addition to counting the neutrophils in the lower compartment after 60 minutes of incubation, samples were taken for γ counts. Composite of 8 experimental values (4 experiments in duplicate). * $p<0.05$, by Student's independent *t*-test.

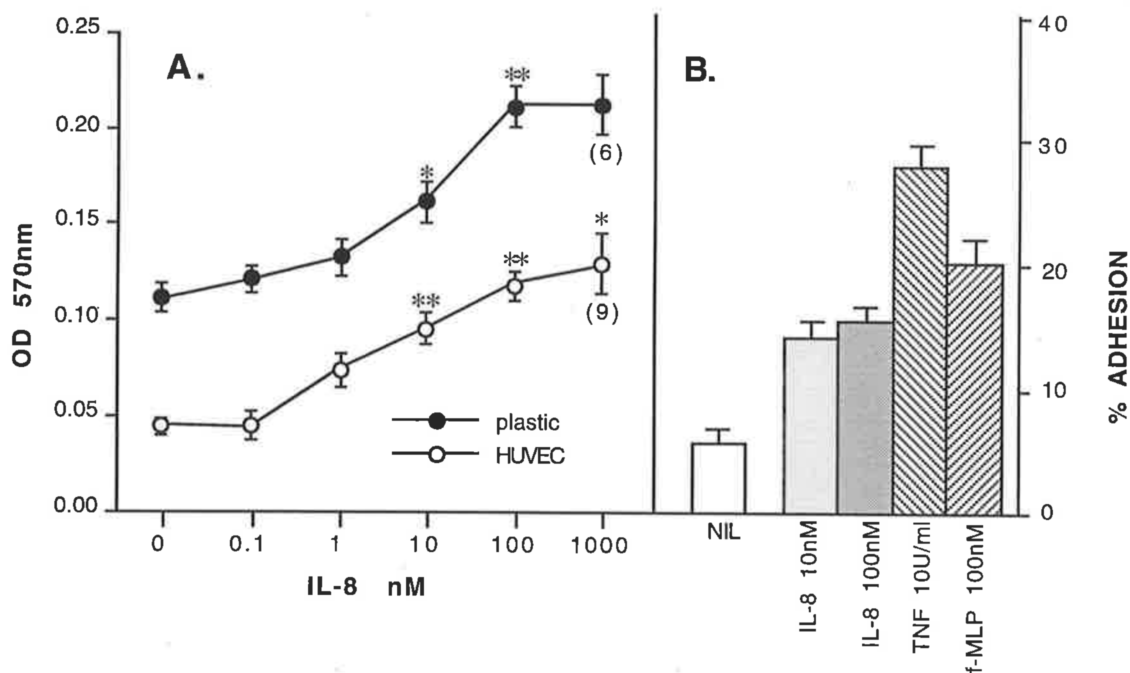


Fig. 3.1 Adhesion of neutrophils to plastic and to endothelium is stimulated by IL-8

(A) Neutrophils (5×10^5) were incubated with varying concentrations of IL-8 in uncoated polystyrene wells or wells containing endothelial monolayers (HUVEC) for 30 minutes. Adhesion is quantitated by Rose Bengal staining as described in Methods. (The *y*-axes represent the directly measured OD_{570}, and the calculated % adhesion, and relate to both graphs A and B.) Each point is a composite of at least 18 experimental values (6 experiments in triplicate) except where indicated by (n). ** $p<0{\cdot}005$, * $p<0{\cdot}05$ compared to 0 IL-8, by independent Student's *t*-test. (B) Preincubation of neutrophils with IL-8 with subsequent washing also increases adhesion to endothelial monolayers in a 10 minute adhesion assay. Each column is a composite of 9 experimental values (3 experiments in triplicate). All stimulants produced significant adhesion compared to nil, $p<0{\cdot}005$ by independent Student's *t*-test.

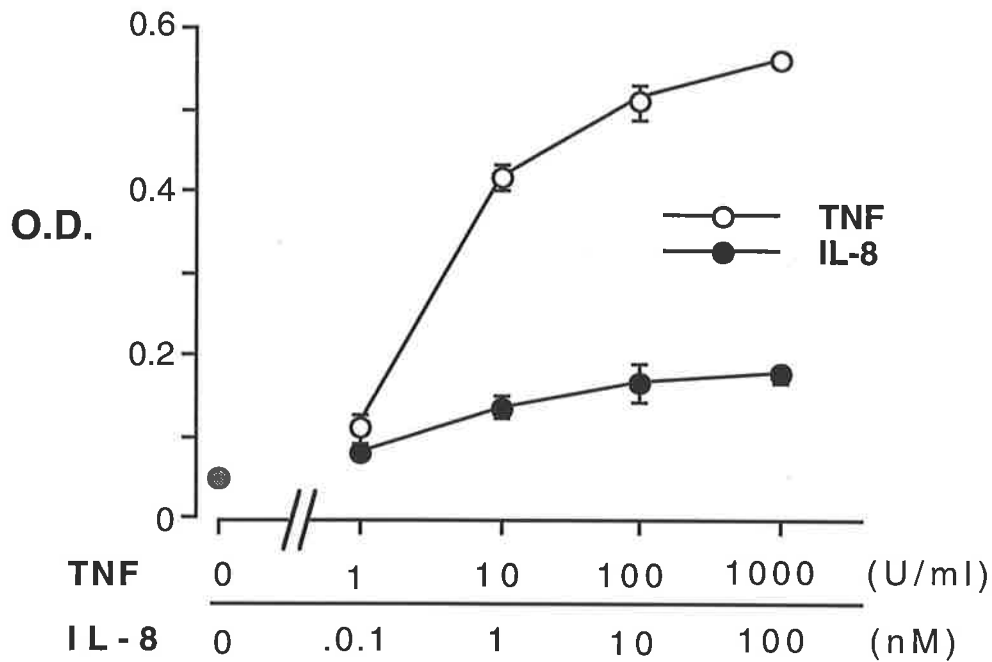


Fig. 3.2 Comparison of the effectiveness of IL-8 and TNF-α at stimulation of neutrophil adhesion to plastic

Neutrophils were co-incubated with IL-8 or TNF-α at the indicated concentrations in polystyrene wells for 30min. Non-adherent cells were then washed off, and adhesion quantitated by OD_{570} of the Rose-Bengal stained cells. Representative experiment, in triplicate. Points are the mean ± SEM OD_{570}.

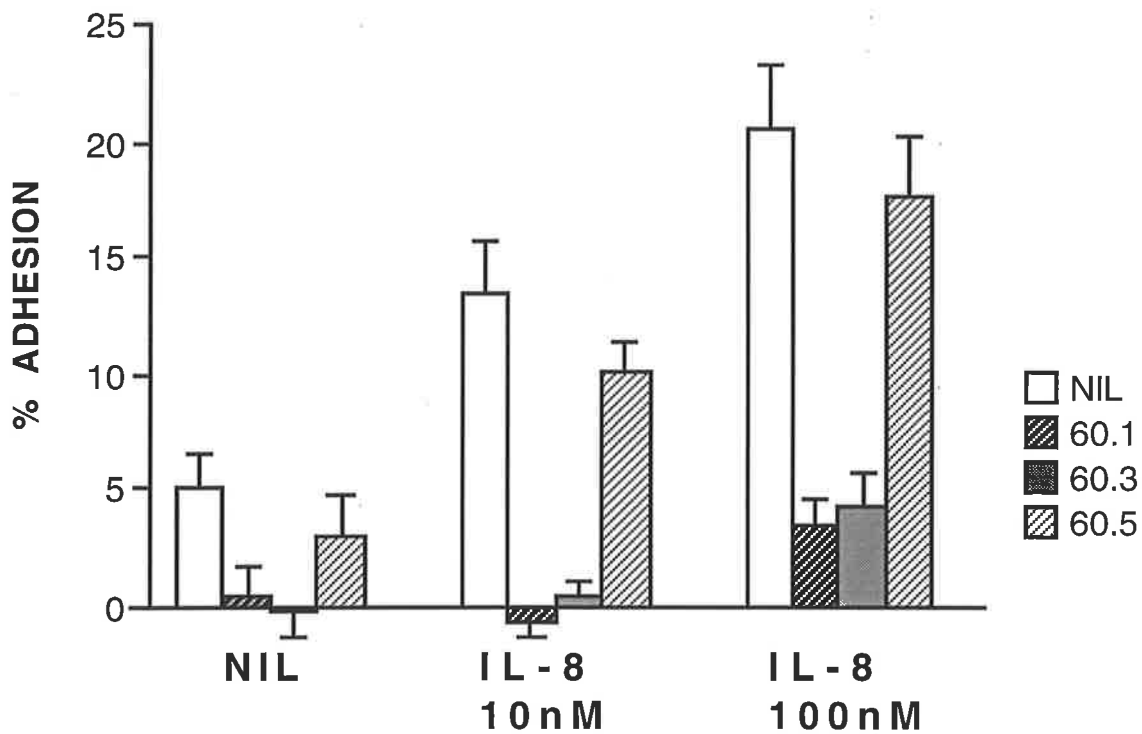


Fig. 3.3 Monoclonal antibodies 60·1 and 60·3 inhibit both IL-8 stimulated and unstimulated adhesion

Neutrophils were preincubated with antibodies for 15min at room temperature, then added to endothelial monolayers in a standard adhesion assay as described in Methods. 60·1 = anti-CD11b, 60·3 = anti-CD18, 60·5 = anti-HLA class I. Composite of 9 experimental values (3 experiments in triplicate). Error bars indicate SEM.

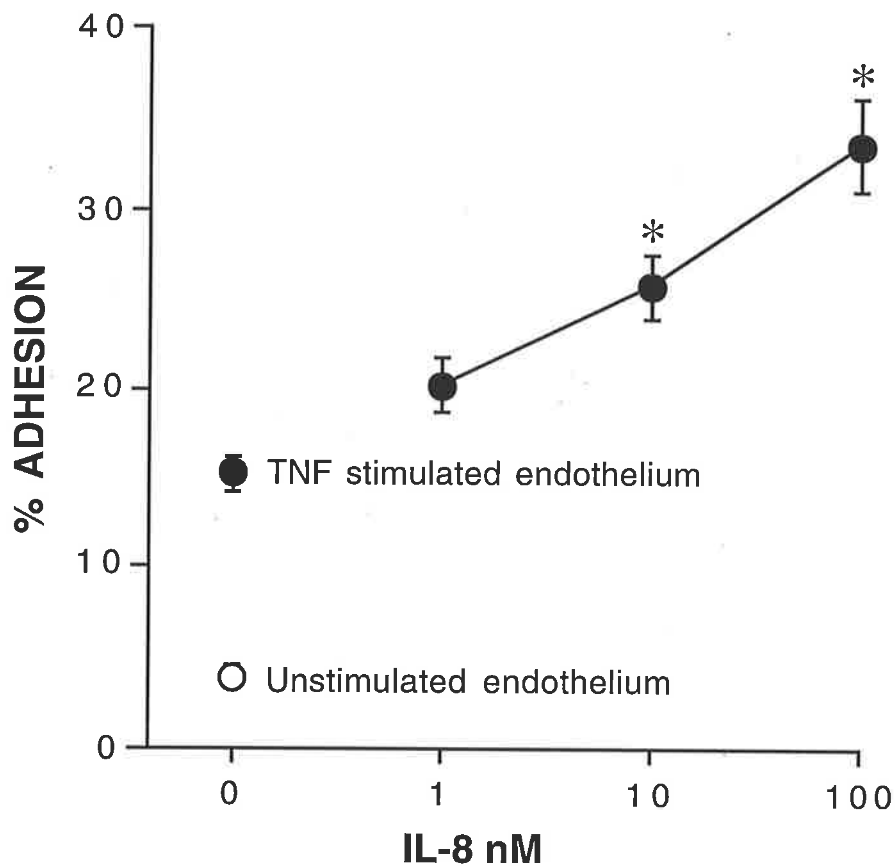


Fig. 3.4 Adhesion of neutrophils to TNF-α stimulated endothelium

Pre-incubation of endothelial monolayers with 100U/ml of TNF-α for 4h increases the adhesion of unstimulated neutrophils, as shown at the 0 IL-8 point on the *x*-axis. This adhesion is further increased by coincubation with IL-8 in a dose dependent manner. Representative experiment (in triplicate) of 6 experiments. * $p<0{\cdot}05$, compared with 0 IL-8, by independent Student's *t*-test.

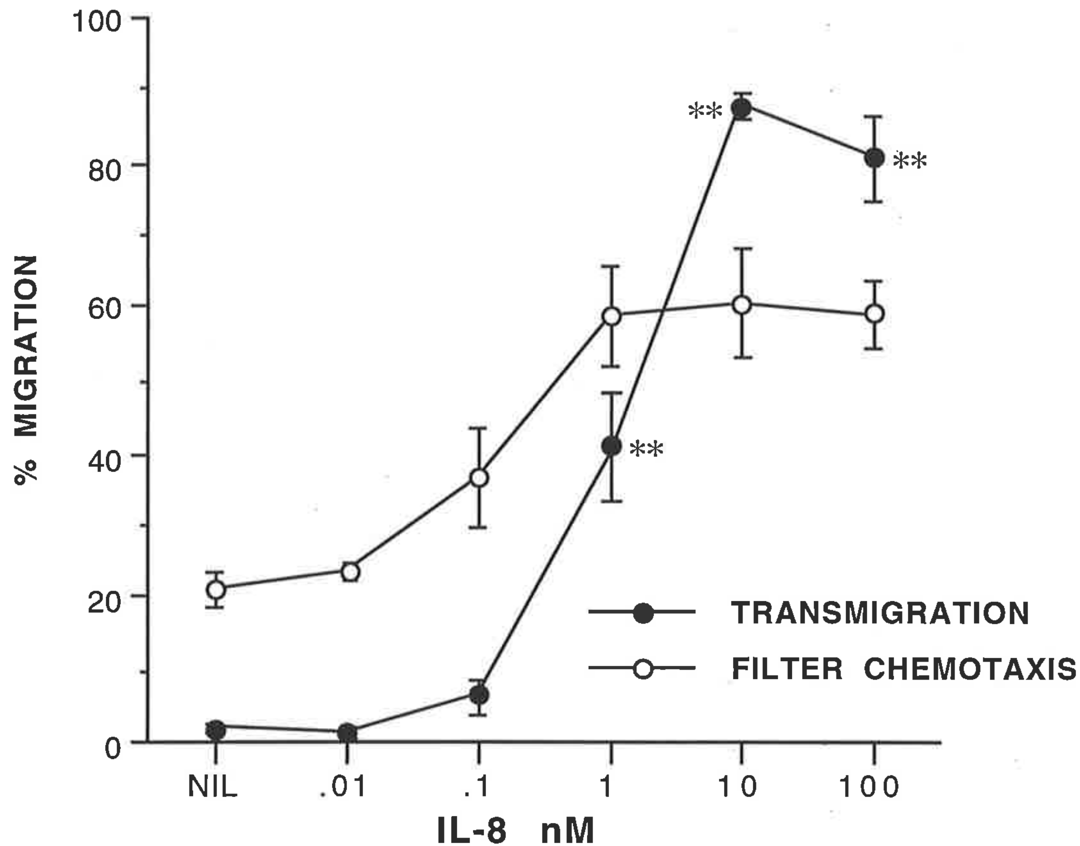
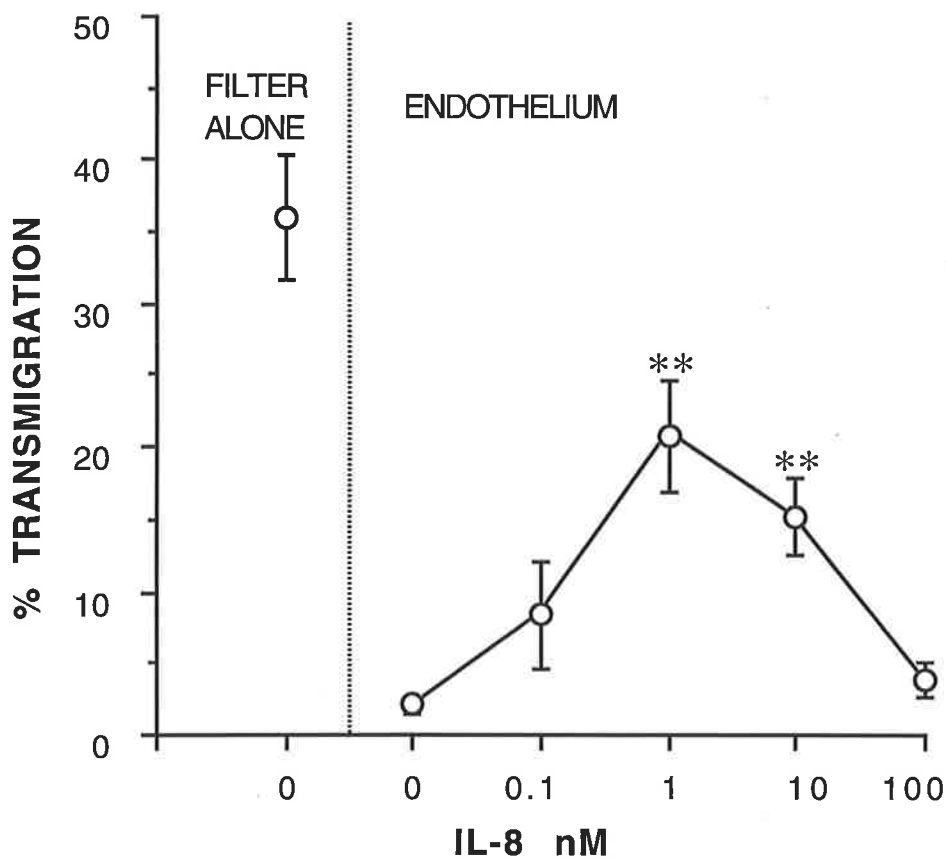


Fig. 3.5 Chemotactic transmigration of neutrophils

Endothelial monolayers were cultured in transwells on polycarbonate filters (3μm pore size). Neutrophil suspensions (10^6 cells) were added to the transwell, and the number of cells migrating through into the lower well after 60 minutes was determined using a Coulter counter, and expressed as a percentage of cells added. The lower well contained IL-8 in the concentrations indicated. Composite of 8 experimental values (4 experiments in duplicate). Significance ** $p<0{\cdot}005$, by Student's independent *t*-test, in comparison with random migration *ie*. no IL-8 in the lower well. Chemotaxis through the filter alone is included for comparison (representative experiment of 3 performed).



<u>Fig. 3.6 Transmigration by IL-8 chemokinesis</u>

Concentration of IL-8 was equal in the upper and lower compartments. 10^6 neutrophils were added to the upper compartment, and the number of cells migrating was counted after a 60 minute incubation. Composite of 6 experimental values (3 experiments in duplicate). Significance ** $p<0{\cdot}005$, by Student's independent *t*-test, in comparison with random migration (no IL-8).

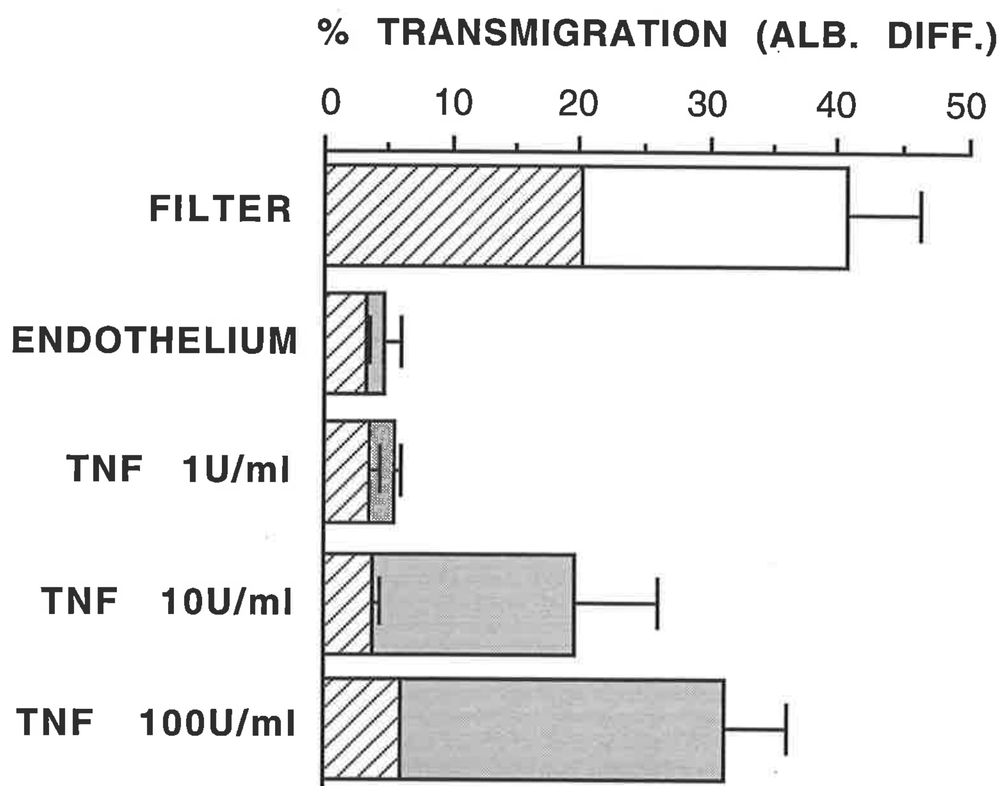


Fig. 3.7 Transmigration of neutrophils after preincubation of endothelium with TNF-α

Endothelium was preincubated for 4 hours in the indicated concentrations of TNF-α, and washed prior to the assay. Diffusion of albumin is indicated by the superimposed hatched columns. Representative experiment of three (each point performed in duplicate); bars indicate SD.

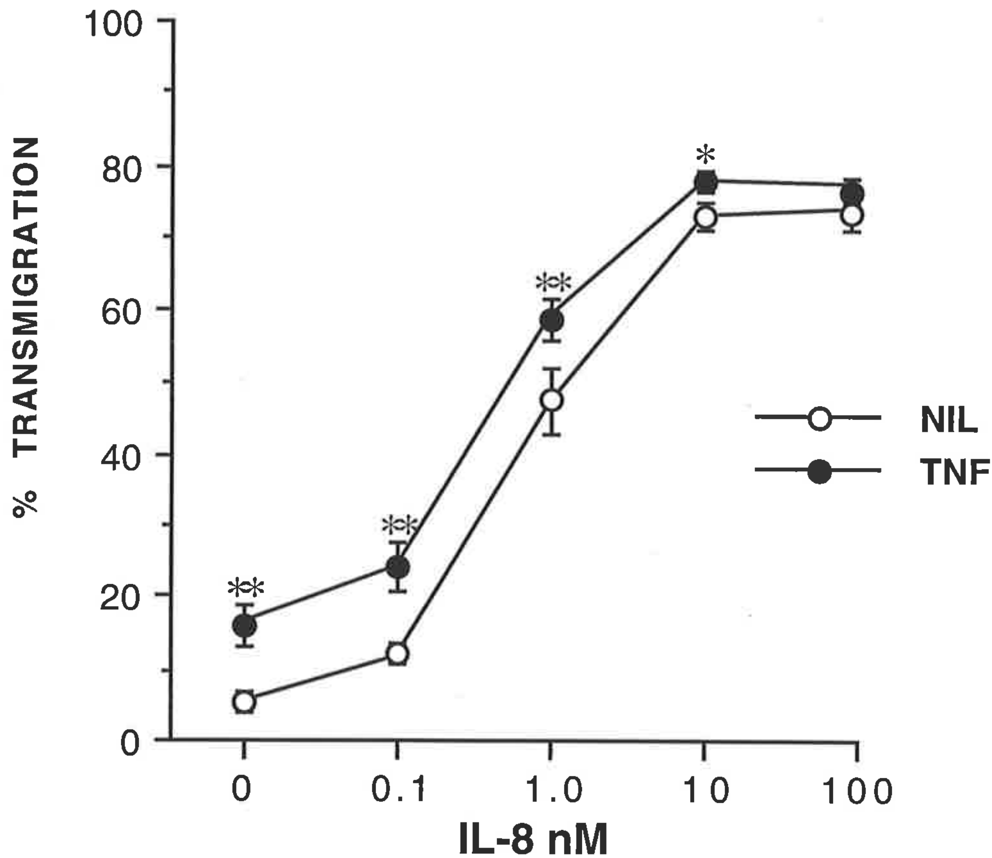


Fig. 3.8 Neutrophil transmigration stimulated by IL-8 chemotaxis through TNF-α preincubated and non-preincubated endothelial monolayers.

Monolayers cultured in Transwells were incubated in medium containing 10U/ml of TNF-α or medium alone for 4 hours prior to the assay, and then washed. 10^6 neutrophils were then added to the transwell, which was placed into a lower well containing medium with IL-8 at the given concentrations. Composite of 10 experimental values (5 experiments in duplicate). Significance refers to comparison of TNF-α preincubated endothelium with endothelium preincubated in medium alone, ** $p<0{\cdot}005$, * $p<0{\cdot}05$ by Student's independent *t*-test.

DISCUSSION

In the context of an examination of the mechanisms of neutrophil transendothelial migration, this chapter examines the role of exogenously added synthetic IL-8 on neutrophil adhesion and transmigration. Adhesion to endothelial cells appears to be necessary for transmigration. This is clearly demonstrated in patients with the leukocyte adhesion deficiency-I (LAD-I) syndrome, whose neutrophils lack the CD18 leukocyte integrins and do not migrate across endothelium either in response to chemotactic stimuli or endothelial activation(305). In addition, MAb which block adhesion also inhibit transmigration(259,306,307). However, adhesion is not sufficient to cause transmigration, and indeed certain types of adhesion, *eg*. Fc mediated binding of neutrophils to endothelium, have been shown not to lead to transmigration(265); and I have found that TNF-α stimulated neutrophils adhere strongly to, but do not transmigrate through, endothelium (data not shown). This suggests that either a chemotactic gradient or active participation of the EC is required. In addition, for neutrophils to migrate across endothelium, de-adhesion from the lumenal surface will be required. It may therefore be important that the adhesiveness of stimulated neutrophils is transient(308).

I have here shown that chemically synthesised 72 amino-acid IL-8 increases the adhesiveness of neutrophils, both to plastic and to cells of the human vascular endothelium, thus confirming the reports of Carveth *et al.*(161), and Detmers *et al.*(162) who used recombinant IL-8. Peak adhesiveness was seen at 30 minutes, in keeping with the time course of CD11b/CD18 expression after IL-8 stimulation demonstrated by Detmers *et al.*(162). It should be noted that the pro-adhesive effect of IL-8 was relatively weak compared to that of another neutrophil stimulatory cytokine, TNF-α (Fig. 3.1(B), 3.2). In direct comparisons, neutrophil adhesion to plastic after IL-8 (100nM) or TNF-α (100U/ml) stimulation was 34% and 90% respectively, and to endothelium 20% *vs*. 52%. Similar stimulation of neutrophil adhesiveness by IL-8 was found using wells coated with collagen, fibronectin and endothelial extracellular matrix prepared by stripping endothelial cells after 2 days of culture (data not shown), also confirming the results obtained by Carveth, *et al*(161).

The stimulatory effect of IL-8 on neutrophil- endothelial adhesion was on the neutrophil, as shown by neutrophil preincubation experiments, where after removal of IL-8 and incubation of the washed neutrophils on monolayers for 10 minutes, adhesion of a similar magnitude to that resulting from coincubation with IL-8 was seen (Fig. 3.1). If the neutrophils were incubated with the monolayer for longer, *ie*. 20-30 minutes, the effect was lost (data not shown), showing that adhesion is transient in the absence of IL-8. Endothelium itself can modulate adhesion, and is likely to possess receptors to the IL-8 related peptide melanoma growth stimulatory activity (MGSA)(185). However, preincubation of endothelium with IL-8 did not enhance adhesion of neutrophils (data not shown), ruling out any pro- adhesive effects of IL-8 on the EC.

Incubation of endothelium with TNF-α increased the adhesion of neutrophils(9), and incubation of neutrophils with IL-8 further increased their adhesion to TNF stimulated monolayers (Fig. 3.4). This would appear to disagree with the findings of Gimbrone *et al.*(309), that endothelium stimulated by TNF-α, IL-1 or LPS secreted a 77 amino-acid variant of IL-8 which inhibited the adhesion of neutrophils to such stimulated endothelium. This may represent an interesting divergence in the properties of these two post-translationally variant molecules, but it should be noted that the *in vitro* adhesion assay used by these authors differed in technique and time course to ours, and so direct comparisons of these molecules will be required to resolve this issue.

The leukocyte integrin glycoproteins LFA-1 and Mac-1 have been implicated in the adhesion of neutrophils to EC(305). I have shown that antibodies to CD11b, specific for the α chain of Mac-1, inhibit adhesion to the same extent as those to the common β chain CD18 (Fig. 3.3), indicating that Mac-1 may be the major integrin molecule responsible for IL-8 stimulated binding. My results with CD18 antibody confirm those of Carveth *et al*(161).

I have shown that IL-8 exerts a potent chemotactic attraction for neutrophils across endothelial monolayers, leading to the transmigration of up to 87·4 ± 2·1% of neutrophils within 1 hour (Fig. 3.5). Although the neutrophil suspensions contained up to 5% eosinophils, no eosinophils were detected in the transmigrated cells when examined in a

stained cytocentrifuged preparation, consistent with the reported specificity of IL-8 for neutrophils. IL-8 also caused increases in transmigration by a chemokinetic effect when concentrations were equal on both sides of the monolayer. At 1nM IL-8, migration increased to 21 ± 3·8%, a significant increase over baseline, but this decreased at higher concentrations, and at 100nM migration was <5% (Fig. 3.6). This implies that IL-8 stimulates the mechanisms of transmigration directly at low concentration, leading to increased transmigration even in the absence of a gradient. This may be by increasing the interaction of surface molecules involved in transmigration, or simply by increasing neutrophil motility (although IL-8 placed in the upper compartment alone did not stimulate transmigration (data not shown)). Higher concentrations do not increase transmigration. Up to 10^{-6}M IL-8 was not toxic to neutrophils, as determined by lactate dehydrogenase (LDH) release assay (data not shown).

The relationship between adhesion and transmigration in these experiments is complex. The concentration of IL-8 required to produce optimal adhesion was 10 fold higher than that optimal for chemotactic transmigration. Chemokinetic migration was reduced at higher concentrations of IL-8, suggesting that excessive adhesion antagonises migration. It was noted that IL-8 was a weaker inducer of adhesion than TNF-α and f-MLP. TNF-α, which strongly induces neutrophil adhesion, does not stimulate and may inhibit(310) neutrophil chemotaxis. Therefore, IL-8 appears to be specialised for induction of chemotactic migration. It is also known, unlike TNF-α and f-MLP, to be unable to stimulate the neutrophil respiratory burst alone(165,166), and to be a weak inducer of degranulation compared to f-MLP(159), suggesting that IL-8 may be able to direct the migration of neutrophils while causing little activation, and hence tissue damage, along the way.

Preincubation of endothelial monolayers with TNF-α led to increased neutrophil transmigration (Fig. 3.7). This was not due to increased permeability of the monolayer, as the diffusion of albumin was not increased by preincubation with 10U/ml TNF-α. IL-8 chemotaxis combined with TNF-α preincubation led to an additive increase in transmigration (Fig. 3.8). Furie *et al.*(266) did not note any consistent additive

transmigration when a chemotactic stimulus was combined with endothelial TNF-α preincubation at optimal concentrations; I found that a concentration of TNF-α which was probably suboptimal for transmigration definitely increased IL-8 chemotactic transmigration, but that this increase was only additive, and was less significant at greater IL-8 gradients. It is possible that part of the mechanism for increased transmigration after TNF-α stimulation of the endothelium is the secretion of IL-8 by the endothelial cells. Although the monolayers were washed prior to the transmigration assay to remove any soluble products, some of the secreted IL-8 may have remained in the subendothelium and act as a chemoattractant. To test this possibility, I preincubated endothelial monolayers with IL-8 at 100nM in the lower compartment and then washed, in an attempt to produce binding to the subendothelial matrix, but no significant increases in transmigration were observed (data not shown). The demonstration that TNF-α increases transmigration even at peak IL-8 gradients implies that additional mechanisms to endothelial secretion of IL-8 are likely to be present.

Leakage of plasma at inflammatory sites is one of the cardinal manifestations of the inflammatory reaction, and is related to increases in small vessel permeability, mediated at least in part through effects on the endothelial cells of inflammatory cytokines, various prostanoids, and LPS. Two *in vivo* studies which have used intradermal injections of IL-8 and found massive accumulation of neutrophils have examined the exudation of labelled plasma protein (albumin) at these sites, and found conflicting results; Colditz *et al.*(171) reported that exudation occurred readily, whereas Foster *et al.*(172) report that coinjection of prostaglandin E_2 with IL-8 was necessary to induce albumin extravasation. My results *in vitro* indicate that albumin does diffuse more readily across the endothelial barrier in the presence of transmigrating neutrophils, albeit at high rates of transmigration. Other *in vitro* studies have shown no increase in permeability of endothelium to albumin when the ratio of transmigrating neutrophils to EC was up to 5:1(263), however the same group later reported that ratios of migrating neutrophils of 20:1 did increase permeability(283). My studies also show that the increase in permeability is dependent on the process of transmigration itself and not endothelial damage from the activation of neutrophils by high concentrations of IL-8 whilst adjacent to the endothelium, since in a chemokinesis

experiment when neutrophils were exposed to equal concentrations of IL-8 but only 15% of the migration occurred compared to chemotaxis, there was no increase in albumin diffusion.

SUMMARY

IL-8 is a potent neutrophil chemotactic stimulant. In this chapter, I have used chemically synthesised IL-8 to investigate its role in human neutrophil adhesion and transendothelial migration. IL-8 enhanced the adhesiveness of human neutrophils to plastic, and to both unstimulated and TNF-α stimulated endothelial monolayers *in vitro*, although it was not as effective as the neutrophil stimulant TNF-α. Using a two compartment model separated by a confluent endothelial monolayer, I have shown that IL-8 chemotactic stimulation induced transmigration across the monolayer of up to 87·4 ± 2·1% of added neutrophils (compared to random unstimulated transmigration of 2·2 ± 0·7%), while chemokinetic stimulation led to transmigration of 21 ± 3·8% of neutrophils. These data suggest that among neutrophil stimulants, IL-8 is specialised for chemotactic migration, and only weakly induces adhesion. Preincubation of endothelium with TNF-α also induced transmigration in this model, and was additive when combined with an IL-8 chemotactic stimulus. Endothelial permeability was increased at maximal rates of chemotactic transmigration, which may correlate with increased permeability of vessels at inflammatory sites *in vivo*.

Transendothelial cell migration is likely to occur by (at least) steps of adhesion, de-adhesion and chemotaxis. The property of IL-8 to stimulate the transient adhesion of neutrophils to endothelium, and the movement of neutrophils across endothelial monolayers *in vitro* supports the concept of a central role for this molecule in the accumulation of neutrophils at inflammatory lesions *in vivo*.

CHAPTER 4

CHEMOTACTIC DESENSITISATION OF NEUTROPHILS DEMONSTRATES IL-8 DEPENDENT AND IL-8 INDEPENDENT MECHANISMS OF TRANSMIGRATION THROUGH CYTOKINE ACTIVATED ENDOTHELIUM

INTRODUCTION

There are at least two mechanisms by which neutrophil transendothelial migration may be accomplished; firstly, a transendothelial gradient of neutrophil chemotactic factors (Ch. 1), and secondly, the activation of endothelium by the "pro-inflammatory" cytokines TNF-α and IL-1 (Ch. 1, (265,266)), which causes the endothelium itself to induce neutrophil trans-migration in the absence of an exogenous chemotactic gradient.

In this chapter, I have investigated the mechanism by which cytokine activated endothelium induces neutrophil transmigration. The previous chapter shown that an exogenous gradient of the potent neutrophil chemoattractant IL-8 induces neutrophil transmigration across non-activated endothelium. EC activated by IL-1 and TNF-α produce IL-8[304]. I have therefore hypothesised that cytokine induced (endogenous) IL-8 secreted by endothelial cells is responsible for neutrophil transmigration, by chemotaxis, across cytokine activated endothelial monolayers. Huber *et al.* recently showed that neutralising anti- IL-8 antibodies partially inhibited transmigration through cytokine activated endothelium[277], offering direct evidence in support of this hypothesis.

To test this hypothesis, I have used the approach of neutrophil desensitisation to IL-8 (and the control chemotactic factor f-MLP), and observed the transmigration response to activated endothelium. My results are consistent with a role for IL-8 in neutrophil transmigration across cytokine activated endothelium, but indicate that other non IL-8 dependent mechanisms are also involved.

RESULTS

4.1 IL-1 preincubation of endothelium induces neutrophil transmigration

Endothelial monolayers cultured in transwells were incubated in IL-1 at varying concentrations for 4h and then washed in assay medium before the addition of 10^6 neutrophils for 60min. IL-1 increased transmigration in a concentration dependent manner (Fig. 4.1), with a plateau occurring at 100U/ml. Similar results were obtained with TNF-α, as shown in chapter 3. IL-1 was used in this chapter to exclude the possibility that the cytokine used to activate the endothelium might also be affecting neutrophil adhesion or motility. 100U/ml IL-1 was used to activate endothelium in most experiments. The level of transmigration induced by this concentration of IL-1 was variable with different endothelial cultures and neutrophil donors, but was usually 3-5 fold greater than baseline. The permeability of the monolayers to radiolabelled albumin was not increased by treatment with 100U/ml IL-1.

IL-1 preincubation of endothelial monolayers cultured on glass slides also resulted in significant increases in both adhesion and transmigration of neutrophils (Fig. 4.5, 4.6)

4.2 Chemotactic transmigration and desensitisation to IL-8 and f-MLP

In order to investigate whether transendothelial migration of neutrophils in response to chemotactic gradients could be desensitised specifically, transmigration in response to gradients of (72 amino acid) IL-8 and f-MLP was tested after preincubation with these agents. 1nM gradients were chosen for testing of desensitisation because this is in the middle of the concentration response curve for these factors, so a positive or negative effect should be easily observed; also, the level of transmigration at these concentrations is similar to that seen with cytokine activated endothelium. Concentration-response experiments indicated that for both factors, 100nM preincubation produced greater homologous desensitisation than 1 or 10nM without affecting the heterologous response (Fig. 4.3); higher concentrations (1μM) reduced transmigration to the heterologous factor (data not shown). Preincubation of neutrophils in 100nM IL-8 reduced their transmigration

to a chemotactic gradient of 1nM IL-8 by 81% (Fig. 4.2). The transmigration response of IL-8 preincubated neutrophils to a 1nM f-MLP gradient was not different to control neutrophils. Neutrophils preincubated in 100nM f-MLP were inhibited by 69% from transmigration to a 1nM f-MLP chemotactic gradient, whereas such neutrophils were not inhibited from migrating towards 1nM IL-8 (Fig. 4.2), or IL-8 at a range of concentrations (Fig. 4.4).

Since it was intended to test the response of desensitised neutrophils to activated endothelium which produces predominantly the 77 amino acid form of IL-8, desensitised neutrophils were also tested for response to gradients of 77 IL-8. f-MLP preincubation did not inhibit neutrophil transmigration in response to 77 IL-8 over a range of concentrations (Fig. 4.4). Preincubation with 72 amino acid IL-8 desensitised effectively to the 77 amino acid form (data not shown).

4.3 Preincubation of neutrophils with either f-MLP or IL-8 inhibits their response to IL-1 activated endothelium.

4.3.1 Transwell assay

Neutrophils preincubated in (desensitised to) 72 aa IL-8 or f-MLP were assayed for transmigration response to IL-1 (100U/ml) activated endothelium (no exogenous chemotactic gradients present). Both chemotactic stimulants inhibited the response of the neutrophils significantly (Fig. 4.5), although the mean inhibition by f-MLP (74%) was less than that by IL-8 (104%) ($p<0{\cdot}05$).

Inhibitory effects of preincubation with either chemotactic factor were maximal at 30 minutes of preincubation (data not shown), and a clear concentration response of preincubation was seen, with 100 nM producing maximal inhibition (Fig. 4.6).

4.3.2 Slide assay

Experiments using the slide transmigration assay illustrate and confirm the inhibition of transmigration after neutrophil preincubation in chemotactic agents. Transmigration through cytokine activated endothelium of 72 IL-8 preincubated neutrophils was virtually

abolished and f-MLP preincubated neutrophils substantially inhibited (Fig. 4.7, 4.8). Inhibition increased progressively with preincubation concentration of IL-8 (Fig. 4.9) and f-MLP (data not shown). The slide assay also demonstrates that chemotactic preincubation did not alter the number of adherent neutrophils.

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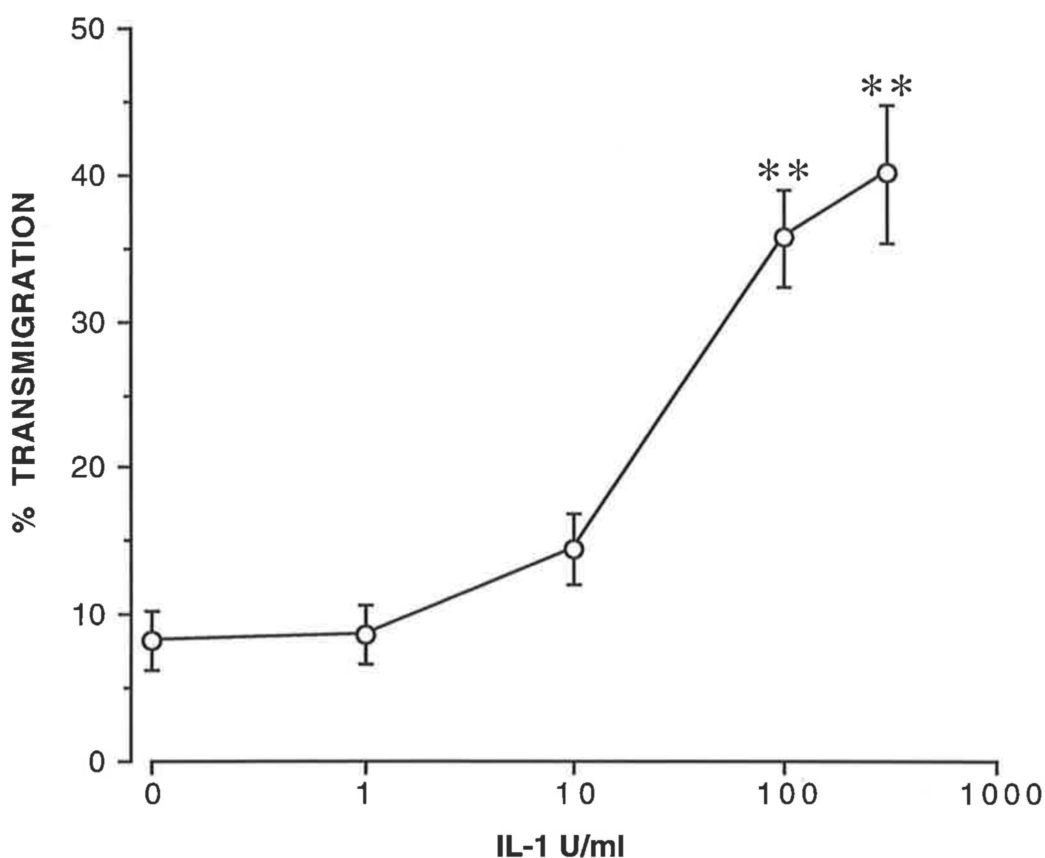


Fig. 4.1 Neutrophil transmigration in response to IL-1 activated endothelium

Endothelial monolayers in transwells were incubated for 4h with IL-1 at the indicated concentrations, and then washed prior to the addition of 10^6 neutrophils. Transmigrating neutrophils were quantitated as described in Materials and Methods. Each point is a composite of 6 experimental values (3 experiments in duplicate). ** $p<0{\cdot}005$ in comparison with 0 IL-1 by independent Student's t-test.

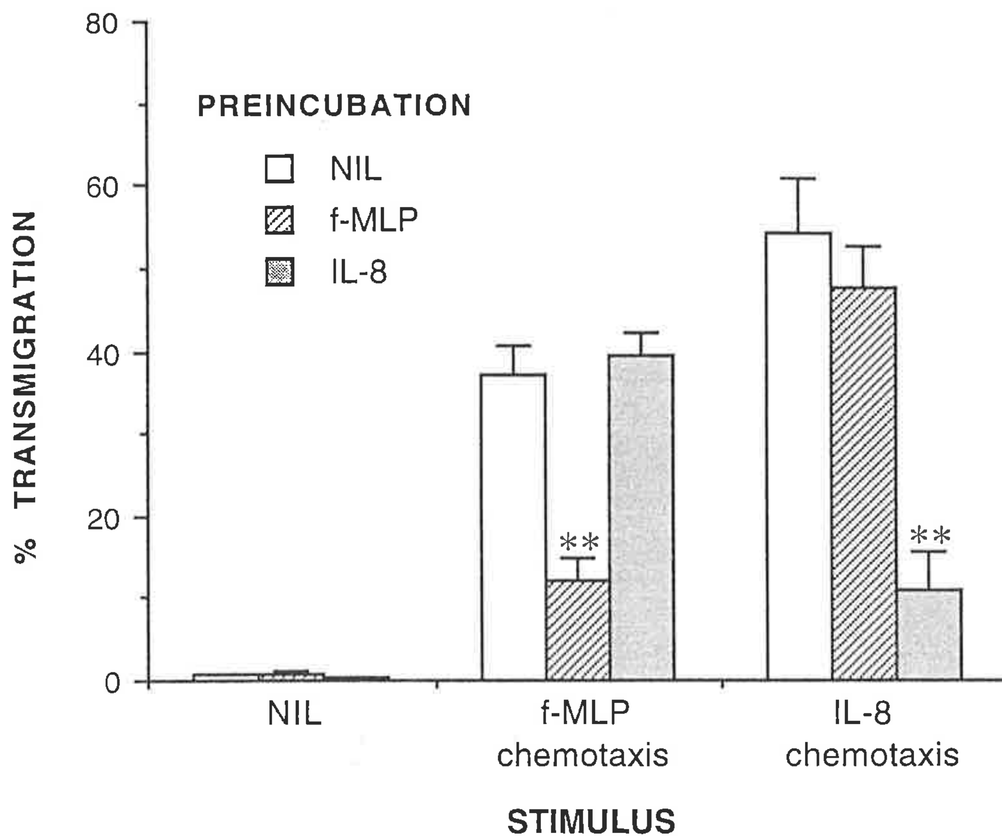


Fig. 4.2 Specific chemotactic desensitisation by preincubation with chemotactic factors

Neutrophils were preincubated with 100nM IL-8, 100nM f-MLP or medium as indicated, for 30min at 37°C. After washing they were placed on endothelial monolayers in trans-wells and subjected to a chemotactic gradient (stimulus) of either nil, 1nM f-MLP or 1nM IL-8. Columns represent the mean ± SEM of 4 experimental values (2 experiments in duplicate). ** $p<0{\cdot}005$ in comparison with preincubation in medium alone, by independent Student's t-test.

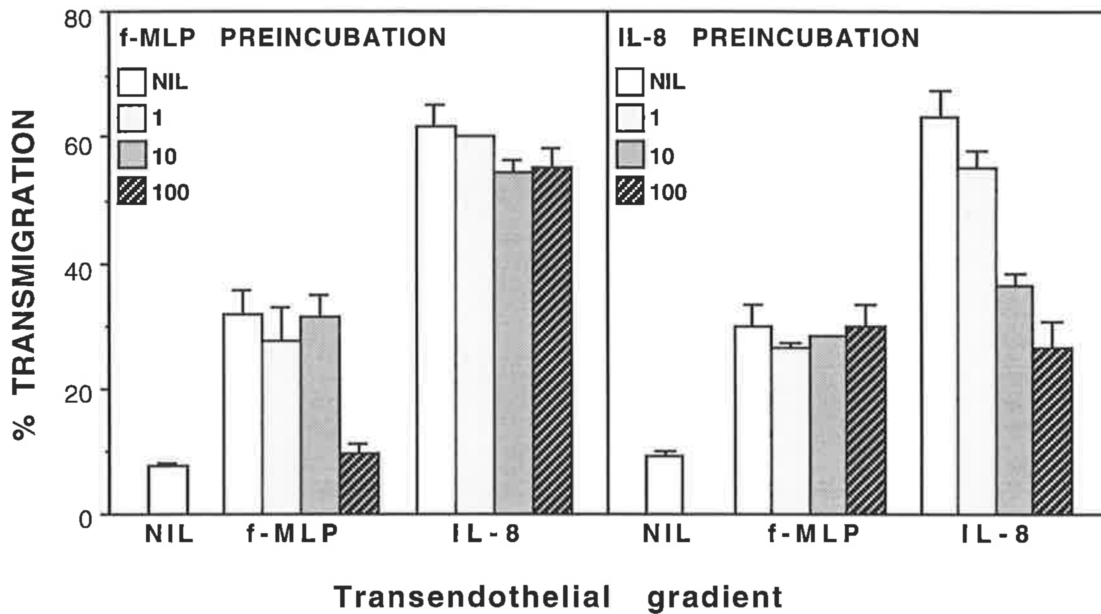


Fig. 4.3 Concentration/response of chemotactic desensitisation

Neutrophils were preincubated with IL-8 or f-MLP at the indicated concentrations, for 30min at 37°C. They were then exposed to a chemotactic gradient of either nil, 1nM f-MLP or 1nM IL-8. Representative experiments.

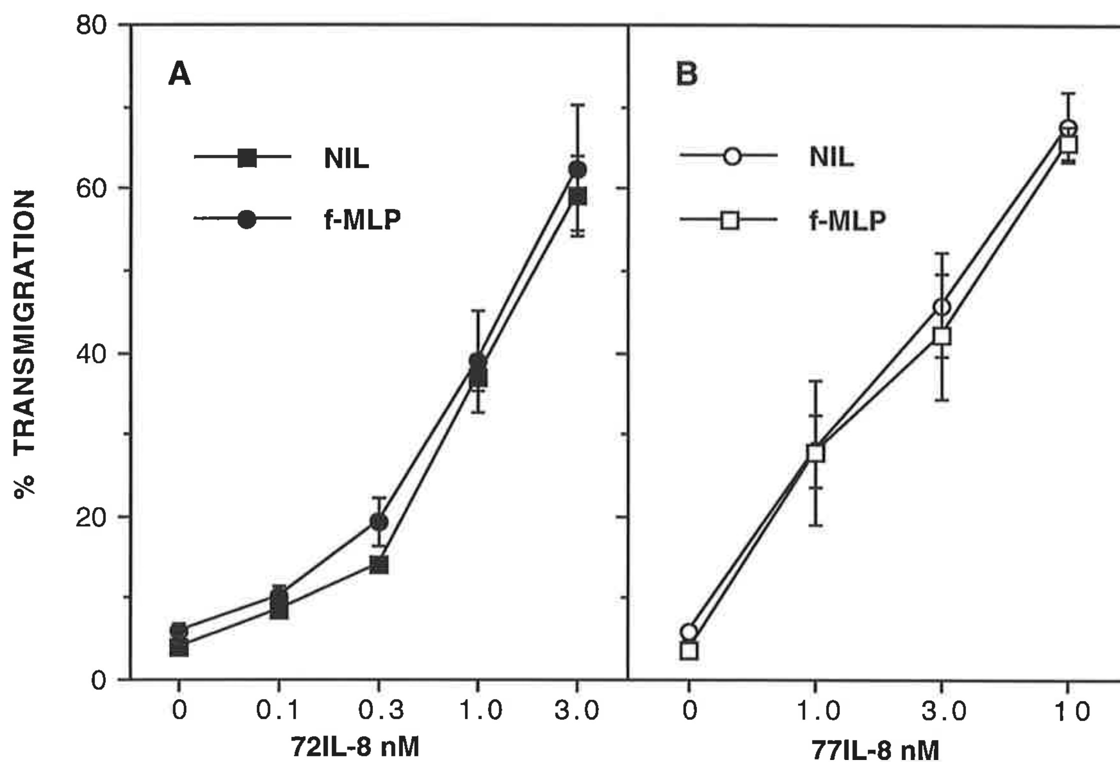


Fig. 4.4 Effect of f-MLP preincubation of neutrophils on their chemotactic transmigration to 72 and 77 amino acid IL-8

Neutrophils were preincubated in 100nM f-MLP or medium alone (nil) at 37°C for 30 minutes and washed. They were then placed on endothelial monolayers in transwells, with the indicated concentration of 72 amino acid (A) or 77 amino acid (B) IL-8 in the lower compartment. Results are the mean ± SEM of 4 experimental values (2 experiments in duplicate). There was no significant difference between f-MLP preincubated and control cell transmigration at any IL-8 concentration.

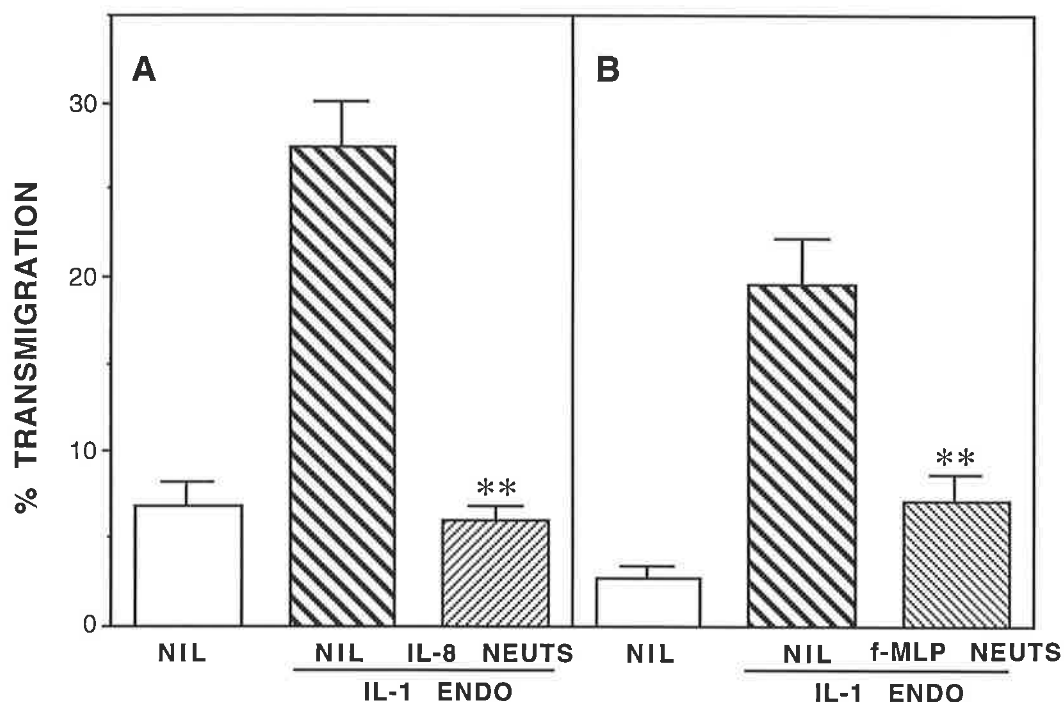


Fig. 4.5 Inhibition of transmigration in response to IL-1 activated endothelium by preincubation of neutrophils with IL-8 or f-MLP

Neutrophils were preincubated for 30 minutes at 37°C in medium alone, IL-8 100nM (A) or f-MLP 100nM (B) (two separate series of experiments). They were then placed onto endothelial monolayers which had been preincubated for 4h in medium alone (nil) or IL-1 100U/ml and then washed. Pooled data from 7 experiments in duplicate (A); 5 experiments in duplicate (B), columns represent mean ± SEM. ** $p<0{\cdot}005$ for comparison of neutrophils preincubated in chemotactic factor with those preincubated in medium alone, by independent Student's *t*-test.

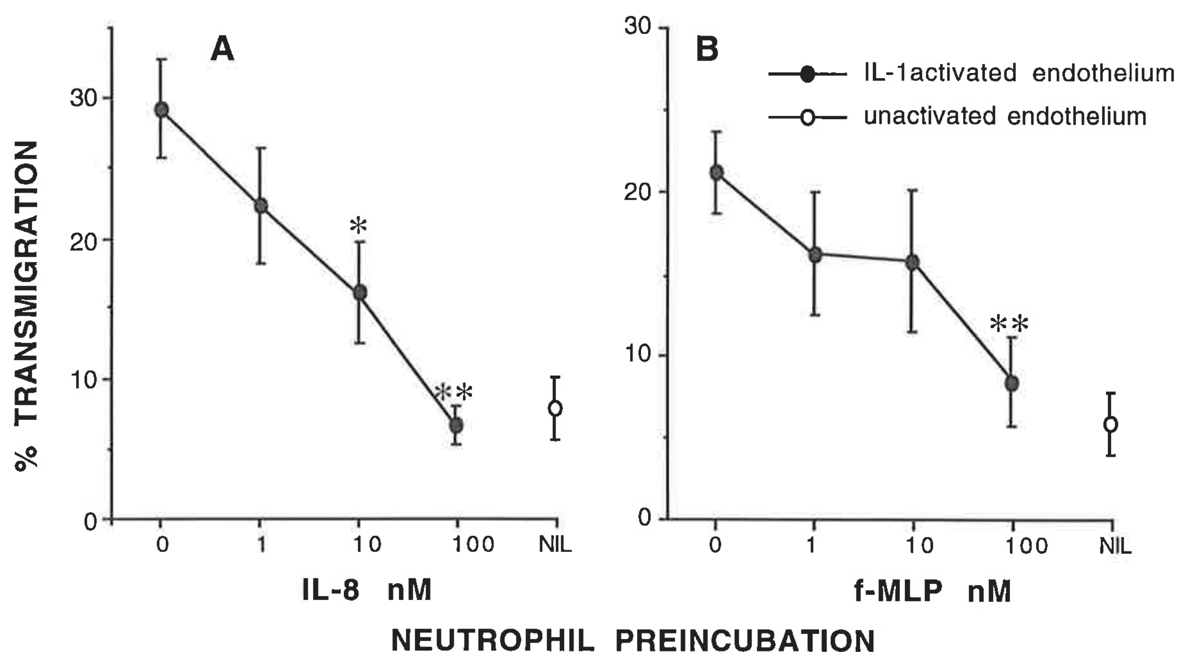
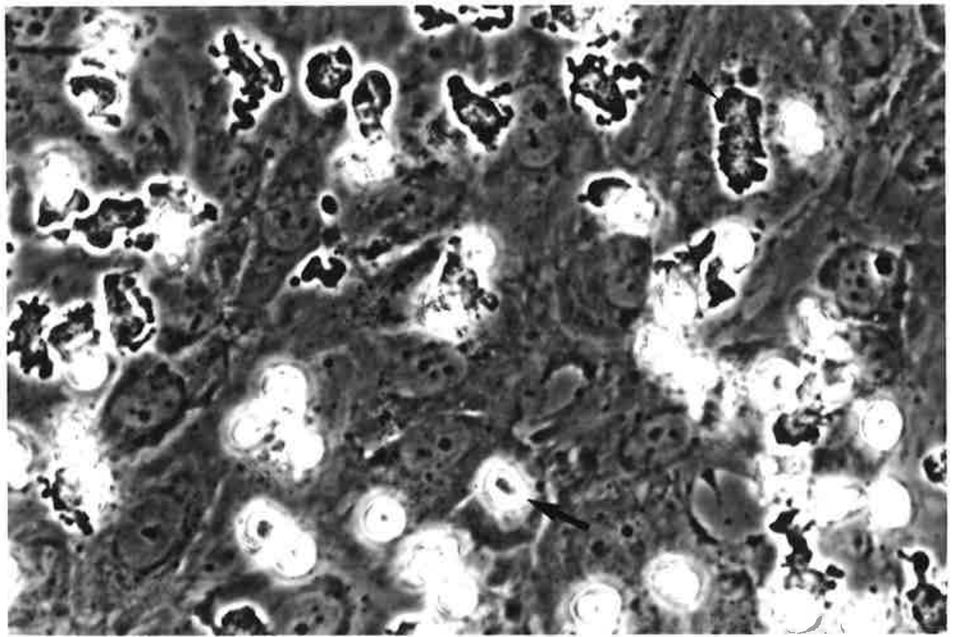


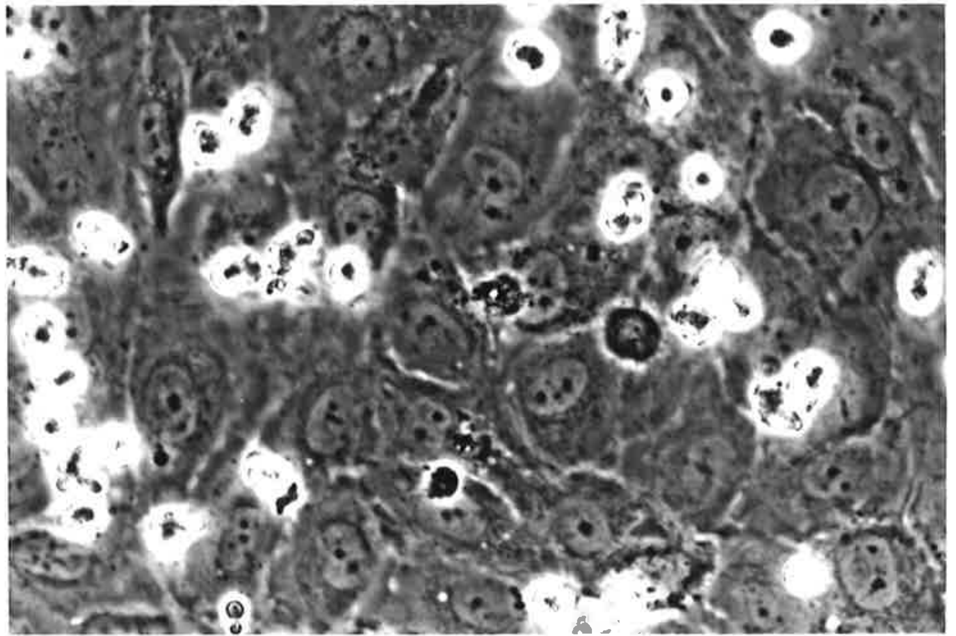
Fig. 4.6 Effect of varying the preincubation concentration of IL-8 or f-MLP on neutrophil transmigration through IL-1 activated endothelium

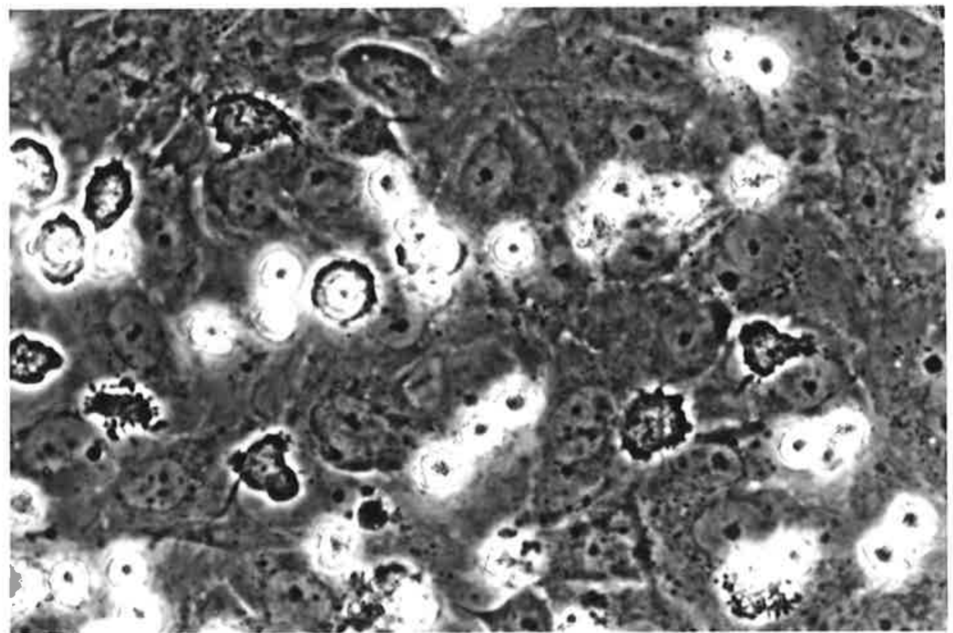
Neutrophils were preincubated for 30 minutes at 37°C in the indicated concentrations of IL-8 (A) or f-MLP (B) and then placed on IL-1 preincubated endothelial monolayers in transwells. Migration of neutrophils preincubated in medium alone through non-activated endothelial monolayers is shown as a baseline. Points represent the mean ± SEM of 8 experimental values (4 experiments in duplicate) (A), or 10 experimental values (5 experiments in duplicate) (B). * $p<0{\cdot}05$, ** $p<0{\cdot}005$ for comparison of neutrophils preincubated in chemotactic factor with those preincubated in medium alone, by independent Student's *t*-test.

Fig. 4.7 Photomicrographs illustrating the adhesion and transmigration of neutrophils on IL-1 activated endothelium

Endothelial monolayers were cultured to confluence on glass chamber slides and pre-incubated in 100U/ml IL-1 for 4h, then washed. Neutrophils were pre-incubated in medium alone (A), IL-8 (B) or f-MLP (C) and then co-incubated with the endothelial monolayers for 20 minutes. Panel (A) shows both adherent (arrow) and transmigrated (arrowhead) neutrophils. Panel (B) shows that after IL-8 preincubation, there are still numerous adherent cells, but none have transmigrated. In Panel (C), f-MLP preincubation has reduced the transmigration but not eliminated it completely. Representative fields, 400× magnification. For quantitation of multiple fields, see Fig. 4.8.







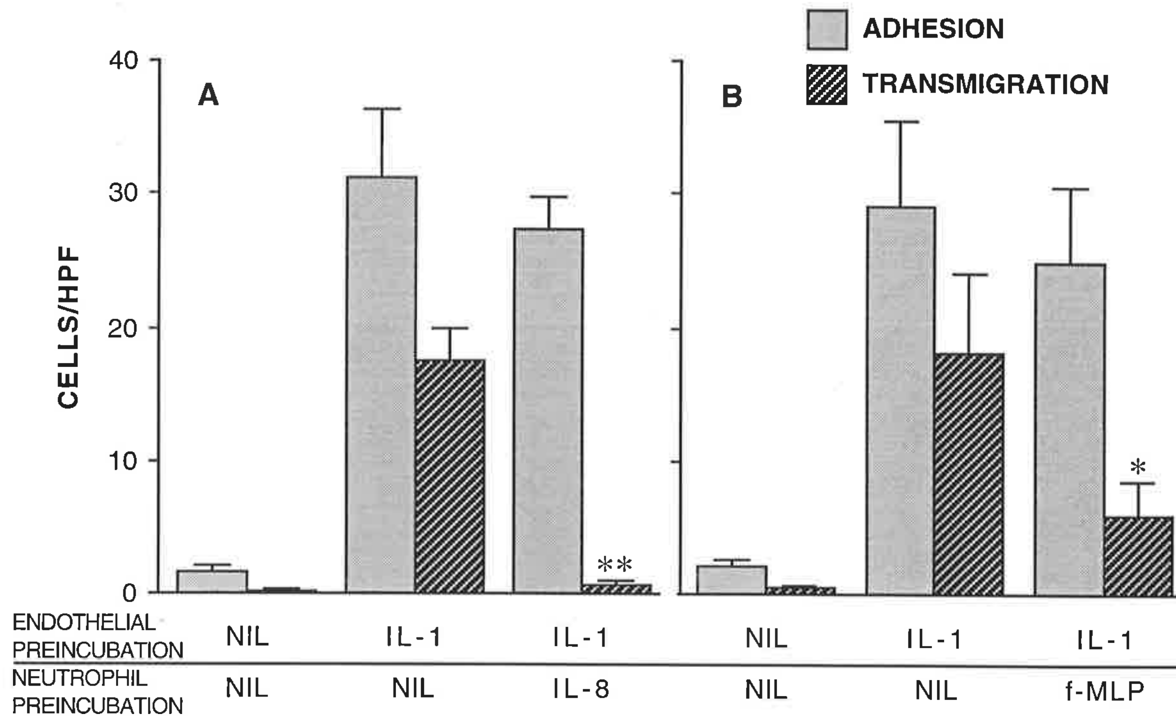


Fig. 4.8 Adhesion and transmigration of IL-8 and f-MLP preincubated neutrophils to IL-1 activated endothelial monolayers cultured on glass slides

Endothelial monolayers were preincubated with 100U/ml IL-1 or medium alone (nil) for 4 hours and washed. Neutrophils preincubated in medium alone (nil) or in 100nM IL-8 (A) or 100nM f-MLP (B) were then added to the endothelial monolayer, and after a 20 minute coincubation, adhesion and transmigration were quantified by microscopy as described in materials and methods. Mean counts ± SEM (in cells/high power field) of 8 slide assays (IL-8), 5 slide assays (f-MLP). Comparisons were made between chemotactic factor preincubated and medium preincubated neutrophils for both adhesion and transmigration, ** $p<0{\cdot}005$, * $p<0{\cdot}05$, by paired Student's *t*-test.

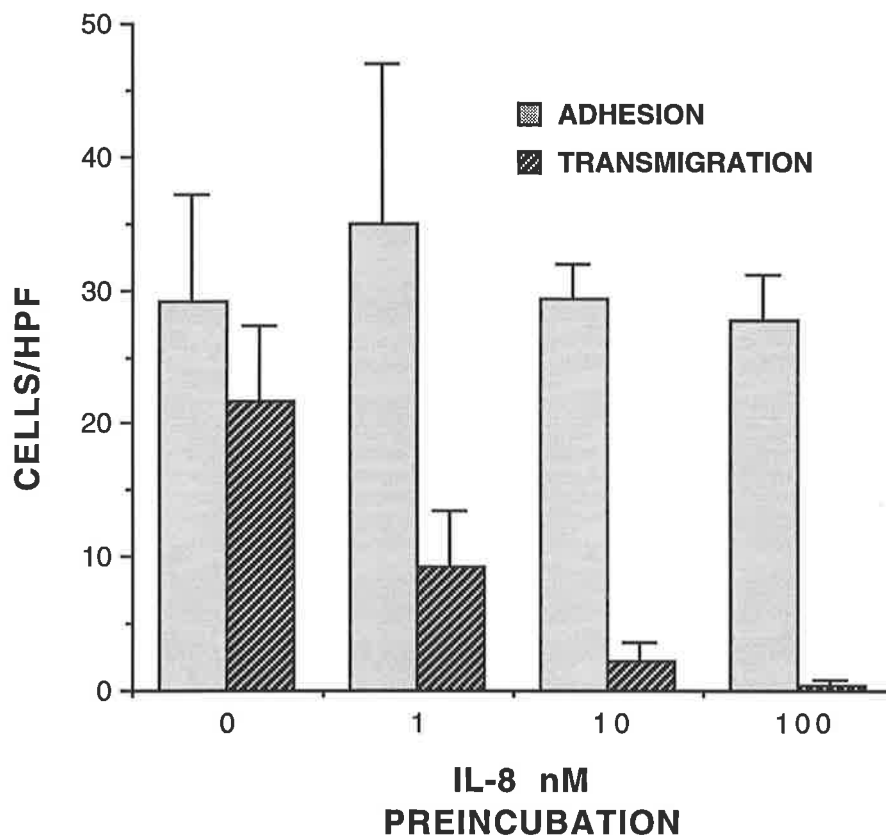


Fig. 4.9 Effect of varying the preincubation concentration of IL-8 on neutrophil transmigration through IL-1 activated endothelium in the slide assay

Endothelial monolayers cultured on glass slides were preincubated in IL-1 100U/ml for 4h and then washed. Neutrophils were preincubated in IL-8 at varying concentrations as shown, and then co-incubated with the monolayers for 20min. Adhesion and transmigration were then quantitated by microscopy, in cells per high power field. Columns represent the mean counts ± SD of 2 experiments.

DISCUSSION

Neutrophils can be desensitised to the effects of various agonists by prior exposure to these agonists. This may occur by receptor down modulation, in which case the desensitisation will be specific to ligands which utilise the particular receptor that has been modulated. Other mechanisms for desensitisation have also been proposed, such as exhaustion of post-receptor intracellular mediators, however this often results in non factor-specific inhibition[106].

In this chapter, I have desensitised neutrophils to the chemotactic effects of IL-8 and a control chemotactic factor, f-MLP. These chemoattractants operate via distinct but related receptors of the G-protein family[111,112]. Despite the similarity of these receptors, others have found that they are not cross-modulated, *ie.* that homologous desensitisation is specific for each of these agents[164]. By incubating neutrophils in suspension with IL-8 or f-MLP at concentrations supra-maximal for chemotactic responses, I was able to selectively desensitise neutrophils for chemotactic transmigration to each agent (Fig. 4.2). They were able to transmigrate normally in response to the heterologous chemotactic stimulus, indicating, importantly, intact motility apparatus and capacity for transendothelial migration. Inhibition of the homologous response was significant but not total in the case of either factor.

Cytokine activated endothelium induces the transendothelial migration of neutrophils[265,266], and IL-8 production by activated EC[304] has been implicated in this (Ch. 3, (277)). I have used an ELISA to confirm that IL-8 is produced by IL-1 activated HUVEC cultured in transwells (Ch. 5). I therefore tested the transmigration response of desensitised neutrophils to IL-1 activated endothelium. Neutrophils which have been preincubated with IL-8 were totally inhibited from transmigrating across IL-1 activated endothelium (by a mean of 104%, Fig. 4.4). Surprisingly, f-MLP preincubation also significantly inhibited transmigration (by 74%), although f-MLP preincubated neutrophils respond normally to gradients of both 72 and 77 amino acid forms of IL-8 over a range of concentrations (Fig. 4.3). This implies that there are mechanisms of transmigration used by activated EC other than an IL-8 gradient, which are inhibited by f-MLP preincubation of

neutrophils. IL-8 preincubation may inhibit the IL-8 independent mechanisms as well as desensitising to the IL-8 dependent mechanism, accounting for the total inhibition. This conclusion is consistent with the incomplete inhibition of transmigration seen after IL-8 neutralisation (Fig. 5.5, (277)).

The role of IL-8 as a mediator of transmigration for cytokine activated endothelium may seem inconsistent with reports of its inhibition of neutrophil adhesion to activated endothelium[309]. I have used direct observation methods to demonstrate that preincubation with IL-8 reduces the number of endothelium associated neutrophils, but this is due to an inhibition of transmigration only, and not of adhesion (Fig. 4.5, 4.6). IL-8 injected intravenously, *ie.* acting on neutrophils on the lumenal side of the endothelium, has been reported to have anti-inflammatory effects[311]. This may correspond to our finding of inhibition of transmigration by preincubation of neutrophils in IL-8; indeed, intravenous f-MLP had similar effects[311]. Therefore, IL-8 stimulation of neutrophils in suspension before their encounter with endothelium will have different effects to IL-8 released locally by activated EC.

The nature of the IL-8 independent mechanism of transmigration that I propose is unknown, but there are several possibilities. Another chemotactic factor in addition to IL-8 may be secreted from the EC which induces neutrophil transmigration in an equivalent manner to IL-8; the neutrophil receptors for this factor may be "cross down-modulated" by incubation of the neutrophils in either IL-8 or f-MLP. However, the majority of the chemotactic activity in the supernatants of activated EC is accounted for by IL-8[145,304] in 72 or 77 amino acid form, or its close relative MGSA (melanoma growth stimulatory activity)[185] which shares the same receptor[186].

A further possible explanation for the IL-8 independent mechanism of transmigration is that the migration response to activated endothelium does not involve chemotaxis in the classical sense. It may be mediated by cell-surface receptor- ligand interactions between neutrophils and activated EC, and the neutrophil surface structures could be altered by stimulation with IL-8 or f-MLP. A candidate molecule is L-selectin (LAM-1), which is shed from the neutrophil surface after stimulation by IL-8[82,312]. However, L-selectin is

currently thought to be involved in the rolling of leukocytes along the endothelial wall, but not in their transmigration[313]. A second candidate is sialyl Lewis^x, the neutrophil ligand for the TNF-α induced endothelial adhesion molecule E-selectin. However, this molecule is not altered in expression after IL-8 treatment of neutrophils[314].

The neutrophil integrins of the CD18 group are clearly essential for transendothelial migration. Neutrophils from patients who congenitally lack this molecule are not able to transmigrate *in vitro*[259], or localise at inflammatory sites *in vivo*[305], and in addition, antibodies to this molecule block transmigration[259]. It seems paradoxical that chemotactic stimulation, which increases expression and activation of the CD18 complexes LFA-1 and Mac-1[82], decreases transmigration. Recently it has been found that contact of neutrophils with E-selectin induces the adhesive conformation of Mac-1[272]. It has been postulated that E-selectin displayed by activated endothelium could act as a solid phase chemoattractant[272]. Thus for transmigration to occur, the localised and sequential activation of Mac-1 may be important. Generalised activation of CD18 complexes by preincubation of neutrophils with chemotactic factors in suspension may antagonise this process.

Current evidence favours a three-step model for the extravasation of neutrophils at inflammatory sites[313]. The first step is the rolling of neutrophils along the endothelial surface, mediated by the selectins; the second is firm adhesion, mediated by the integrin/ICAM interaction; and the third is the transendothelial migration of cells into the tissues. The production of IL-8 by activated EC suggests that an IL-8 gradient may be one of the mechanisms of the transmigration step. My finding that desensitisation of neutrophils to IL-8 reduces their transmigration in response to activated endothelium supports this. However, the finding that f-MLP desensitisation, which does not reduce the IL-8 response, also reduces transmigration, points to other, non IL-8 dependent mechanisms of transmigration through activated endothelium.

In vivo, neutrophils which are activated by cytokines intravascularly are shown to be defective in migration into inflammatory sites[311,315,316]. There are now several possible explanations for this. Shedding of neutrophil surface L-selectin after chemotactic factor

stimulation may reduce the ability of endothelial adhesion molecules to capture the neutrophil from the circulation[312]. As shown in Fig. 4.2, factor specific desensitisation of neutrophils may inhibit transmigration in response to transendothelial chemotactic gradients of the same factor. I have also now shown that pre-activation of neutrophils may inhibit their transmigration through cytokine activated endothelium.

SUMMARY

In chapter 1, it was shown that an exogenous gradient of IL-8 induces the transendothelial migration of neutrophils. Treatment of endothelium with the cytokines IL-1 or TNF-α also causes neutrophil transmigration, and recent evidence suggests that this may be due to endogenous IL-8 produced by the endothelium. I have used specific chemotactic desensitisation of neutrophils to investigate the role of IL-8 in transmigration through cytokine activated endothelium.

Preincubation of neutrophils with IL-8 reduced their chemotactic transmigration response to an IL-8 gradient by 81%, demonstrating desensitisation. Transmigration in response to cytokine activated endothelium was inhibited by 104% after IL-8 preincubation, thus tending to support the role of IL-8. However, preincubation with another neutrophil chemotactic factor f-MLP, which did not affect the IL-8 response, also inhibited transmigration, by 74%. This suggests that f-MLP preincubation acts to inhibit a non-IL-8 dependent mechanism of transmigration through cytokine activated endothelium. Chemotactic factor pre-treatment of neutrophils did not reduce their adhesion to activated endothelium, but specifically blocked the transmigration step.

I have therefore shown that chemotactic transmigration can be subjected to factor- specific desensitisation, and have used this to provide evidence supporting a role for IL-8 in transmigration through cytokine activated endothelium, as well as suggesting a further IL-8 independent mechanism. These data also provide a mechanism for the observed defect in accumulation of neutrophils at inflammatory sites when chemotactic factors are infused intravenously.

CHAPTER 5

PRODUCTION OF IL-8 BY ENDOTHELIAL CELLS AND ITS ROLE IN NEUTROPHIL TRANSENDOTHELIAL MIGRATION

INTRODUCTION

A central theme of this thesis is the finding that vascular endothelium, when "activated" by pro-inflammatory cytokines, is able to induce the migration of resting neutrophils across the monolayer, in the absence of exogenous chemotactic gradients, and without increasing the permeability of the monolayer (Ch. 3, 4). This observation was also made by others in 1989[265,266], and in that year it was also reported that activated endothelium synthesised and secreted a neutrophil chemotactic factor, identified as IL-8[304]. I and others hypothesised therefore that IL-8 was the mediator of neutrophil transmigration through cytokine activated endothelium.

Initial studies (Ch. 3) indicated that IL-8, when applied exogenously as a gradient across a non-activated endothelial monolayer, was able to induce neutrophil transmigration by chemotaxis, and when placed in equal concentrations on both sides of the monolayer, weakly induced chemokinetic transmigration. An early experiment (Fig. 3.8) designed to test the above hypothesis showed that even at a maximal IL-8 chemotactic gradient, transmigration could be further increased by cytokine activation of the endothelium, *ie*. that endothelial activation and IL-8 chemotaxis were additive, suggesting that although IL-8 may be a mediator, there were mechanisms in addition to IL-8 used by activated endothelium to induce neutrophil transmigration. Chemotactic desensitisation experiments (Ch. 4) led to a similar conclusion, *ie*. the results were consistent with a significant role for IL-8 as a mediator, but suggested that there was also another mechanism.

The hypothesis that IL-8 is a mediator of neutrophil transmigration induced by cytokine-activated endothelium was confirmed by another group, who used a polyclonal antibody to neutralise IL-8 in an *in-vitro* transmigration assay[277]. These experiments used endothelium cultured on a collagen gel to form a monolayer, and neutrophils were incubated with the monolayers. Activation of the endothelium with TNF-α led to an increase in neutrophils associated with the endothelium (adherent and transmigrated) from a baseline of $9 \pm 3\%$ to $81 \pm 6\%$. IL-8 neutralising antibodies reduced this increment by $71 \pm 6\%$. The reason for an incomplete inhibition of the TNF-α mediated increase may be that the antibody was not able to neutralise IL-8 sequestered in the subendothelial collagen

matrix, or alternatively, that another mediator of this effect exists in addition to IL-8. This latter interpretation would be consistent with the results of desensitisation experiments reported in chapter 4.

Many questions remain regarding the mode of action of IL-8 as a mediator of neutrophil transmigration induced by cytokine stimulated endothelium. It has not been formally demonstrated that production of IL-8 by EC in response to cytokine stimulation parallels transmigration in term of concentration/response and time course. It was not clear whether IL-8 is acting by chemotaxis or chemokinesis, *ie*. is the endothelium able to secrete IL-8 in a polarised fashion so as to create a transendothelial gradient. It has been suggested that IL-8 may be displayed on the apical surface of the endothelium, and act to induce neutrophil motility via a surface-bound chemotactic gradient (referred to as haptotaxis)[133], but this has not been proven. Finally, the nature of the other, non-IL-8 mediator(s) of transmigration is not known, although reports suggest that endothelial PAF may play a part[278].

These questions are addressed in this chapter by direct measurement of IL-8 production by EC and determination of its relationship to neutrophil transmigration, and by neutralisation of IL-8 produced by cytokine activated endothelium. IL-8 was quantitated by ELISA, developed using a high-titre rabbit antiserum to IL-8 which was a kind gift of Dr. S.L. Kunkel, Ann Arbor, Michigan (Ch. 2). This antiserum has efficient neutralising capabilities, and was used to confirm and extend the findings of Huber *et al.*[277], referred to above. These studies have revealed that IL-8 production by cytokine activated EC follows a stimulus concentration/response relationship which correlates with neutrophil transmigration, and that secretion is not polarised, *ie*. it is released both above and below the monolayer. Furthermore, neutralisation of IL-8 on both sides of the monolayer inhibits neutrophil transmigration through TNF-α activated endothelium by a mean of 40%, but neutralisation of IL-8 on either side alone may increase or decrease transmigration, and the significance of this will be discussed.

A further issue is the role of adhesion molecules themselves in the step of transmigration. Neutrophil adhesion to endothelium does not necessarily lead to transmigration[265], but the

leukocyte integrin adhesion molecules, at least, are required for transmigration to occur[259,306]. Although the major function of the selectins appears to be to mediate leukocyte rolling, the inducibility of E-selectin on EC by inflammatory cytokines has suggested the possibility of a role in transmigration. A plausible mechanism for this was provided by the finding that E-selectin was able to activate neutrophil CD18/CD11b adhesion function, and indeed soluble E-selectin was chemotactic for neutrophils[272]. However, it has subsequently been shown that antibodies to E-selectin did not block transmigration induced by cytokine-activated endothelium, in a static assay[271]. I have confirmed this result, using anti-E-selectin FAb_2 fragments which are demonstrated to be able to block adhesion. Furthermore, I have carried out time-course experiments to examine the correlation between transmigration and the endothelial expression of ICAM-1 and E-selectin, measured in parallel. These studies address the question of whether the steps of rolling, adhesion and transmigration can be separated, whether IL-8 is a mediator of transmigration, and whether adhesion molecules also play a role in the transmigration step.

RESULTS

5.1 TNF-α and IL-1 stimulate endothelial cell IL-8 production in a concentration dependent manner

Endothelial cells cultured in 24 or 96 well cluster dishes for 1-2 days (to reach confluence) were activated by adding TNF-α or IL-1 at a range of concentrations, for 4h. Medium was then exchanged, and collected after 1h. IL-8 was quantitated by ELISA. Although EC lines varied in their sensitivity to TNF-α and in the quantity of IL-8 produced, representative concentration/response curves for TNF-α and IL-1 are shown in Fig. 5.1A,C. For both of these factors, IL-8 production was generally detectable at 1U/ml and maximal at 100U/ml. Transmigration of neutrophils through endothelial monolayers activated by TNF-α or IL-1 for 4h showed very similar concentration response relationships (Fig. 5.1B,D).

5.2 Correlation between the time course of IL-8 production and adhesion molecule expression by activated endothelium and induction of neutrophil transmigration

EC monolayers were activated by TNF-α for 4, 24 and 48h and IL-8 production was measured. In parallel, EC derived from the same primary line were used for a transmigration experiment. IL-8 output was maximal at 4h, but continued with only a small diminution at 48h after TNF-α stimulation (in 3 experiments, the time course of IL-8 production was similar, with levels at 48h always >50% of the early maximum). Transmigration showed an almost precise correlation with IL-8 production (r=0.99)(Fig. 5.2). There was no obvious correlation with the patterns of expression of either E-selectin or ICAM-1.

5.3 IL-8 production by activated EC is not polarised

HUVEC were cultured in transwells of 35mm diameter, and activated with TNF-α 100U/ml. Larger transwells were used to allow increased numbers of EC, to ensure that levels of IL-8 in the supernatants would be in the measurable range, and also so that the

volume of medium in the upper and lower compartments would be closer to equal (volume ratio upper/lower, 6·5mm transwell 0·21, 35mm transwell, 0·52) Medium was sampled from compartments above and below the monolayers for IL-8 quantitation. Levels in the upper and lower compartments were not significantly different (Fig. 5.3), indicating that secretion of IL-8 by activated EC is not polarised. Although the volume in the lower well was greater than the upper, correcting for well volume still did not lead to significant differences, and the measured amounts as shown represent the exposure of neutrophils in the transwell transmigration assay.

5.4 The effect of neutralisation of IL-8 activity on neutrophil transmigration through TNF-α activated endothelium

Antiserum raised against IL-8 was shown to have neutralising activity in a chemotaxis assay. IL-8 1nM was placed into wells of a 24 well cluster dish, and incubated for 15min with medium or antiserum at 1:250 and 1:500 dilution. Transwells without endothelial monolayers, containing 10^6 neutrophils, were placed into the wells, and neutrophil migration across the filters was quantitated (Fig. 5.4). Antiserum alone (1:250) had no effect on migration, but inhibited the IL-8 induced chemotactic migration completely. Antiserum neutralised IL-8 at dilutions out to 1:1000, but did not neutralise the chemotactic effects of f-MLP (data not shown). 1:500 dilution was used in subsequent experiments.

The effect of neutralisation of IL-8 on neutrophil transmigration through TNF-α activated endothelium was then determined. Antiserum was added 15min prior to addition of neutrophils, to either the upper, lower or both compartments. Complete neutralisation of IL-8 (both compartments) reduced neutrophil transmigration induced by activated endothelium, by a mean of $36 \pm 7{\cdot}5\%$ ($p = 0{\cdot}006$). Antiserum placed in the lower compartment only, reduced transmigration by a greater amount, $72 \pm 30\%$ ($p = 0{\cdot}005$), whilst if antiserum was added to the upper compartment only, there was a trend towards an increase in transmigration- $70 \pm 55\%$ (NS), although there was some variability between experiments (Fig. 5.5).

5.5 The effect of blocking the adhesion molecules E-selectin and CD18 on neutrophil transmigration through TNF-α activated endothelium

Antibodies against E-selectin were raised in our laboratory. For assays of inhibition of neutrophil adhesion to E-selectin, FAb_2 fragments were used, to avoid adhesion through neutrophil Fc receptors. Fig. 5.6 shows that whilst MAb 5H11 and 3G3 only partially inhibited neutrophil adhesion to TNF-α activated HUVEC when used alone, both MAb together inhibited substantially. (These results were confirmed in other adhesion assays, J. Gamble, unpublished data). In the same assay, HB203, a blocking CD18 antibody, inhibited adhesion partially. When the same antibodies were used in an assay of transmigration through TNF-α activated endothelium, no inhibition was seen with anti E-selectin antibodies, either individually or combined (there was a trend towards an increase), while HB203 inhibited completely (Fig. 5.7).

5.6 The effect of the PAF antagonist WEB2086 on transmigration through TNF-α activated endothelium

10nM PAF in the lower compartment of the transwell induced neutrophil transendothelial migration by chemotaxis, and this was inhibited by treating the neutrophils with the PAF receptor antagonist, WEB2086 (Fig. 5.8). In separate experiments, WEB2086 did not impair neutrophil transmigration to IL-8 chemotaxis, indicating specificity (data not shown). Nor did WEB2086 increase baseline unstimulated transmigration (Fig. 5.8). WEB2086 did not have any inhibitory effect on neutrophil transmigration in response to endothelium activated by TNF-α 100U/ml, nor did it add to the partial inhibitory effect of IL-8 neutralising antiserum. The magnitude of migration in response to PAF (WEB2086-inhibitable) in this experiment was not as great as that in response to TNF-α activated endothelium with IL-8 antiserum (putative endogenous PAF-induced), but in other experiments, it was of similar magnitude, yet only the exogenous PAF was inhibited.

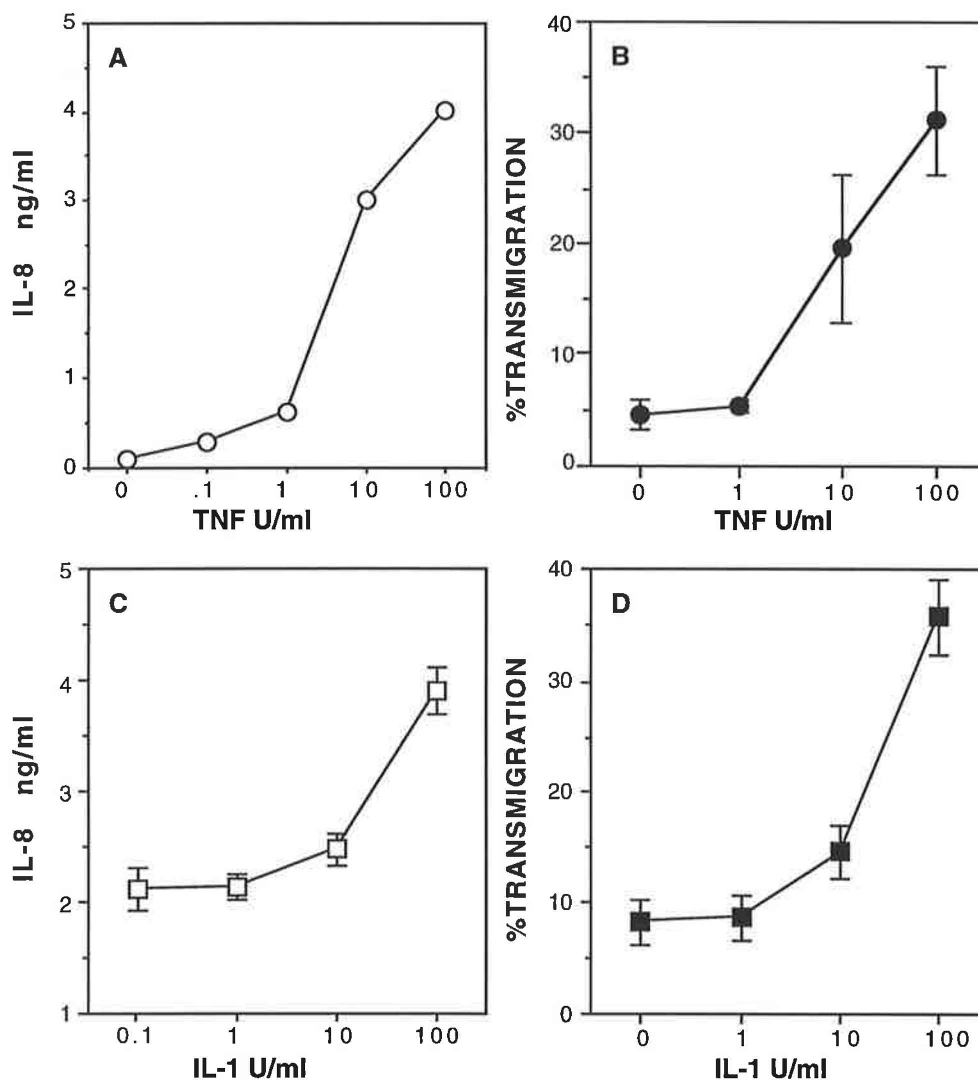


Fig. 5.1 Production of IL-8 and induction of neutrophil transmigration by endothelium activated by TNF-α or IL-1

Endothelial monolayers were cultured in 24 well cluster dishes, and incubated with TNF-α (A) or IL-1 (B) at varying concentrations for 4h. The monolayers were then washed, the medium replaced, and after 1h it was collected, and IL-8 concentration quantitated by ELISA. Endothelial monolayers in transwells were preincubated in medium alone or in TNF-α (C) or IL-1 (D) at the indicated concentrations. Neutrophil transmigration was quantitated as described in Ch. 2. Representative experiments, see also Chapters 3, 4, 7 and 9 for TNF-α and IL-1 concentration/response experiments of endothelial IL-8 production and induction of neutrophil transmigration.

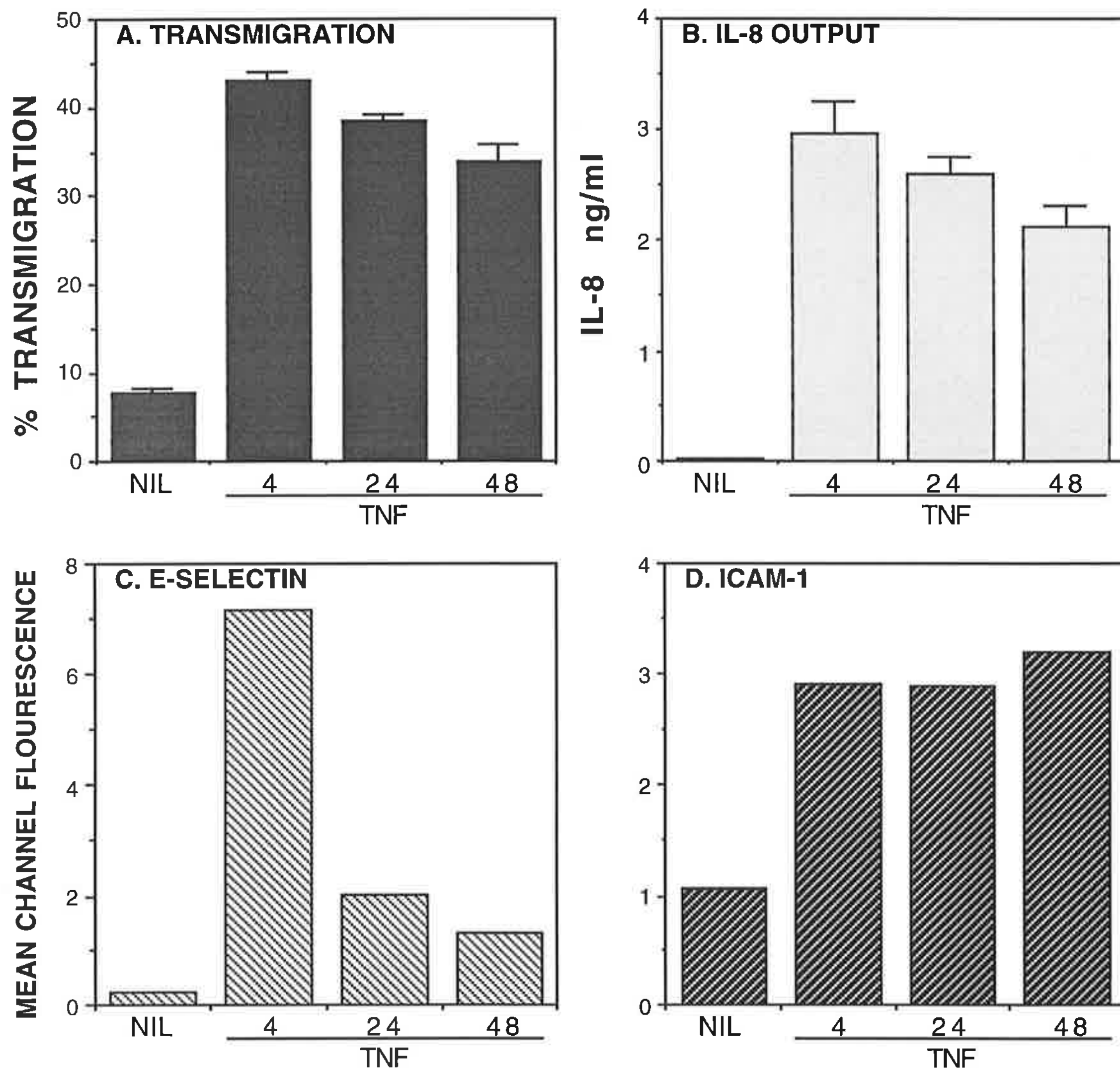


Fig. 5.2 Endothelial induction of neutrophil transmigration, production of IL-8, and expression of E-selectin and ICAM-1 with varying times of TNF-α activation

Endothelium was incubated with TNF-α for 4, 24 or 48h. Monolayers in transwells were used for quantitation of the induction of neutrophil transmigration (A). Monolayers in 24 well cluster dishes were used for measurement of IL-8 production (B) as described in Fig. 5.1, and the same EC were then stained with antibodies against E-selectin (C) or ICAM-1 (D) and the surface expression of each was quantitated by flow cytometry. Mean fluorescence intensity is shown on the vertical axis. The same endothelial cell line was used for part (A) and parts (B-D).

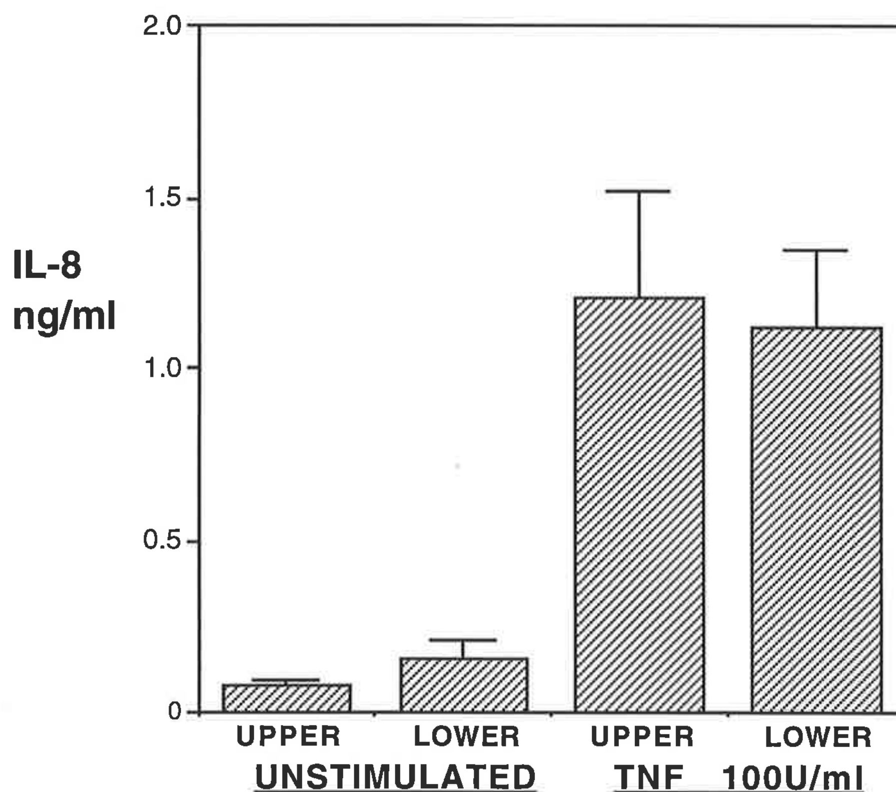


Fig. 5.3 Quantitation of IL-8 production above and below the endothelial monolayer

HUVEC were cultured in 35mm transwells and preincubated with TNF-α for 4h. Medium was exchanged in the compartments above and below the monolayer, and collected after 1h. IL-8 was quantitated by ELISA. Values are the mean ± SEM of 9 experimental determinations, ie. 3 experiments with triplicate determinations.

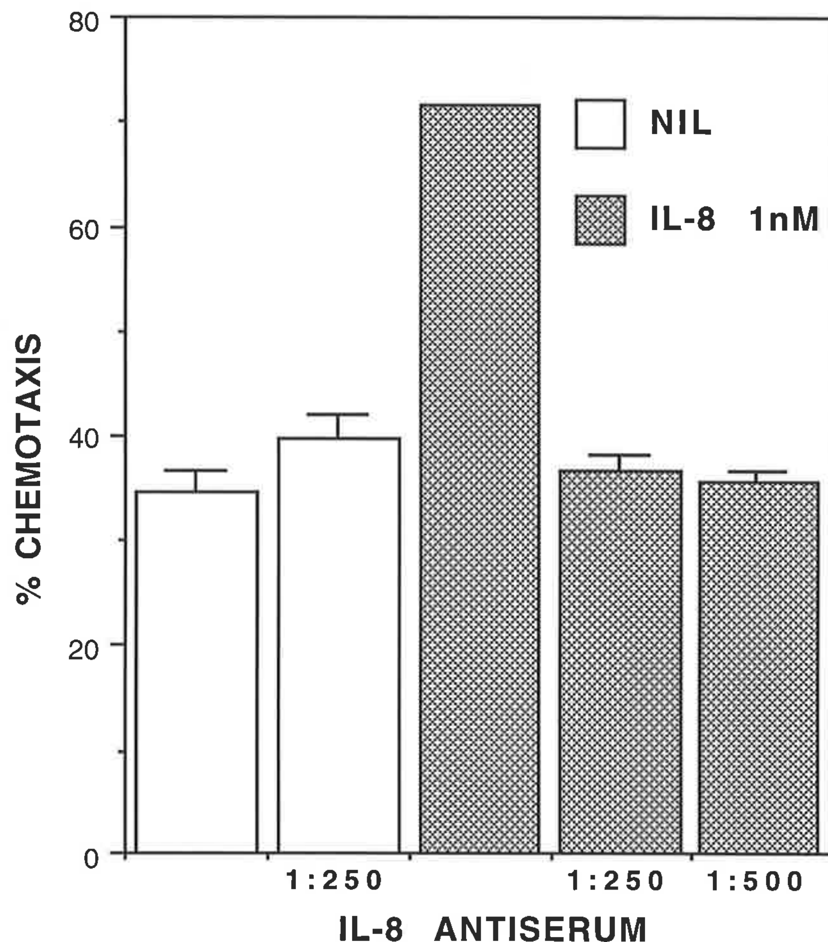


Fig. 5.4 Antiserum raised against IL-8 neutralises the effects of IL-8 in a chemotaxis assay

Antiserum against IL-8 was raised by immunisation of rabbits (obtained from Dr. S. Kunkel, Ann Arbor, MI, USA). This antiserum was incubated with IL-8 1nM in medium at varying ratios, for 15min in a 24 well cluster dish. Transwells without endothelium, containing 10^6 neutrophils, were placed into these wells and incubated for 45min. Neutrophil migration was then quantitated (Ch. 2). Medium with no IL-8, and medium with antiserum alone were negative controls, and medium with IL-8 1nM alone was the positive control. Experiment performed in duplicate, columns are the mean ± SD of the percentage migration for each stimulus.

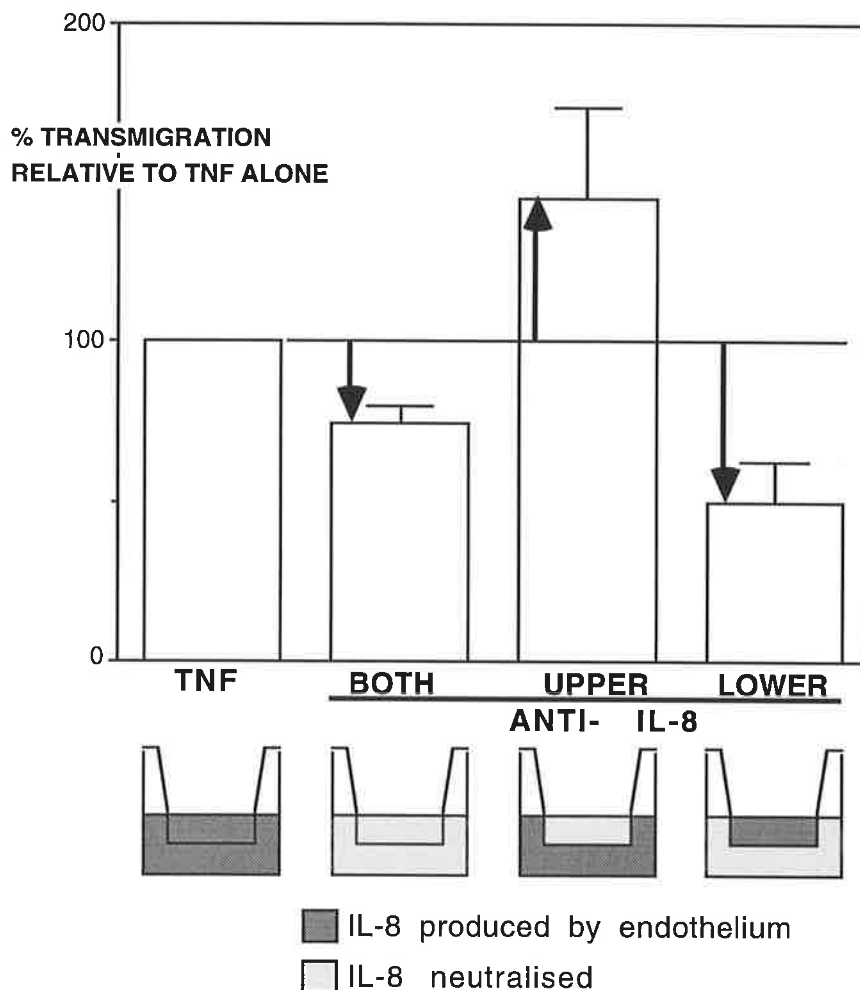


Fig. 5.5 The effect of neutralisation of IL-8 on neutrophil transmigration induced by TNF-α activated endothelium

Endothelial monolayers in transwells were preincubated with TNF-α for 4h, then medium was replaced with medium with or without IL-8 antiserum at 1:500 dilution, in either the top, bottom or both compartments, and incubated for 15min. 10^6 neutrophils were then added to the top compartment, with the antibody remaining present. Neutrophil transmigration was quantitated in the usual way. TNF-α enhanced neutrophil transmigration as shown previously; the effect of IL-8 antiserum, as a percentage change of the TNF-α stimulated level of transmigration (relative to baseline) is shown on the vertical axis. Columns represent the mean ± SEM percentage change. Percentage calculated from each of 7 experiments in duplicate (both sides) or 5 experiments in duplicate (upper, lower) separately, and then pooled together.

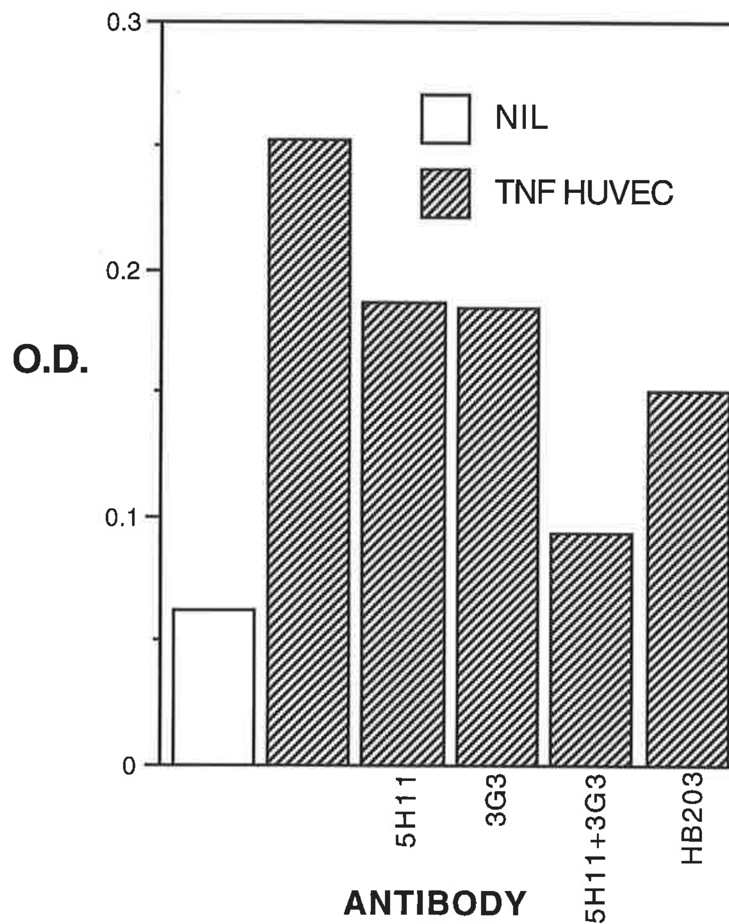


Fig. 5.6 The effect of antibodies to E-selectin and CD18 on neutrophil adhesion to TNF-α activated endothelium

HUVEC were cultured on 96 well cluster dishes, and preincubated with TNF-α 100U/ml for 4h. The monolayers were then washed and the antibodies HB203 (anti-CD18, IgG1, 50μg/ml) and 3G3 and/or 5H11 (anti E-selectin, FAb, 20μg/ml) incubated with the monolayers for 15min before the addition of 10^5 neutrophils. After 30min incubation, adhesion wasquantified by reading the OD_{570} of the Rose-Bengal stained neutrophils, as described in Chapter 2. Representative experiment, columns are the mean of triplicate wells.

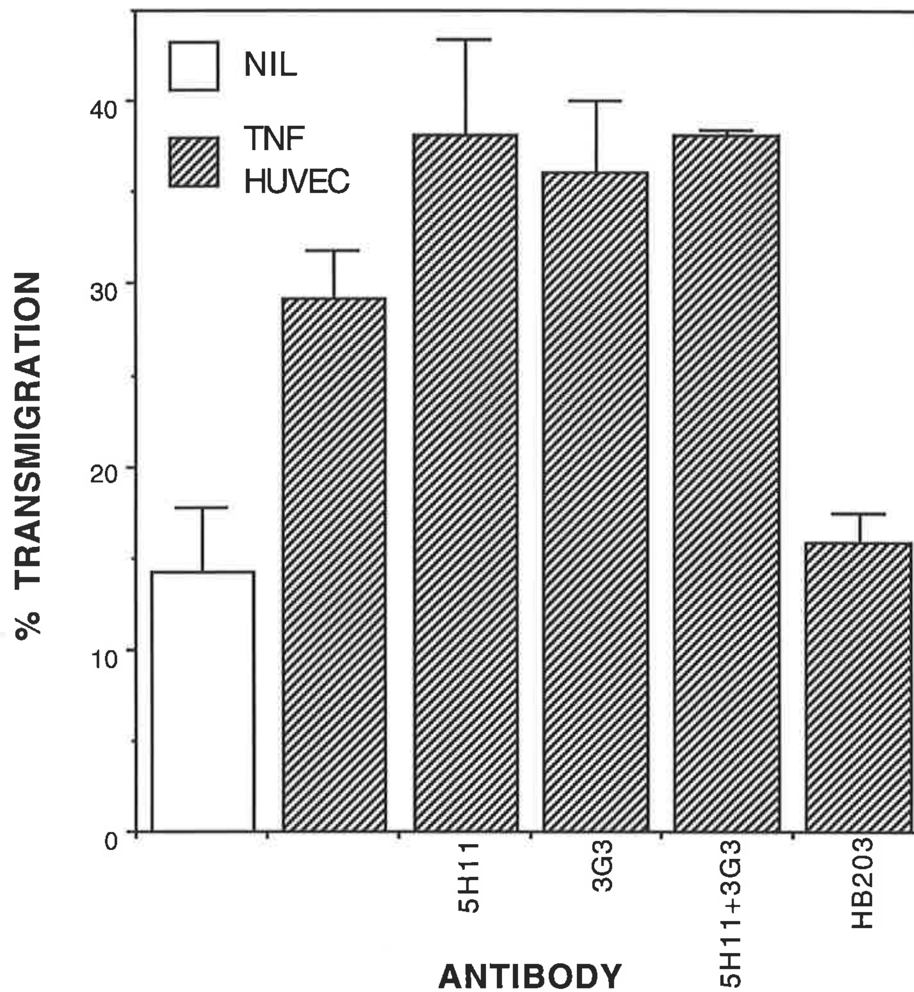


Fig. 5.7 The effect of E-selectin blocking antibodies on neutrophil transmigration through TNF-α activated endothelium

Endothelial monolayers cultured in transwells were preincubated with TNF-α 100U/ml for 4h, washed, and incubated with the antibodies 3G3, 5H11 and HB203 as in Fig. 5.6. Neutrophil transmigration was then quantitated as described in chapter 2. Experiment performed in duplicate, columns represent the mean ± SD.

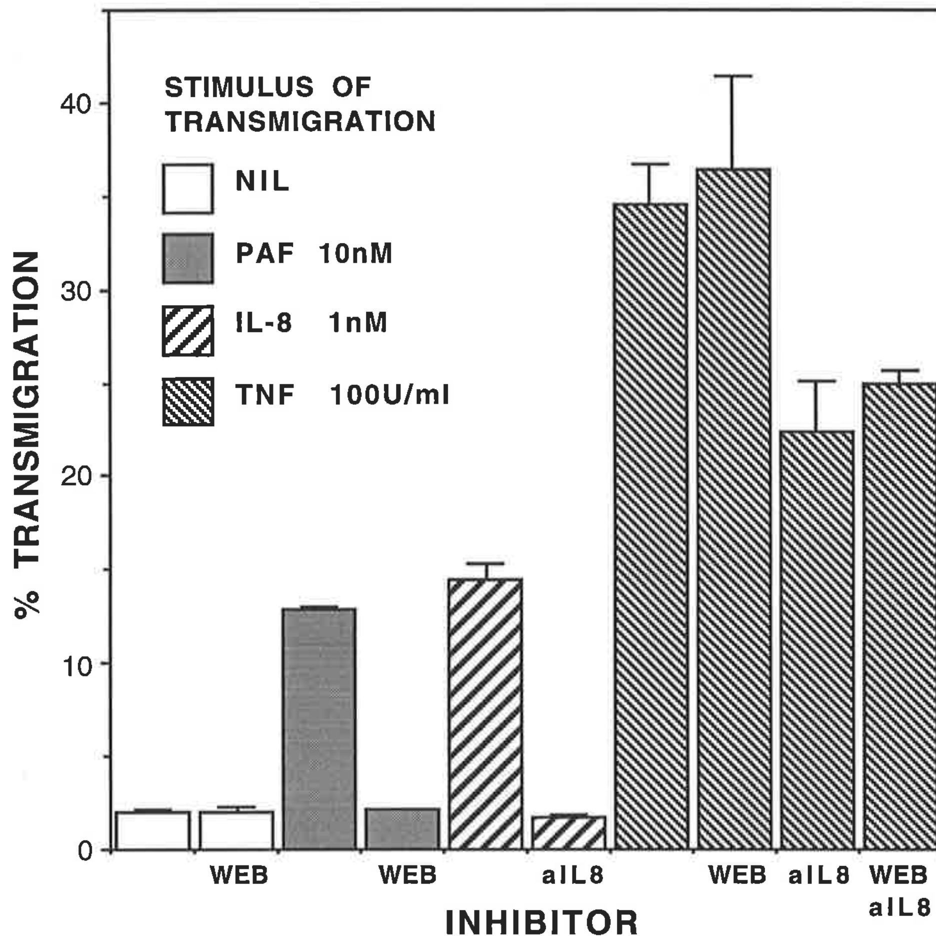


Fig. 5.8 The effect of PAF inhibition on transmigration through TNF-α activated endothelium

IL-8 at 1nM or PAF 10nM were placed in the lower compartment of the transwells, as indicated, as chemotactic stimuli of transmigration. Antiserum to IL-8 was added to one of the pairs of wells containing IL-8, prior to the experiment. 4 pairs of wells were preincubated with TNF-α 100U/ml for 4 h. After washing, 2 of the the TNF-α preincubated wells had medium replaced which contained IL-8 antiserum and the others were replenished with medium alone. The PAF receptor inhibitor, WEB2086 (WEB) was added to some wells, to a final concentration of 10μM, immediately prior to the addition of neutrophils, as indicated. 10^6 neutrophils were then added to each transwell, and transmigration quantified. Representative experiment, in duplicate, of 3 performed.

DISCUSSION

Experiments described in this chapter further explore the role of IL-8 as a mediator of neutrophil transmigration induced by cytokine-activated endothelium. An ELISA for IL-8 was developed in order to investigate the correlation between IL-8 production by endothelium and the ability of endothelium to induce neutrophil transmigration. TNF-α and IL-1 activation of endothelium induced production of IL-8 which was dependent on the concentration of stimulus. The concentration/response relationship was similar for IL-8 production by endothelium, and for endothelial induction of neutrophil transmigration, in independent experiments. The production of IL-8 by EC activated for 4, 24 and 48h by TNF-α was identical to the induction of transmigration in parallel experiments, whereas the expression of the adhesion molecules E-selectin and ICAM-1 did not correlate. Further examples of the correlation between IL-8 production and transmigration will be provided in later chapters. The immunoreactive IL-8 produced by activated EC and measured here is biologically active, since supernatants of activated EC have been shown to induce neutrophil chemotaxis across filters which was anti-IL-8 inhibitable(304). Blocking of E-selectin mediated adhesion of neutrophils to activated EC in the transwell assay did not inhibit transmigration, confirming the findings of Furie *et al.*(271), and refuting the contention that E-selectin, by activating neutrophil β_2 integrins, is an important mediator of transmigration(272). In addition, contrary to the findings of Kuijpers *et al.*(278), I did not find any inhibition of transmigration with the PAF receptor antagonist WEB2086, despite the clear activity of this compound in inhibiting transmigration to an exogenous gradient of PAF. Therefore my evidence does not support their suggestion of a role for PAF expression by cytokine activated endothelium in its induction of neutrophil transmigration.

An IL-8 transendothelial chemotactic gradient is able to induce up to 90% transmigration of neutrophils (Ch. 3). I hypothesised that the secretion of IL-8 by cytokine activated endothelial monolayers may be polarised, such that more IL-8 was secreted basally, in order to create a concentration differential between the upper and lower compartments (there are precedents for polarisation of endothelial secretion, *eg.* von Willebrandt's factor(317), endothelin-1(318)). However, it should be noted that transmigration through

cytokine activated endothelium is generally only in the range of 20-40%. This may be due to insufficient quantities of IL-8 being produced, or may be because IL-8 is produced on both sides of the monolayer, such that it is inducing transmigration by chemokinesis, which is far less effective (Fig. 3.6). To test this formally, IL-8 concentrations in the upper and lower compartments of a transwell were measured, and shown not to be significantly different (Fig. 5.2). Therefore, in this *in vitro* system at least (the situation may well be different *in vivo*, as discussed below), if IL-8 is a mediator of transmigration, it acts by chemokinesis.

In order to conclusively show that IL-8 is (at least partially) responsible for the transmigration of neutrophils induced by cytokine activated endothelium, it is necessary to specifically neutralise IL-8, and test whether transmigration still occurs. This could be done by adding IL-8 neutralising antibodies, or conceivably by using antisense oligonucleotides to block the production of IL-8 by removing the mRNA. The latter approach has not yet been attempted, but the results of the former were first reported by Huber *et al.* in 1991[277]. I have also used neutralising antibodies, but used a different approach which has produced some novel results. The transwell system allows the antibodies to be applied to either side of the monolayer. When IL-8 is completely neutralised, *ie.* on both sides of the monolayer, then transmigration is reduced, by a mean of 36%. This is less than the 71% reduction reported by Huber *et al.*, which may be due to the different experimental system used, but is in agreement insofar as the inhibition was subtotal, confirming our earlier evidence indicating that other mediators or mechanisms may also be present. An alternative explanation for the incomplete inhibition of transmigration is that the antibodies were not reaching IL-8 in the subendothelial space, bound to the matrix.

When antiserum was added to the lower compartment only, transmigration was further reduced. Taken in conjunction with the result shown in Fig. 5.2, that IL-8 would normally be present both above and below the monolayer, it can be seen that this would result in the creation of a reverse chemotactic gradient. Interestingly, antiserum added to the upper compartment only, tended to increase transmigration, perhaps not surprisingly, since

neutralisation of IL-8 above the monolayer would be expected effectively to increase the concentration gradient, favouring transmigration. However, this finding has several important implications. If IL-8 bound to the apical endothelial surface were important in neutrophil transmigration, acting to stimulate neutrophil motility in a haptotactic fashion as has been proposed[133], neutralisation of this by antibody in the upper compartment would be expected to reduce transmigration. More importantly, this manoeuvre (neutralisation of IL-8 secreted apically) may approximate more closely the situation that exists *in vivo*, for several reasons. The upper compartment represents the vascular space, and IL-8 secreted into the vessel would be diluted by plasma, and washed away by flowing blood, whilst that secreted into the subendothelial space would remain for longer since tissue fluids turn over more slowly, and would accumulate and increase concentration. Furthermore, it has been shown that red blood cells are able to adsorb and neutralise IL-8[208], through specific chemokine receptors[209], and even more interestingly, that normal individuals have specific neutralising IL-8 antibodies in serum[220]. Therefore, although secretion of IL-8 by EC *in vitro* is not polarised (despite which neutrophil transmigration still occurs), *in vivo*, apically secreted IL-8 would be neutralised, enhancing the chemotactic gradient, and as this experiment demonstrates, increasing the effectiveness of endothelial induction of transmigration.

A model of neutrophil transmigration *in vivo* can therefore be constructed, in which the endothelium plays a central role. Activated EC express E-selectin, which in a situation of flow, slows or stops the neutrophil on the endothelial surface. (This step is not necessary *in vitro* in a static assay.) An adhesive step between neutrophil LFA-1 and/or Mac-1 is essential for transmigration *in vitro* and *in vivo*. The β_2 integrins require activation for adhesion to their ligands, ICAM-1 or 2, and IL-8 (secreted by the endothelium) could perform this function, as shown in chapter 3. IL-8 is not necessarily the mediator of integrin activation at the apical endothelial surface however, since neutralisation of IL-8 in the top compartment did not reduce transmigration (Fig. 5.5). It is conceivable that E-selectin[272] or PECAM[56] could activate the leukocyte integrins when IL-8 is neutralised, but this was not tested. The third step is the actual migration of the neutrophil between the EC junctions to reach the other side of the monolayer, and this is dependent at

least in part on the production of IL-8 by the endothelium, as the neutralisation experiments clearly show. Other mediators of transmigration may exist in addition to IL-8, since the neutralising antibodies do not block completely, and this is under investigation (see also the following chapter). Experiments have shown that IL-8 is far more effective at induction of transmigration when acting by chemotaxis rather than by chemokinesis (87% *vs*. 21%, figs. 3.4, 3.5). *In vitro*, activation of endothelium with TNF-α or IL-1 induces migration of only up to 50% of neutrophils, and in most experiments, only 20-40%. This is explained by the finding that IL-8 is secreted by the EC into both compartments, and therefore can act only by chemokinesis. Converting this to a chemotactic gradient by neutralisation of IL-8 in the upper compartment tends to increase transmigration. Returning to the model of transmigration *in vivo*, IL-8 produced by the endothelium is likely to be acting by chemotaxis, since apically secreted IL-8 will be neutralised in the circulation. Each of the three steps of initial rolling or adhesion, firm adhesion, and transmigration can therefore be controlled by the endothelium, and IL-8 plays a critical role, mediating transmigration by its potent chemotactic effects.

SUMMARY

In chapter 3, it was shown that the neutrophil chemoattractant IL-8 could mediate neutrophil transendothelial migration by chemotaxis. The finding that endothelium activated by TNF-α or IL-1 can itself induce neutrophils to transmigrate was reported in chapters 3 and 4, and desensitisation studies, described in chapter 4, were consistent with the hypothesis that IL-8 produced by activated endothelium was a mediator of this transmigration. In this chapter, I firstly describe the use of an ELISA to measure IL-8 production by activated EC. IL-8 was produced by EC stimulated by TNF-α or IL-1 in a concentration-dependent fashion, and was sustained for 48h. The quantity of IL-8 produced correlated with the level of induction of transmigration. Expression of the adhesion molecules E-selectin and ICAM-1 by TNF-α activated endothelium did not correlate with transmigration. Blocking neutrophil adhesion to E-selectin did not reduce transmigration, suggesting that this molecule does not play a direct role in transendothelial migration.

Further evidence for the role of IL-8 in the induction of neutrophil transmigration by cytokine activated endothelium came from blocking antibody experiments. Neutralisation of IL-8 reduced transmigration through cytokine activated endothelium by 36%, proving that IL-8 is at least partially responsible. There may be other mediators accounting for the residual transmigration, or the antibody may not have adequately neutralised IL-8 sequestered in the subendothelium. Platelet activating factor is another putative mediator of neutrophil transmigration through cytokine-activated endothelium, but effective PAF inhibition did not further reduce transmigration when combined with IL-8 neutralisation, indicating that endogenous PAF has no effect in this system.

IL-8 produced by activated endothelium works in the transwell assay system by chemokinesis rather than by chemotaxis, since IL-8 was present at approximately equal concentrations in the upper and lower compartments. Neutralisation of IL-8 in the upper compartment converts the IL-8 effect to one of chemotaxis, and increased transmigration in some experiments. *In vivo*, since plasma flow, erythrocytes and endogenous neutralising antibodies may neutralise IL-8 secreted above the endothelium, it is likely that endothelium

derived IL-8 forms a chemotactic gradient across the monolayer, and therefore acts as a potent mediator of transmigration.

CHAPTER 6

THE ROLE OF G- AND GM-CSF IN NEUTROPHIL TRANSENDOTHELIAL MIGRATION: COMPARISON WITH IL-8

INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF) were originally isolated on the basis of their effects on myelopoietic progenitor cells[319]. They also have a range of effects on mature neutrophils, most of which could be considered pro-inflammatory, such as enhancement of phagocytosis, degranulation, and priming for and activation of the respiratory burst[320,321,322]. In addition, they induce up-regulation of neutrophil surface adhesion molecules of the β_2 integrin group CD11b/CD18, and down regulation of L-selectin[323]. GM-CSF induces neutrophil adhesion in-vitro[324,325], while G-CSF is without effect[323]. Both G- and GM-CSF have been reported to be chemotactic for neutrophils[326], but other studies have not revealed chemotactic activity[327], and this point remains controversial. It has been postulated that G and/or GM-CSF may, through their effects on neutrophil motility, play a role in attracting neutrophils to the inflammatory site, along with such known chemotactic mediators as C5a and IL-8[328]. I have shown in chapter 3 that IL-8 induces neutrophil transendothelial migration, a prerequisite to tissue infiltration. As shown in chapter 5, IL-8 also acts as a mediator for neutrophil transmigration induced by cytokine activated endothelium.

I hypothesised that G- and GM-CSF may cause neutrophil transmigration by chemotactic activity, and furthermore, since these factors are also produced by cytokine activated endothelium[329,330], that they may be co-mediators (along with IL-8) of endothelium-driven transmigration. I have investigated this by firstly testing the chemotactic effects of these CSF in a polycarbonate filter assay system, and subsequently testing their ability to induce transmigration through endothelial monolayers cultured on these filters, and comparing these effects with IL-8. I have used GM-CSF antibodies to determine whether neutralisation of this factor would reduce transmigration through TNF-α activated endothelium. The findings suggest that contrary to expectations, the CSF do not play a prominent role in the migration of neutrophils across endothelium *in vitro*. I have also confirmed recent reports that GM-CSF treatment of neutrophils reduces their migration through cytokine activated monolayers, but show that G-CSF does not have the same effect.

RESULTS

6.1 Gradients of G- and GM-CSF increase migration of neutrophils through polycarbonate filters

G- and GM-CSF were placed in the lower compartment of a transwell assembly, without endothelium. Both factors increased migration of neutrophils across the filter, reaching a peak at concentrations of 0·1-1nM (Fig. 6.1). Migration baseline was 31 ± 2·9%, and increased to a maximum at 0·1nM GM-CSF of 59·4 ± 1·5% (p<0·005). By comparison, 10nM IL-8 in the lower compartment increased migration to a peak level of 79·5 ± 3% (p<0·005). Chemotactic indices (*ie*. stimulated/baseline migration) were therefore 1·9 and 2·6 respectively.

6.2 G- and GM-CSF increase neutrophil migration on filters independently of gradient

G- and GM-CSF were placed in the upper and/or lower compartments of transwells, at 0·1 and 1, or 0·01 and 0·1nM respectively, in a checkerboard experiment. Table 6.1 shows that both G- and GM-CSF stimulated migration to the same extent whether they were in the lower, both or the upper compartment only. By comparison, IL-8 stimulated maximum migration when placed in the lower compartment only (chemotaxis), was less effective when placed in both compartments (chemokinesis), and did not stimulate migration at all when placed in the upper compartment only. These findings indicate that in this assay system, G- and GM-CSF stimulate neutrophil motility in a gradient independent fashion, *ie*. were chemokinetic and not chemotactic.

Table 6.1 GM-CSF and G-CSF cause similar increases in neutrophil migration across filters towards or against a gradient

G-CSF

		UPPER COMPARTMENT		
	nM	0	0·1	1·0
LOWER CPT.	0	20·3 ± 1·1	36·5 ± 3·0	46·3 ± 0·6
	0·1	42·3 ± 2·2	40·0 ± 1·6	39·1 ± 0·3
	1·0	44·6 ± 3·1	46·1 ± 0·4	41·7 ± 4·3

GM-CSF

		UPPER COMPARTMENT		
	nM	0	0·01	0·1
LOWER CPT.	0	26.0 ± 2.7	41·5 ± 4·3	43·0 ± 1·4
	0·01	44·7 ± 6·2	42·4 ± 2·0	41·9 ± 3·7
	0·1	41·8 ± 3·1	44·7 ± 1·2	39·0 ± 5·8

IL-8

		UPPER COMPARTMENT		
	nM	0	1	10
LOWER CPT.	0	18·8 ± 1·4	20·7 ± 2·7	23·5 ± 2·2
	1	77·6 ± 5·4	75·7 ± 1·8	43·7 ± 4·8
	10	86·3 ± 2·8	84·7 ± 3·9	67·9 ± 8·1

GM-CSF was *e.coli* derived. The factors were placed in lower, upper or both compartments of transwells in a "checkerboard" at the concentrations indicated. Representative experiment, in duplicate, of 3 (GM- and G-CSF) or 2 (IL-8) experiments performed.

6.3 Effect of G- and GM-CSF on neutrophil transendothelial migration

Similar experiments to the above were carried out with a confluent endothelial monolayer cultured on the transwell filter. Neutrophils accumulating in the lower compartment had migrated through the endothelium, reproducing the process of diapedesis which occurs *in vivo*. Fig. 6.2 shows that in the absence of any stimulus, the baseline transmigration is far lower than that through filter alone, indicating an effective barrier function of the endothelial monolayer. As shown previously in chapter 3, IL-8 placed in the lower compartment was very effective at inducing neutrophil transendothelial migration by chemotaxis. By contrast, gradients of G- and GM-CSF (both *e.coli* (non-glycosylated) and CHO cell (glycosylated) forms) produced only minimal (although statistically significant) transmigration, despite optimal concentrations being reached (Fig. 6.2). The potency of non-glycosylated GM-CSF is seen to be 10-fold higher than the glycosylated form in this system, which is in agreement with other assays. Its potency, defined by peak of effectiveness, is 100-fold greater than IL-8, however the maximum increase of transmigration over baseline is only 1·8 fold, compared to 10·8 fold for 10nM IL-8. G- and GM-CSF increased transmigration to a similar degree when present on the upper only, or both sides of the monolayer. Combinations of the CSF with IL-8 produced no greater than additive effects, which were evident only at low IL-8 concentrations (data not shown).

6.4 Neutralising GM-CSF antibodies do not affect the induction of neutrophil transmigration by activated endothelium

Transmigration of neutrophils was increased by preincubation of endothelium with TNF-α (data not shown). Neutralising antibodies against IL-8 inhibited this response by a mean of 44·9 ± 9·8%, which is in general agreement with previous findings (Ch. 5) and those of other groups[277]. To determine whether GM-CSF may be a co-mediator, with IL-8, of transmigration induced by cytokine activated EC, I used the anti GM-CSF neutralising antibody HGM2/3.1. This antibody reduced the motility stimulating effects of 0·01nM GM-CSF (*e.coli*) in a filter migration assay (Fig. 6.3A), but did not significantly inhibit the migration of neutrophils through cytokine activated endothelium (Fig. 6.3B). Anti- G-CSF neutralising antibodies were also used, but were of low titre and required high

concentrations to neutralise G-CSF, at which in some experiments the control antibody also reduced migration; however, the antibody did not reduce migration through TNF-α activated endothelium any more than control (data not shown).

6.5 GM-CSF but not G-CSF inhibits neutrophil transmigration through TNF-α activated endothelium

G- and GM-CSF placed in the upper compartment at the same time as addition of the neutrophils increased transmigration through resting endothelium, but GM-CSF reduced migration in response to TNF-α activated endothelium. Inhibition showed a concentration-response relationship (Fig. 6.4) and was maximal at 0·1nM. It was seen in 6/6 experiments, with mean inhibition of 54 ± 13%. (Inhibition was calculated as the decrease in transmigration on TNF-α activated monolayers, as a percentage of the transmigration on TNF-α activated monolayers minus background unstimulated transmigration). Interestingly, whilst G-CSF stimulated neutrophil motility to a similar degree as GM-CSF (Figs. 6.1, 6.2), it did not inhibit transmigration through activated endothelium (Fig. 6.4); at 0·1nM G-CSF small increases were seen in 3/5 and small decreases in 2/5 experiments, with the mean transmigration no different to control neutrophils.

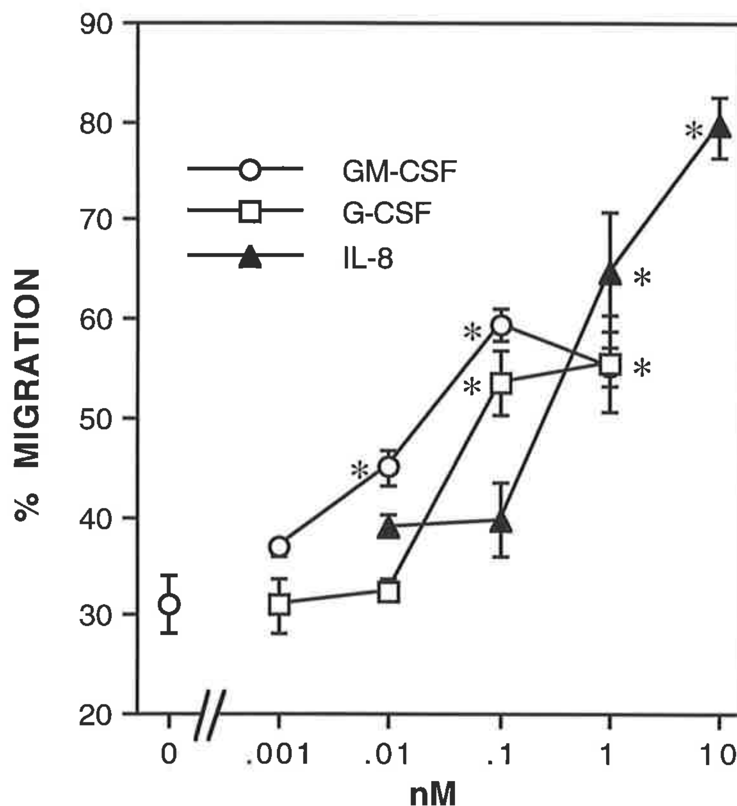


Fig. 6.1 Migration of neutrophils through filters without endothelium (chemotactic migration) in response to gradients of IL-8, GM-CSF or G-CSF.

IL-8, GM-CSF (CHO-derived) or G-CSF were placed in the lower compartment of the transwell apparatus at the indicated concentrations, and 10^6 neutrophils were placed into the transwell insert, which did not have an endothelial monolayer cultured on it. Migration into the lower compartment was quantified after 45 minutes. For GM-CSF, points are the mean ± SD of 6 experimental determinations (3 experiments in duplicate); for G-CSF and IL-8, representative experiments of 3, each point in duplicate. * $p<0{\cdot}005$, compared with unstimulated migration baseline, by independent Student's t-test.

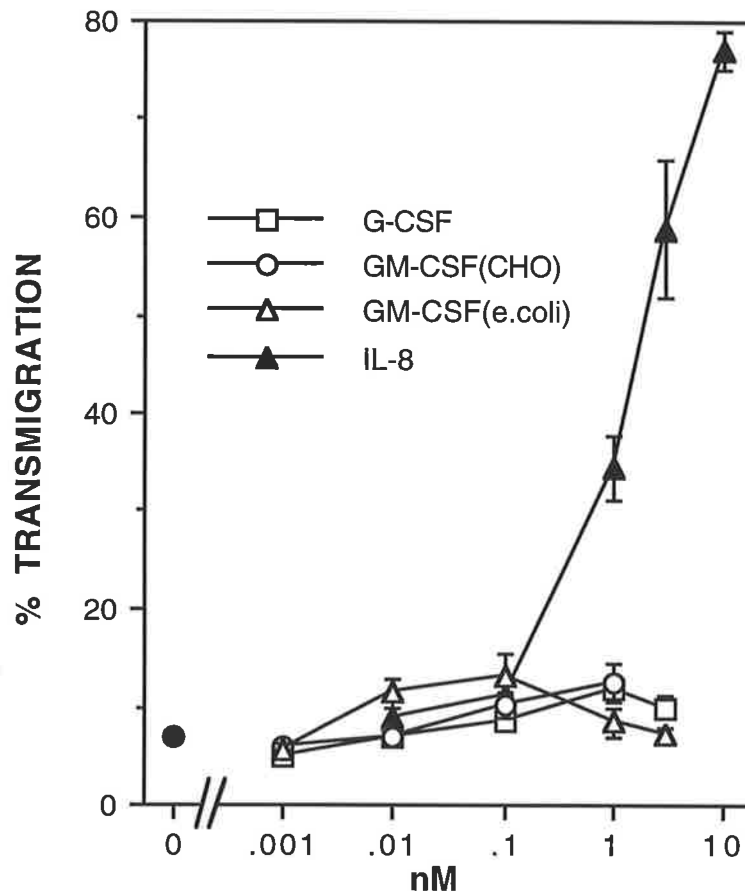


Fig. 6.2 Migration of neutrophils through filters with endothelium (chemotactic transmigration) in response to gradients of IL-8, GM-CSF or G-CSF

As in fig. 1, except neutrophils migrated through a monolayer of endothelium cultured on the transwell filter to reach the lower compartment. Points are the mean ± SEM of 6-10 experimental determinations for all factors, ie. 3-5 experiments in duplicate. The following points were significantly different to unstimulated migration by independent Student's *t*-test: $p<0{\cdot}005$- IL-8 0·1-10nM, G-CSF 1·0nM, GM(CHO) 1·0nM, GM(*e.coli*) 0·01, 0·1nM; $p<0{\cdot}05$- GM(CHO) 0·1nM.

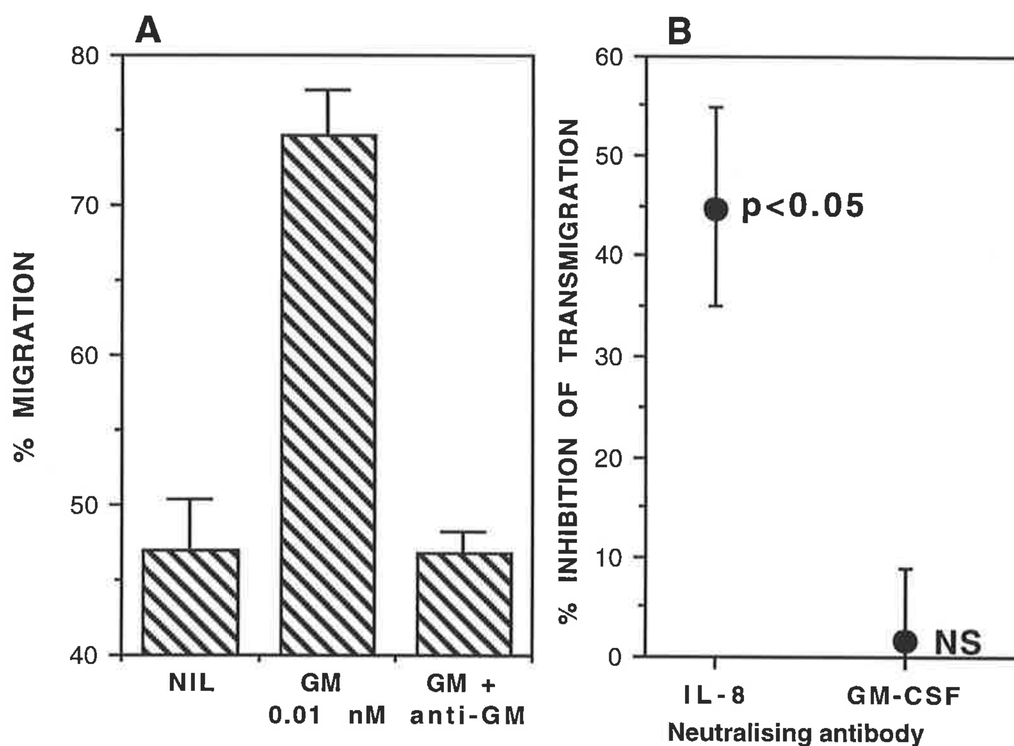


Fig. 6.3 Effect of neutralising antibodies on neutrophil transmigration in response to cytokine activated endothelium

(A) Antibody HGM2/3.1 blocks the stimulatory effect of GM-CSF on neutrophil migration through the filter alone. GM-CSF 0·01nM (*e.coli*) was preincubated with the antibody at 0·5µg/ml for 30 minutes before being placed in the lower compartment of the transwell apparatus, without endothelium. (B) Neutrophil transmigration through cytokine-stimulated endothelial monolayers is not inhibited by HGM2/3.1. Endothelial monolayers were preincubated for 4h in 100U/ml TNF; this resulted in an enhancement of neutrophil transmigration from 7·4±1·5% to 31·7±7·8%. Neutralising antiserum (1:500) to IL-8 or HGM2/3.1 at 0·5µg/ml were added to both sides of the monolayer 20min prior to the assay. Transmigration induction by activated endothelium was reduced by the indicated percentage, relative to baseline. Inhibition percentage was calculated for 4 separate experiments each in duplicate, and pooled. Points represent mean ± SEM; * $p<0{\cdot}05$, by paired Student's *t*-test.

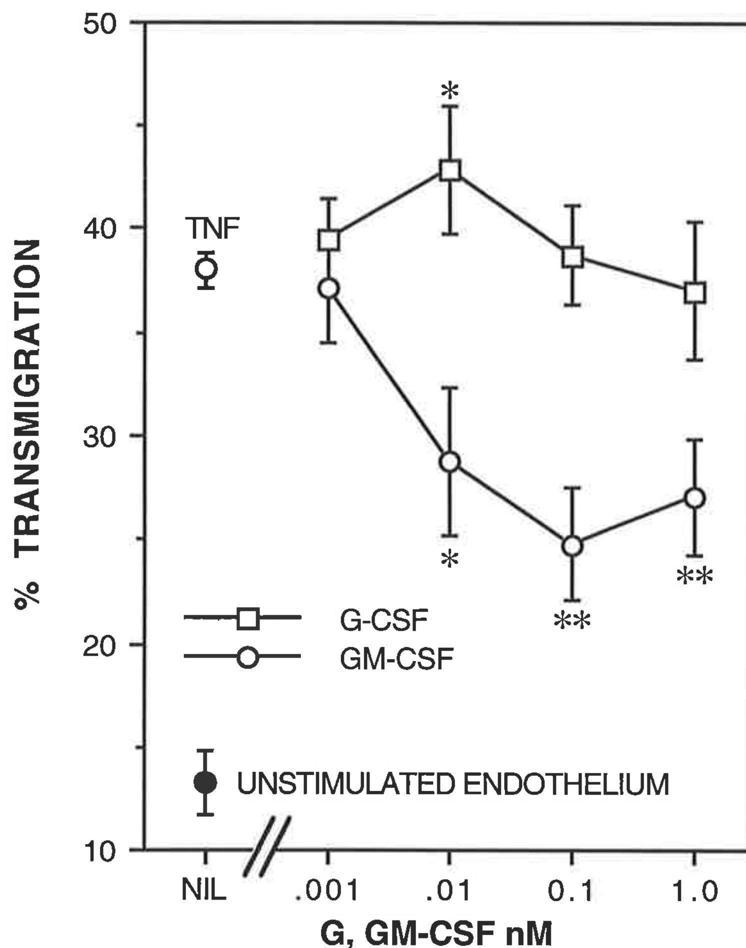


Fig. 6.4 Exposure of neutrophils to GM- but not G-CSF inhibits neutrophil migration through TNF-α stimulated endothelial monolayers

Endothelial monolayers were preincubated for 4h in 100U/ml TNF-α, which increased migration as shown (compare unstimulated endothelium). Increasing concentrations of GM (*e.coli*) or G-CSF were added to the top compartment with the neutrophils at the start of the transmigration assay, and transmigration quantified at 45 minutes. Each point is the mean ± SEM of 4-10 (G-CSF) or 6-12 (GM-CSF) separate determinations (ie. 2-6 experiments in duplicate). * $p<0{\cdot}05$, ** $p<0{\cdot}005$, comparison with no G- or GM-CSF, by independent Student's *t*-test.

DISCUSSION

The accumulation of neutrophils in tissues is a prerequisite step in the acute inflammatory response. This is dependent on their adhesion to, and transmigration through endothelium (diapedesis). After the arrest of the neutrophil by adhesion at the apical endothelial surface, mediated by the selectins, transendothelial migration may occur through two mechanisms; firstly, the attraction of the neutrophil to chemotactic factors produced in the tissues, and secondly, the induction of neutrophil transmigration by "activated" endothelium. I have shown in chapter 3 that IL-8, a neutrophil chemoattractant produced by a variety of tissue cells stimulated by inflammatory mediators, is a potent effector of the first mechanism, chemotactic transmigration. In the second mechanism, endothelium activated by the cytokines IL-1 and TNF-α induces the transmigration of unstimulated neutrophils, in the absence of any added chemotactic gradients, and without any increase in endothelial permeability[265,266]. Activated EC produce IL-8, and this has recently been shown to mediate the transmigration of neutrophils[277], although it is only partially responsible (Ch. 4, 5, (277)).

I have therefore sought to determine what other factors might mediate transmigration induced by cytokine activated endothelium. G- and GM-CSF are candidates, since they would seem to share many properties with IL-8. All factors have been reported to have chemotactic activity for neutrophils, they may be produced by stimulated tissue resident cells, and they are produced by EC activated by IL-1 and TNF-α. I hypothesised that like IL-8, G- and GM-CSF may attract neutrophils across endothelium when produced in the tissues, and also that they may be co-mediators, with IL-8, of transmigration induced by cytokine activated endothelium.

In order to assess the chemotactic activity of the CSF, I used polycarbonate filters in transwells, since this is a parallel system to that which I use for determining endothelial transmigration. This is essentially a Boyden chamber using a polycarbonate filter with 3μm pores. Another feature of this assay was that I counted cells in the lower compartment, *ie*. "drop off" cells, since it was found that these represented the vast majority of cells which had migrated. It is possible that those who measure only cells

adherent to the undersurface of the filter may be underestimating migration, at least in the case of neutrophils.

These experiments showed that neutrophil migration could be stimulated by G- and GM-CSF in the upper compartment only, *ie.* against a concentration gradient, to the same extent as with the gradient. This was not the case with the prototypic chemotactic factor, IL-8. Therefore in this system at least, G- and GM-CSF are chemokinetic and not chemotactic. However, the degree of migration across filters which occurred in the presence of a CSF gradient was of a similar order of magnitude to that occurring with an IL-8 gradient. It was surprising then that when an endothelial monolayer was present, G- and GM-CSF were far less effective at inducing transendothelial migration than IL-8. In chapter 3, it was shown that the presence of the endothelial barrier actually increases the chemotactic effectiveness of IL-8, presumably by maintaining the gradient and preventing it from being diluted by diffusion into the upper compartment. The CSF and IL-8 are all of low molecular weight, and IL-8 and GM-CSF diffuse similarly across endothelium (data not shown). Therefore there appear to be fundamental differences in the motility stimulating effects of the CSF and IL-8 which are reflected in the above assays.

Given these findings, it appeared unlikely that G- and GM-CSF could play a significant role as mediators of transmigration induced by activated endothelium. This was further confirmed when addition of neutralising GM-CSF antibodies failed to reduce transmigration through TNF-α activated endothelium (Fig. 6.3B). Furthermore no additional decrease in transmigration was seen when GM-CSF antibodies were combined with IL-8 antibodies, above that produced by IL-8 antibodies alone (data not shown). Neutralising G-CSF antibodies are difficult to produce due to the close homology between murine and human G-CSF, however although there were technical difficulties, it appeared that G-CSF antibodies (alone and in combination with IL-8 and GM-CSF antibodies) also had no effect (data not shown).

When neutrophils were exposed to GM-CSF above the endothelial monolayer, migration in response to TNF-α activated endothelium was reduced. This has recently been described by others[331,332]. Several other factors with neutrophil activating properties also have the

effect of reducing transmigration through cytokine activated endothelium (Ch. 4, (332)). It was therefore surprising to find that G-CSF, which is similar to GM-CSF for the properties examined in this paper, did not reduce this form of transmigration. The mechanism of the inhibition by GM-CSF and other activating factors is not known, but does not appear to be related to an alteration in adhesion molecules[(332)], or the responsiveness of neutrophils to IL-8 (Ch. 4). It may be related to alterations in neutrophil cytoskeletal properties which are essential for the response to activated endothelium[(332)]. In-vivo studies have shown a reduction in migration of neutrophils to inflammatory sites during treatment with GM-CSF[(315)], which would be at least partially explained by the above findings, but I am not aware of reports of similar reductions during G-CSF treatment, again consistent with my results.

This study has examined the motility responses of neutrophils in-vitro, and suggests that the CSF are not likely candidates for endogenous direct mediators of neutrophil diapedesis at sites of inflammation *in-vivo*. I have also found that there are differences in the effect of the CSF on the neutrophil transmigration response to activated endothelium which might have relevance to their therapeutic use.

SUMMARY

GM- and G-CSF have pro-inflammatory effects on mature neutrophils. Both factors have been reported to cause neutrophil chemotaxis, and are produced by cells stimulated by inflammatory mediators, including endothelial cells. In this chapter, I have tested the hypothesis that these factors might mediate neutrophil transendothelial migration, either by forming a gradient across the endothelial monolayer, or through the production of CSF by activated endothelium. Studies of neutrophil migration across filters without endothelium showed that migration was promoted in the presence of a gradient of either of the CSF, but was equally promoted against the gradient, *ie.* the CSF are chemokinetic but not chemotactic. The CSF promoted migration of neutrophils across endothelial monolayers cultured on filters, but the magnitude of this effect was very small compared with a prototypic neutrophil chemoattractant, IL-8 (migration index *ie.* stimulated/unstimulated, 1·8 fold for GM-CSF, 10·8 fold for IL-8). Activation of endothelial monolayers by preincubation with TNF-α increased neutrophil transmigration significantly; neutralising antibodies to IL-8 inhibited this increase by 44%, whereas neutralising anti-GM-CSF antibodies did not inhibit. These data suggest little role for the CSF in neutrophil diapedesis at inflammatory sites in-vivo. Exposure of neutrophils to GM-CSF decreased their migration through TNF-α activated monolayers whereas G-CSF did not. This may have implications for the therapeutic administration of these factors.

CHAPTER 7.

TNF RECEPTOR USAGE ON ENDOTHELIAL CELLS

INTRODUCTION

Tumour necrosis factor α (TNF-α) was originally characterised on the basis of two predominant types of activity. As the name suggests, this factor has direct cytotoxic effects on tumour cells[333], although only a limited range of tumours are susceptible. The other initially characterised activity is that of the induction of a general wasting syndrome or cachexia, the causative factor of which was first named cachectin[334], but was later found to be identical to TNF-α. Subsequently many additional activities have been ascribed to this highly pleiotropic cytokine, but those of particular interest are the pro-inflammatory effects of TNF-α on many cell types. In particular, TNF-α activates inflammatory functions in neutrophils[335], eosinophils and monocytes, such as production of oxygen radical species, phagocytosis of micro-organisms, degranulation with release of proteolytic enzymes, and cell adhesion (reviewed in [336]).

TNF-α acts upon EC to produce an "activated" state, characterised by enhanced or new expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin), with consequent adhesiveness for leukocytes[9]; the induction of pro-coagulant activity; production of IL-6 and GM-CSF[336]; and as demonstrated in previous chapters, production of IL-8 and consequent induction of neutrophil transmigration. The pro-inflammatory effects of TNF-α have significantly limited its clinical effectiveness as an anti-tumour agent for therapeutic use.

There are two separate receptors for TNF-α, p55 and p75 (reviewed in [337]), which were cloned in 1990[338,339,340,341]. The extracellular portions of these two molecules are homologous to each other, and both are able to bind TNF-α (and TNF-β or lymphotoxin) with high affinity. The intracellular portions are not homologous to each other, suggesting that the two receptors may employ different signalling pathways. Since these cytoplasmic domains have no homology to any known signalling proteins, the transduction pathways remain unknown. It is likely that additional proteins are required to associate with these receptors to initiate signal transduction. The distribution of the p55 receptor appears to be ubiquitous, while the p75 may be relatively more restricted to cells of haematopoietic

lineage. Whether the two receptors mediate different functions has been highly controversial[342,343]. Separation of the anti-tumour from the pro-inflammatory effects would obviously be of great potential therapeutic benefit.

Endothelial cells were initially reported to bear only p55 receptors[344], however many studies have now shown that they express both receptors[345,346], including analysis carried out in our own laboratory[347]. Several groups have used receptor specific blocking antibodies to determine the role of each of the receptors in mediating the TNF-α effect on EC, with inconclusive results[345,346]. We obtained mutant TNF-α molecules with amino-acid substitutions in the receptor binding region, which reduced their binding to either of the receptors separately, producing relatively receptor-specific agonists. Mutants were produced by site-directed mutagenesis of TNF-α cDNA, and expressed in *e.coli*. The mutants R32W (arginine at position 32 substituted by tryptophan), E146K (glu 146 $\rightarrow$ lys) and R32W/S86T (R32W + ser 86 $\rightarrow$ thr) show only very slight reductions in affinity for p55 relative to wild type (WT) TNF-α, but have moderate (R32W) or marked (E146K, R32W/S86T) reductions in affinity for p75, such that they are relatively p55 specific, while D143F (asp 143 $\rightarrow$ phe) binds only to p75. Table 7.1 shows the receptor binding affinities of the mutant molecules, relative to wild-type TNF-α[347]. TNF-α mutants were then compared with WT TNF-α for their ability to activate a range of EC responses. Functions tested included expression of E-selectin, production of IL-8, and induction of neutrophil transmigration.

This chapter describes work undertaken by myself as part of a collaborative project with others in the Division of Human Immunology, IMVS. Jeffrey A. Barbara tested the mutant molecules on tumour cell lines and neutrophils, and Angel F. Lopez supervised the project. Xaveer van Ostade and Walter Fiers from Ghent in Belgium (University of Ghent, and Roche Research, respectively) produced and kindly made available the TNF-α mutant molecules for these experiments.

Table 7.1 TNF-α mutants- receptor affinity and specificity

TNF-α mutant	relative p55 affinity	relative p75 affinity	specificity
WT	1	1	p55=p75
R32W	0.7	0.002	p55>p75
E146K	0.5	0.0003	p55>>p75
R32W/S86T	0.45	0	p55
D143F	0	0.03	p75

RESULTS

7.1 Receptor p55 specific mutants show reduced potency in activation of EC compared to WT TNF-α

The mutants R32W, E146K and R32W/S86T were tested for their ability to activate EC functions of E-selectin expression, quantitated by flow cytometry (using a MAb raised in our own laboratory, 9D9), IL-8 production, quantitated by ELISA, and neutrophil transendothelial migration quantitated by transwell assay. In each case, EC were activated by the addition of TNF-α, WT or mutant, at varying concentrations 4h before the measurement of function.

Fig. 7.1a shows the effect of R32W on E-selectin expression by EC, and 7.1b the effect of R32W on EC induction of neutrophil transmigration. R32W was only marginally less potent than WT TNF-α. Potency was determined by the EC50, or concentration of TNF-α required to produce 50% of the maximum effect. The mean relative potency of R32W compared to TNF-α WT is shown in Table 7.2. At peak concentrations, the effectiveness of R32W was equal to WT.

E146K and R32W/S86T were markedly less potent than WT. Fig. 7.2a shows concentration/response curves for induction of E-selectin. Since the levels of expression of different HUVEC lines was variable, the Y-axis is scaled as an index of maximal E-selectin expression at optimal concentration of WT, so that the results of separate experiments could be pooled. R32W/S86T has identical effect on HUVEC E-selectin expression to E146K. Greater concentrations of either mutant are required to stimulate the same level of expression as WT. Fig. 7.2b shows EC production of IL-8 after stimulation with TNF-α WT or E146K, also scaled as an index. R32W/S86T was similar to E146K for activation of IL-8 production, in 2 experiments (data not shown). Transmigration of neutrophils through endothelium activated by TNF-α WT or E146K is shown in Fig. 7.3, and is the pooled results of 3 experiments. The mean relative potencies of E146K and R32W/S86T are shown in Table 7.2. Although not proven in every experiment (the

concentration of TNF-α mutant used was not always high enough), at maximal concentrations of E146K and R32W/S86T, effectiveness was equivalent to WT TNF-α.

Table 7.2 Relative potencies of TNF-α mutants compared to WT TNF-α in assays of EC function.

	E-selectin induction		IL-8 induction		Neutrophil transmigration	
	n		*n*		*n*	
TNF-α WT		1		1		1
R32W	4	0·38	3	0·27	3	0·62
E146K	7	0·008	6	0·02	3	0·065
R32W/S86T	3	0·006				

n=number of assays performed. Potency calculated as EC50 (effective concentration producing 50% of the maximum activity) of WT/mutant.

7.2 A p75 specific TNF-α mutant is inactive on EC

TNF-α mutant D143F has a reduced affinity at p75, but no binding at all to p55, thus it is p75 specific. This mutant was tested for activation of EC by E-selectin expression and IL-8 production. In 3 separate experiments, D143F showed no activity at all for either of these functions. Fig. 7.4a shows a representative experiment.

As a positive control to ensure that D143F was active, Dr. J Barbara in our laboratory tested it on PC60 cells[347]. This is a rat/mouse T cell hybridoma cell line which is 100-500 fold less sensitive to human TNF-α than to murine TNF-α. When transfected with human p75, these cells respond to human TNF-α as well as to murine, and produce GM-CSF. D143F stimulation caused significant GM-CSF production in p75 transfected cells (approximately 1/3 as much compared to the same concentration of WT TNF-α, in keeping

with the reduced affinity of this mutant for p75) (E146K caused no production at all). Therefore, D143F not only binds to but is an agonist at p75, in responsive cell types.

Since D143F binds to p75 but does not stimulate function in EC, I tested the hypothesis that excess D143F might block p75 and prevent WT TNF-α from binding to it, therefore reducing its potency to that of E146K. Fig. 7.4b shows that D143F at 1000ng/ml did not significantly antagonise WT TNF-α at 0·01 or 0·1 ng/ml (compare E146K in the same figure). Nor did binding of p75, with D143F 30ng/ml and binding of p55 with E146K 1ng/ml reproduce the potency of WT.

7.3 Antibodies to p55 activate EC

Antibodies against p55 were obtained which show no cross-reactivity with p75. Some p55 antibodies block TNF binding without activating the receptor(HTR-5[(346)]), but MAb HTR-9 and HTR-1 have agonist activity at the p55 receptor. These antibodies were used to activate EC E-selectin expression. HTR-1 was considerably more potent, presumably because it is an IgM and can therefore extensively cross-link receptors (although cross-linking has not formally been proven to be necessary for TNF receptor signalling). Fig. 7.5 shows that these antibodies can efficiently activate EC, although interestingly, despite peak concentrations being reached, HTR-1 does not increase the MFI for E-selectin to as high levels as does WT TNF-α.

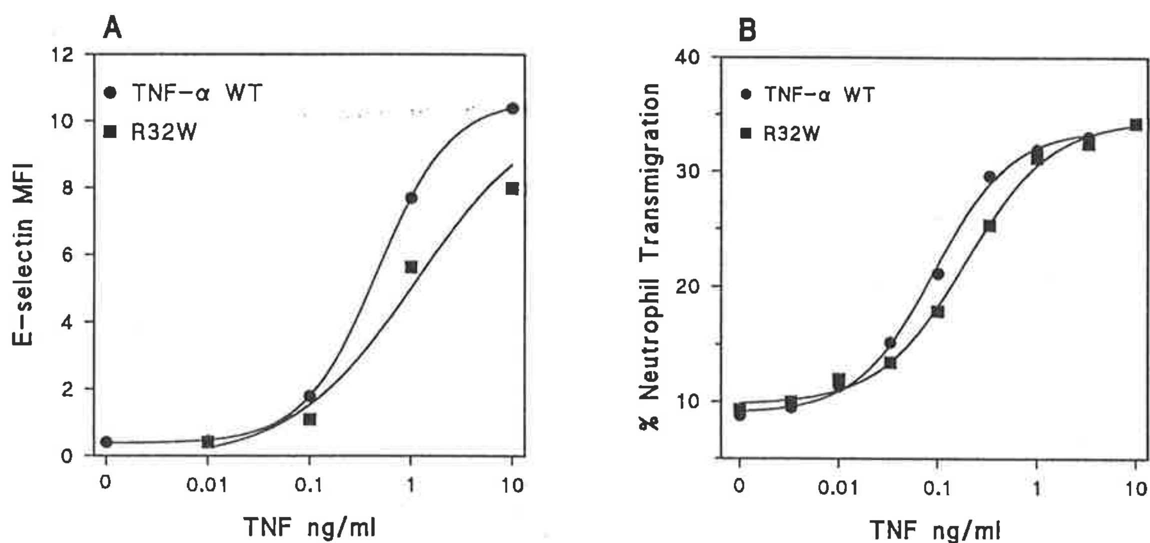


Fig. 7.1 R32W is slightly reduced in potency of induction of E-selectin and neutrophil transmigration compared with WT TNF-α

(a) HUVEC in 24 well dishes were treated with TNF-α WT or mutant R32W for 4h at the indicated concentrations, then stained with MAb 9D9 against E-selectin and processed for flow cytometry. MFI=mean flourescence intensity of 10,000 cells counted. Representative experiment of 4. (b) HUVEC monolayers in Transwells were activated by preincubation with TNF-α WT or R32W at the indicated concentrations for 4h, and neutrophil transmigration through the monolayers was then quantitated. Composite of three experiments.

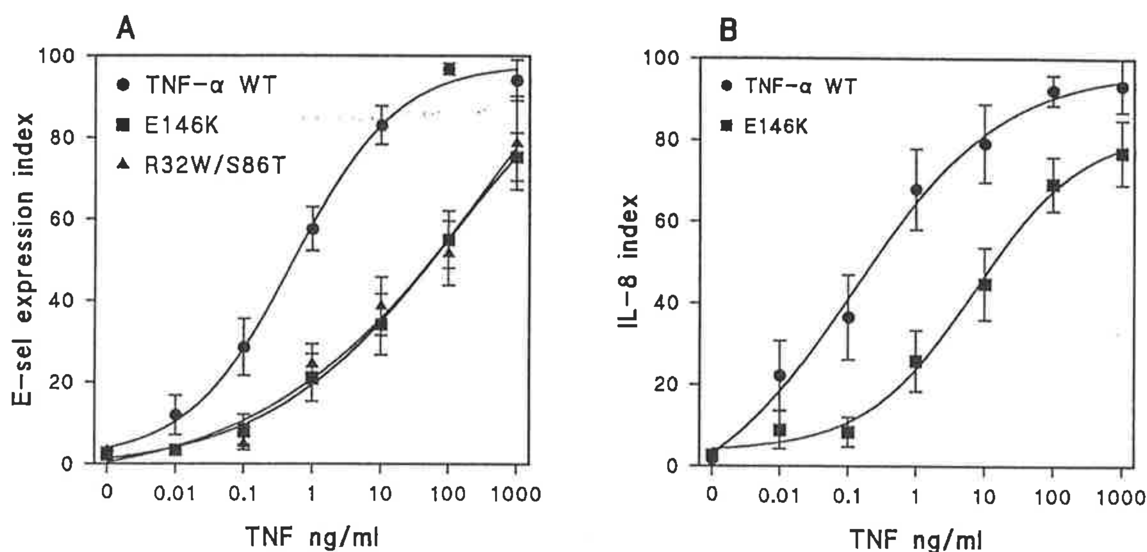


Fig. 7.2 Highly p55-specific TNF-α mutants show markedly reduced potency in activation of HUVEC E-selectin expression and IL-8 production

(a) HUVEC in 24 well dishes were treated with TNF-α WT or mutants E146K or R32W/S86T for 4h at the indicated concentrations, then stained with MAb 9D9 against E-selectin and processed for flow cytometry. MFI was determined for each point, and values for each experiment were expressed as an index of the maximum value obtained at the optimal concentration of WT TNF-α. The effects of both mutants were significantly different to WT, $p<0.001$, by ANOVA (two factor with replication). (b) HUVEC in 24 or 96 well dishes were treated with TNF-α WT or E146K for 4h, medium exchanged, then collected after 1h, and IL-8 quantitated by ELISA. 6 experiments were performed in duplicate or triplicate. The mean value for each point was expressed as an index of the maximal IL-8 level produced by optimal concentrations of WT TNF-α for each experiment. The mean ± SEM of these 6 values are shown. The effects of E146K were significantly different to WT, $p<0.001$, by ANOVA (two factor with replication).

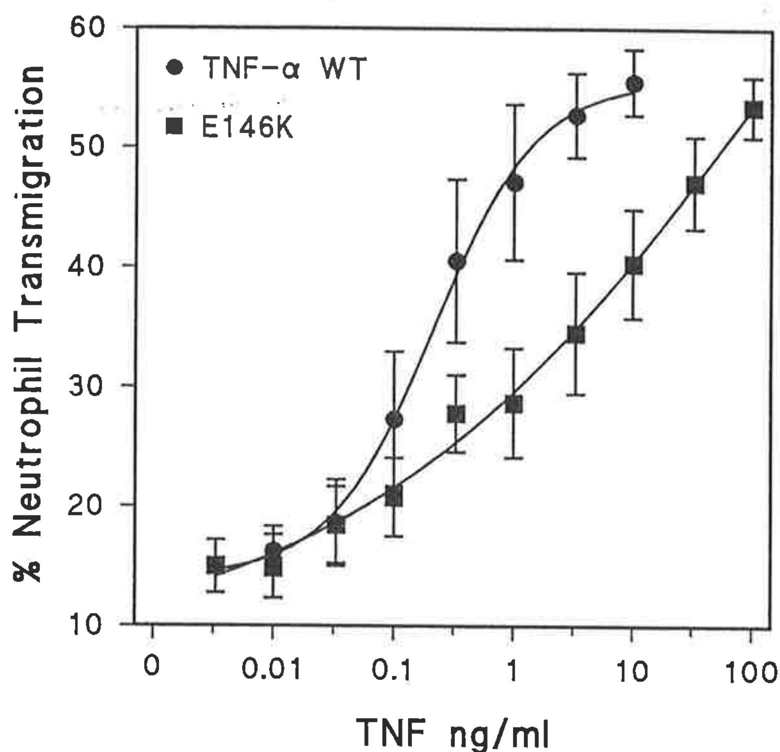


Fig. 7.3 E146K is markedly less potent at stimulating EC to induce neutrophil transmigration than WT TNF-α

HUVEC monolayers in Transwells were activated by preincubation with TNF-α WT or E146K at the indicated concentrations for 4h, and neutrophil transmigration through the monolayers was then quantitated. Each point is the mean ± SEM of three values, from three separate experiments. The effects of WT TNF-α and E146K were significantly different, $p<0.001$, by ANOVA (two factor with replication).

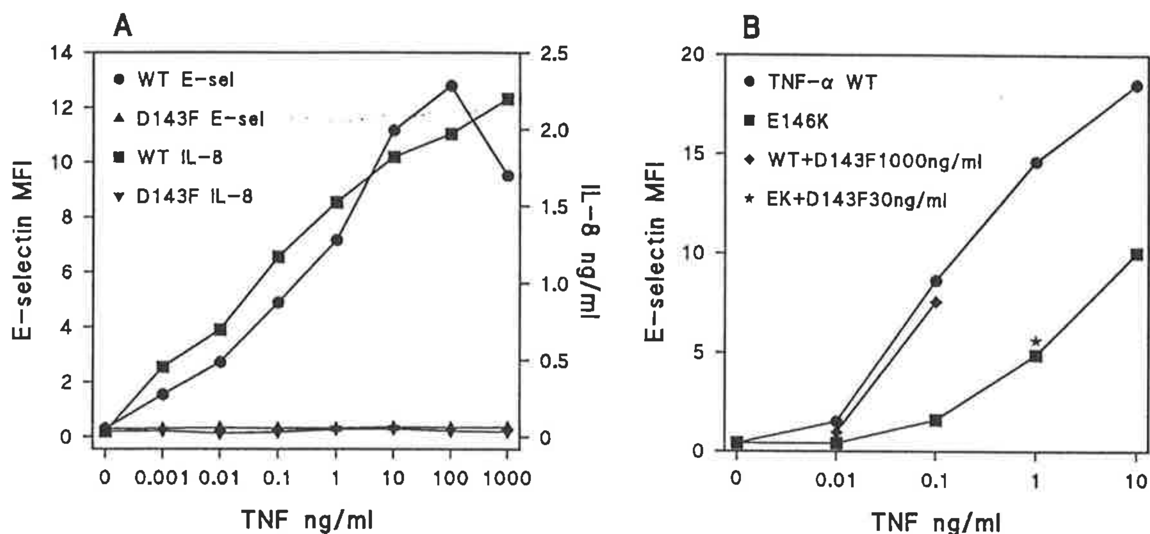


Fig. 7.4 The p75 specific mutant D143F does not activate EC

(a) WT TNF-α or D143F was incubated with EC at varying concentrations and E-selectin expression and IL-8 production after 4h was quantitated as in the preceding figures. E-selectin MFI and IL-8 production in ng/ml are both shown, on separate Y axes. Representative experiment of 3. (b) EC were activated by varying concentrations of WT TNF-α or E146K alone, or in combination with D143F, for 4h, and expression of E-selectin then measured. Representative experiment of 2.

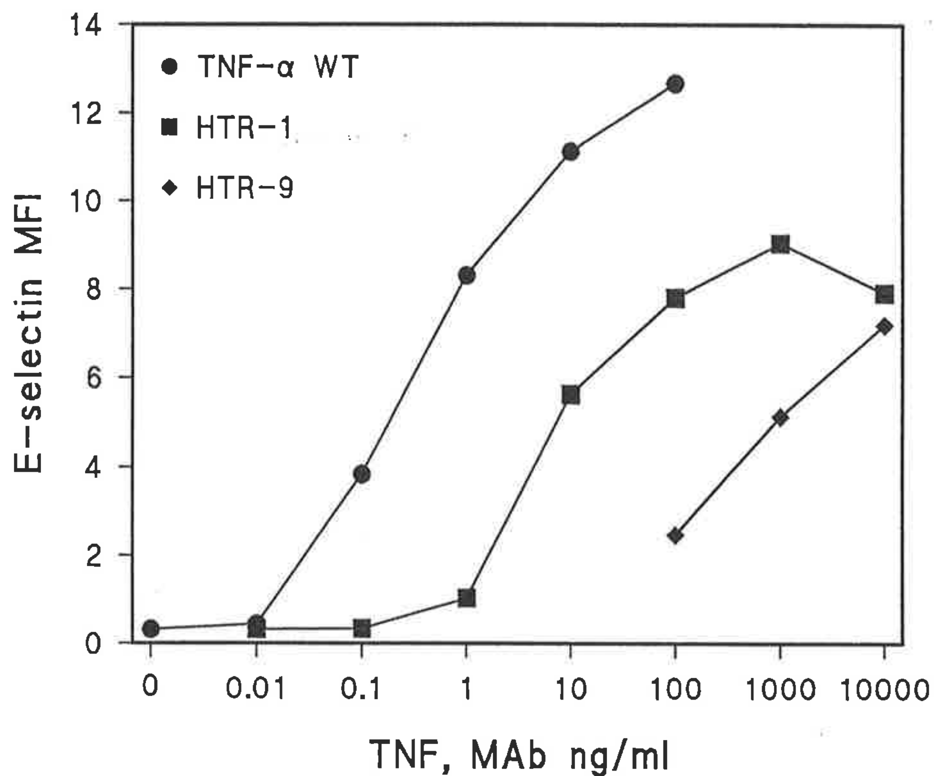


Fig. 7.5 p55 monoclonal antibodies stimulate HUVEC E-selectin expression

MAb's HTR-9 (IgG1) and HTR-1 (IgM), and TNF-α WT, were incubated with HUVEC monolayers cultured in 24 well dishes for 4h at the indicated concentrations. E-selectin expression was then quantitated by flow cytometry using the MAb 9D9. Anti-receptor MAb's alone without 9D9 increased MFI due to their binding to the p55 receptor, but in this case the MFI was not greater than 0·8. Representative of 2 experiments.

DISCUSSION

In the experiments described in this chapter, I have used receptor specific TNF-α mutant molecules, as well as TNF receptor antibodies, to determine the roles of the p55 and p75 TNF receptors in the activation of HUVEC by TNF-α. A close correlation was found between the EC functions of induction of expression of E-selectin, production of IL-8, and ability to induce neutrophil transmigration, for each of the TNF-α variants, so these functions will not be discussed individually. Because the major difference between WT TNF-α and the mutants was found to be in their potency as agonists, with shifts seen in the concentration/response curves, the figures in this chapter have been prepared with a program (Fig.P) that allows mathematical curve fitting of data, so that EC50 can be calculated for each mutant (Table 7.2).

A TNF-α mutant with moderate reduction in affinity for p75 (R32W) showed similar effects to WT TNF-α, with only a slight reduction in potency. Taken alone, this could be interpreted to be due to the slight reduction in affinity of R32W for p55. However, mutants with the same affinity for p55 but more marked reductions in p75 affinity (E146K, R32W/S86T) were 16-160 fold less potent, suggesting a significant role for p75 binding in WT TNF-α activity. All mutants were able to activate EC to the same degree as WT when used at high enough concentrations. This suggests that binding to p55 alone is fully able to activate HUVEC, but that p75 plays an accessory role, conferring enhanced potency on WT TNF-α which binds both receptors. The p75 specific mutant D143F had no effect even at high concentrations, showing that this receptor alone is not able to activate EC. Activating antibodies to p55 confirm that p55 is able to activate HUVEC by acting alone. Although the antibodies showed a reduction in degree of activation relative to WT, this may have been due to a qualitative difference in the mode of activation of p55, and not due to lack of activation of p75.

Previous studies on this matter have led to conflicting results. Shalaby *et al.*[346] used blocking p55 and p75 antibodies, and showed that either could reduce the activity of TNF-α on EC, and that both together blocked completely, leading to the conclusion that

binding to both receptors was responsible for TNF-α effects. Mackay *et al.*(345) showed that EC activation could be mediated through p55 using the receptor specific agonists HTR-1 and R32W/S86T, and that p75 agonist antibodies had no effect, therefore concluding that p55 alone was responsible for TNF-α effects on EC. Neither of these groups, however, carried out concentration/response curve experiments. Analysing these studies in the light of our work, the first set of results would be expected if moderate concentrations of TNF-α were used (indeed this group used 1-10U/ml (0·02-0·2ng/ml) of TNF-α), and the second set at high agonist concentrations (10ng/ml of WT and mutant TNF-α were used). Therefore, differing results were obtained by these groups, both of which are consistent with our work, and explained by the different concentrations of TNF-α used. The opposing conclusions of these groups are therefore both incorrect, since the interaction of p55 and p75 are dependent on the concentration of agonist.

Recently, Slowik *et al.*(348) carried out similar studies to those of Shalaby *et al.*(346) discussed above. It was found that the effects of low concentration (0·2ng/ml) WT TNF-α could be inhibited by blocking MAb against either p55 or p75. This group also used R32W, and showed that the (reduced) effects of this agonist at the same concentration were inhibited by p55 but not p75 MAb. They also showed that preincubation of EC with R32W could desensitise them to subsequent exposure to WT TNF-α suggesting that, at high concentrations, the major receptor was p55. They therefore reached conclusions similar to ours, *ie*. that p55 was the principal activating receptor, but that p75 contributes to activation at low TNF-α concentrations.

The manner of interaction between p55 and p75 in EC activation is unknown. One hypothesis is that since TNF-α is a heterotrimer and able to bind three molecules of receptor, the simultaneous binding and therefore aggregation of mixed sets of p55 and p75 by each TNF-α ligand may be more active in signalling. However, this would be expected to be evident at both high and low concentrations of ligand. Furthermore, consideration of the tertiary structure of the TNFR suggests that each TNF-α trimer should be able to bind either three molecules of p55 or three molecules of p75 (homocomplexes) but not both together(heterocomplexes)(347). Cross-linking experiments, which readily detect TNF-

receptor homocomplexes on cells incubated with TNF-α, do not detect heterocomplexes[349]. Another possibility is that the intracellular signals of the two receptors converge, such that p75 signalling interacts with and facilitates signals transmitted via p55. I tested this hypothesis by combining the p55 agonist E146K and the p75 agonist D143F (at 30× higher concentration, to account for lower receptor affinity)(Fig. 7.5), but no enhancement of E146K activity was seen. Also it was recently shown[349] (albeit using different cell types) that antibodies which are agonists at p75 could block the effects of p75 in enhancing the potency of TNF-α at low concentrations, suggesting that the generation of a p75 signal is not involved.

Another hypothesis was recently suggested by Tartaglia *et al.* It was shown that the association rate for TNF-α with p75 was 20× more rapid than with p55, but that TNF-α also dissociated rapidly from p75. However, the presence of both p75 and p55 together on a cell increased the association of TNF-α with p55 by approximately 10×. The rate of dissociation from p55 was very slow. Taken together, these results suggest a model in which p75 captures TNF-α rapidly and facilitates its binding to p55 ("passing on"), where it remains bound and signals[349]. This model would allow the possibility of TNF-α binding to p55 in the absence of p75, but suggest that this would occur less efficiently. In qualitative terms, this hypothesis accounts well for the potency-enhancing effects of p75 binding which we have demonstrated, although it is interesting that a 500 fold reduction in binding to p75 (R32W) does not reduce potency by much, whereas a 5000 fold reduction (E146K, R32W/S86T) has a far greater effect.

The primacy of the p55 TNFR is demonstrated in gene deletion mice which lack this receptor[350]. These mice show a total lack of effect of TNF-α on lymphocytes, and are resistant to systemic administration of LPS, the toxicity of which is TNF-α-mediated. These mice show normal expression of p75 and only slightly reduced binding of TNF-α to cells. This confirms that p75 alone cannot mediate inflammatory functions, but does not rule out a role for p75 in other functions. Indeed, p75 was found to specifically and solely mediate the TNF-α induced up-regulation of TGF-α mRNA in a malignant epithelial cell

line, whereas p55 mediated up-regulation of epidermal growth factor receptor mRNA in the same cells[351].

In parallel studies, it was shown in our laboratory that the roles of p55 and p75 in the stimulation of neutrophil inflammatory functions by TNF-α were similar to their roles on EC[347]. However, the cytotoxicity of TNF-α for HEp-2 malignant epithelial cells, and cytostasis for U937 myeloid leukemia cells was fully mediated by p55 alone, with no potency enhancing function of p75. This appears to represent a divergence of the anti-tumor and pro-inflammatory effects of TNF-α, *ie*. although both are mediated ultimately by p55, the pro-inflammatory effects are dependent on p75 at low TNF-α concentrations. A consequence of this is that p55 specific TNF-α mutants could be expected to have anti-tumour effects equivalent to WT TNF-α, while on a same-concentration basis, they should have reduced pro-inflammatory toxicity. The potential of this for therapy is illustrated in the mouse model. Human TNF-α interacts with murine p55 but not p75, unlike murine TNF-α which interacts with both. Human TNF-α retains anti-tumour effectiveness in mice, but is 50× less toxic than murine TNF-α, enabling much higher doses to be given, with higher therapeutic efficacy. The TNFR-55 specific mutants described here may offer an equivalent effect in the human, by similarly widening the therapeutic window between desirable anti-tumour effects and undesirable pro-inflammatory toxicity.

SUMMARY

TNF-α activates the EC inflammatory functions of E-selectin expression, IL-8 production, and induction of neutrophil transmigration. There are known to be two separate high affinity receptors for TNF-α, p55 and p75. The role of each of these receptors in EC activation by TNF-α is not known. I have used mutant TNF-α molecules, which bind with relative specificity to either p55 or p75 to investigate their roles.

The mutant R32W which has moderately reduced binding to p75 was only slightly less potent in EC activation than WT TNF-α, but E146K and R32W/S86T which have markedly reduced affinity for p75 and are therefore p55 specific were 16-160 fold less potent than WT. At high concentrations, these mutants were able to fully activate EC, suggesting that the differences were quantitative and not qualitative. D143F, specific for p75, did not activate EC functions even at high concentrations. Antibodies specific for p55 were also able to activate EC. All parameters of activation (E-selectin expression, IL-8 production and neutrophil transmigration) were affected similarly by each of the agonists.

These results imply that EC pro-inflammatory activation by TNF-α is mediated principally by the p55 receptor. The p75 receptor does not signal but enhances the activity of TNF-α on p55, which is evident as increased potency. The mechanism of this may be a capture and "passing on" of TNF-α by the more rapidly binding p75 to the slow binding p55. It has been demonstrated by others in this laboratory that the anti-tumour effects of TNF-α are mediated solely by p55 with no involvement of p75. Therefore, TNF-α mutant molecules may retain anti-tumour effect, whilst by reduced binding to p75, show attenuated pro-inflammatory effects. This suggests potential therapeutic utility of these mutants, with equivalent anti-tumour effects to TNF-α but reduced toxicity.

CHAPTER 8

TNF-α ACTIVATED ENDOTHELIAL CELLS EXPRESS RECEPTORS FOR INTERLEUKIN-3 WHICH MEDIATE IL-8 PRODUCTION AND NEUTROPHIL TRANSMIGRATION

INTRODUCTION

The number of factors which activate endothelial cells to stimulate IL-8 production and neutrophil transmigration is limited. The prototypic cytokine activators are TNF-α and IL-1, both of which have been investigated in the preceding chapters. LPS also activates EC to produce a similar range of effects[277,304]. Other cytokines, such as IL-4 and IFN-γ, produce a different range of effects on EC, inducing different adhesion molecules (*ie.* VCAM rather than E-selectin by IL-4[352]) and different chemotactic factors[353], which may lead to migration of a different subset of leukocytes. These variations are of great current interest and are the subject of ongoing research in our own and other laboratories, but will not be discussed further here.

Interleukin-3 was not previously known to act upon EC. This factor was isolated on the basis of its effect of stimulating colony formation in a range of immature haematopoietic precursor cells (previous name multi-colony stimulating factor)[319]. However, no IL-3 has been detected in bone marrow *in vivo*, raising questions as to its role. IL-3 has also been found to stimulate the functions of mature monocytes, eosinophils and basophils, suggesting a role for this factor in inflammation. IL-3 is produced by T cells and activated mast cells[354], and this together with its targeting of eosinophils suggests that it may be involved in allergic inflammation.

The receptor for IL-3 (IL-3R) is a heterodimer, consisting of an α-chain unique to this cytokine, and a β chain which is common to the receptors for IL-3, GM-CSF and IL-5. Whilst the β-chain has been found on a variety of cells, particularly in the company of the GM-CSFR α-chain, the IL-3 receptor was previously thought to be restricted to cells of myeloid origin, and EC had previously been shown not to respond to this factor[248]. It was discovered by others in our laboratory that mRNA for the α and β chains of the IL-3 receptor were present in HUVEC, and while small amounts only could be detected in unstimulated EC, activation of the cells with TNF-α (as well as IL-1, LPS[249] and IFN-γ (Korpelainen *et al.*, ms. in preparation)) resulted in significant increases. The common β-chain (β_c) was also present, but α-chains for the receptors for GM-CSF and IL-5 could not

be detected. This is of particular interest since GM-CSF has been reported to have effects on EC, but these findings have been controversial, and they are now further called into question.

Expression of the receptor mRNA was accompanied by specific binding of IL-3; scatchard analysis of binding data suggested the presence of ~1500 high affinity receptors per cell[(249)]. No binding of GM-CSF was detected. I sought to further verify the expression of this receptor on EC, by flow cytometry using MAb against the IL-3Rα, and also used these MAb for immunoflourescence to study the distribution of the receptor on the cell surface. A functional role for the receptor was demonstrated on TNF-α activated EC by the ability of IL-3 to enhance IL-8 production, and the transmigration of neutrophils. Others in our laboratory demonstrated that IL-3 was also able to enhance the expression of E-selectin on TNF-α activated EC.

This chapter describes work undertaken by myself as part of a collaborative project with others in the Division of Human Immunology, IMVS. E. Korpelainen initially demonstrated receptor mRNA using RNAse protection assays, M. Dottore carried out binding studies, S. Qiyu developed monoclonal antibodies to IL-3Rα, and Dr. A. Lopez supervised the project.

RESULTS

8.1 Demonstration of α chain for the IL-3 receptor on endothelial cells by flow cytometry

Three MAb were developed against the IL-3Rα- 9F5, 6H6 and 7G3. Cultured HUVEC were preincubated either in medium alone or with TNF-α 100U/ml for 24h, a concentration and time which binding studies had demonstrated to be optimal for surface up-regulation of IL-3Rα, before staining with the MAb 7G3. Representative histograms are shown in Fig. 8.1. The curve is broad for 7G3 expression on both unstimulated and TNF-α activated HUVEC, indicating variable intensity of staining, and overlaps with the negative control antibody curve, indicating that some cells do not express receptor. Nevertheless, the MFI of the cells is higher for 7G3 than negative control antibody, and is increased by TNF-α activation of the HUVEC.

MFI values for up-regulation of anti-IL-3Rα staining were low, so to confirm that the staining was reproducible, it was repeated several times on different HUVEC lines and the results pooled and subjected to statistical analysis (Fig. 8.2). The average MFI of cells stained with a negative control antibody or no primary antibody was $0{\cdot}31 \pm 0{\cdot}01$ on unstimulated HUVEC, and $0{\cdot}42 \pm 0{\cdot}01$ after 24h TNF-α 100U/ml. The value for unstimulated HUVEC stained with 7G3 was $0{\cdot}53 \pm 0{\cdot}03$ ($p<0{\cdot}001$ for difference from negative antibody) and for TNF-α activated HUVEC it was $1{\cdot}1 \pm 0{\cdot}04$ ($p<0{\cdot}001$ for comparison with negative control antibody, and comparison with 7G3 on unstimulated HUVEC). Although the value for MFI of unstimulated HUVEC was very low, this was consistent with the finding of small amounts of mRNA for IL-3Rα in these cells. The average MFI of HUVEC stained with 8G6, an antibody against the α-chain of the GM-CSF receptor, was $0{\cdot}34 \pm 0{\cdot}02$ and $0{\cdot}45 \pm 0{\cdot}04$ for unstimulated and TNF-α activated cells respectively (not significantly different to negative control antibody in either case).

8.2 Visualisation of IL-3 receptor α chains on EC by laser scanning immunofluorescence microscopy

HUVEC were cultured on glass slides, and either untreated or activated by incubation with TNF-α 100U/ml for 24h. The cells were stained for immunofluorescence with antibodies to GM-CSFRα as a control, or a cocktail of all three antibodies to IL-3Rα. Standard immunofluorescence microscopy revealed very dim fluorescence even on the activated cells (as expected given the low fluorescence values on flow cytometry), and while there was a discernible difference between positive and negative samples, this was not easily demonstrated on photographs. I therefore used laser scanning microscopy (in non-confocal mode to capture maximum available signal) to image the cells, and enhanced the computer captured images by increasing contrast and brightness. This produced clear differences between negative (GM-CSFRα) and positive antibodies, and a clearly brighter signal from activated *vs.* unactivated cells (Fig. 8.3), with staining uniformly across the cell surface. It can be seen that, as predicted by FC analysis, the brightness of staining is variable for different cells. It is important to note that the computer software allows a precise quantitative control of image manipulation, and exactly the same degree of enhancement was applied to each of the frames.

8.3 Increase in IL-8 mRNA in IL-3 stimulated TNF-α activated HUVEC

EC responses to IL-3 were sought in order to confirm that the receptor detected by several means, as described above, was indeed capable of transducing a signal, and to determine its functional significance. RNA was prepared from passaged HUVEC (75cm^2 flasks) which had been either unstimulated, activated by TNF-α 100U/ml for 24h, incubated with IL-3 30ng/ml for 6h, or subjected to both treatments. The RNA was run on a formaldehyde/agarose gel, transferred to a nitrocellulose filter (northern blotting) and probed with a ^{32}P-labelled IL-8 cDNA, then exposed on a phosphor storage screen. The same filter was then stripped of the labelled probe and re-probed with cDNA for glyceraldehyde phosphate dehydrogenase (GAP-DH), to control for quantity of RNA loaded.

Fig. 8.4 shows a filter probed for IL-8 alone (this filter was not probed with GAP-DH). Ultraviolet photography of the filter showed approximately equal amounts of RNA (18s & 28s ribosomal) in each lane. Note the absence of hybridisation signal for IL-8 in unstimulated and IL-3 stimulated EC. Fig. 8.5 shows a separate experiment, in which the filter was probed for both IL-8 and GAP-DH. No RNA from HUVEC stimulated with IL-3 alone was present in this experiment. No mRNA for IL-8 was detected in the unstimulated HUVEC, while IL-8 mRNA was present in TNF-α activated HUVEC, and a higher intensity band was seen with TNF-α and IL-3 together. The digital images were analysed by integration of the intensity of the area of each specific band, and a ratio of intensity of IL-8/GAP-DH was derived, which gave an indication of the relative quantity of IL-8 mRNA present in each lane. This revealed that there was a 36% increase in IL-8 mRNA in TNF-α/IL-3 stimulated HUVEC compared to TNF-α alone.

8.4 Enhancement of IL-8 production by addition of IL-3 to TNF-α activated EC

HUVEC treated with IL-3 alone (30 ng/ml for 6 or 24h) did not produce IL-8, as measured by ELISA in cell supernatants (baseline IL-8 production $0{\cdot}04 \pm 0{\cdot}01$ ng/ml/hr, IL-3 stimulated EC, $0{\cdot}06 \pm 0{\cdot}02$, NS). Production of IL-8 was increased by activation of the EC by TNF-α (100U/ml, 24h), a treatment which also results in up-regulation of receptors for IL-3. Addition of IL-3 (30ng/ml, 6h) to TNF-α activated EC significantly augmented IL-8 output, from $2{\cdot}62 \pm 0{\cdot}6$ ng/ml/hr to $5{\cdot}02 \pm 0{\cdot}98$ ($p<0{\cdot}01$) (Fig. 8.6A).

8.5 Increase in neutrophil transmigration through TNF-α activated endothelial monolayers by addition of IL-3

As demonstrated in chapters 4 and 5, IL-8 is a major mediator of neutrophil transmigration through cytokine-activated endothelium. It should therefore be expected that the increase in IL-8 production described above should result in increased transmigration. However, a feature of the *in vitro* system used to assay transmigration may limit this increase. As determined in chapter 5, when EC are activated by TNF-α, IL-8 is produced both above and below the endothelium, so that transmigration is stimulated by IL-8 by chemokinesis.

This mechanism, as shown in chapter 3 (Fig. 3.6), results in only low levels of transmigration, and furthermore, at higher levels of IL-8, increasing the concentration led to decreases in migration. In fact, using a standard transmigration assay as described in chapter 2, little or no increase in transmigration through TNF-α activated endothelium was seen with the addition of IL-3.

Increases in IL-8 output are more likely to be paralleled by increases in transmigration if chemotactic rather than chemokinetic migration mechanisms are used (Fig. 3.5). For these experiments, medium was exchanged in both compartments of the transwell 2 hours prior to the addition of neutrophils, while the medium in the upper compartment only was exchanged when the neutrophils were added, thereby allowing EC to secrete IL-8 into the lower compartment for longer than the upper, creating an IL-8 gradient across the monolayer. In these circumstances, clear increases in neutrophil transmigration were observed when IL-3 (30ng/ml, 6h) was added to TNF-α activated (100U/ml, 24h) EC monolayers. In fact, this manipulation of the assay system may bring it closer to a representation of the situation *in vivo*, since as pointed out in the discussion section of chapter 5, IL-8 secreted into the bloodstream (the upper compartment) from the apical endothelial surface is continually washed away and diluted by plasma flow and neutralising mechanisms of blood, whilst IL-8 secreted into the subendothelial region is likely to be removed much more slowly, thus creating a gradient and allowing chemotaxis to occur.

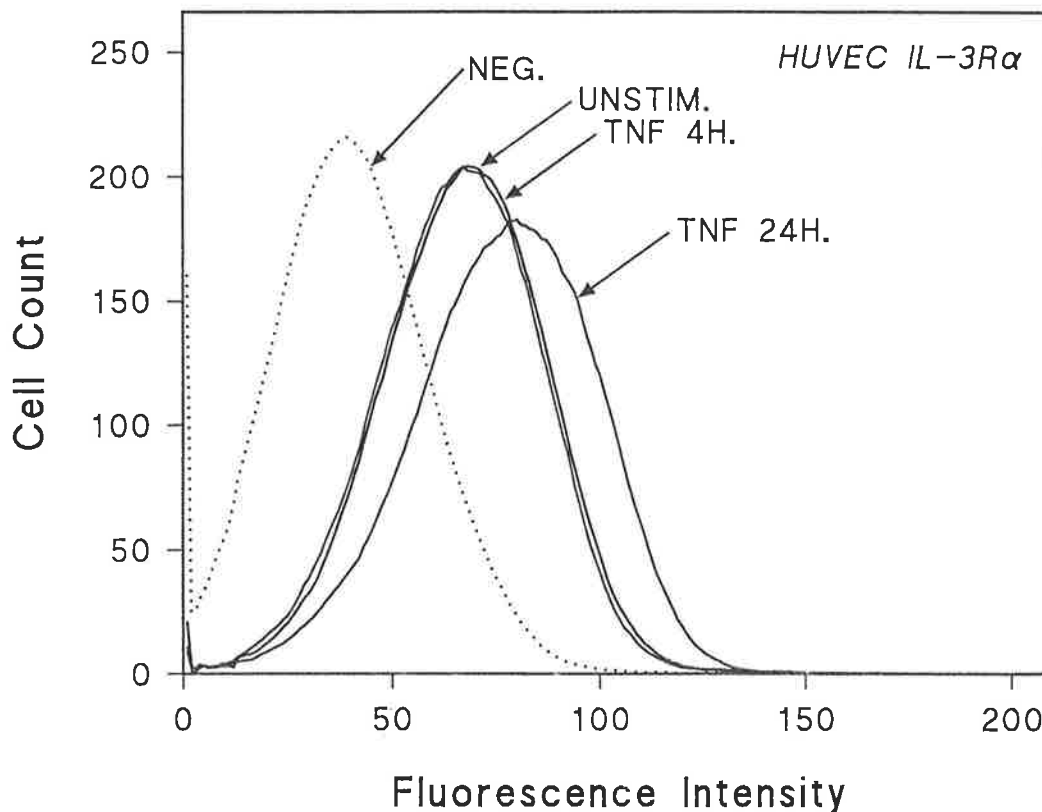


Fig. 8.1 Expression of IL-3Rα by HUVEC- flow cytometry histograms

Flow cytometry histogram profiles of HUVEC stained with negative control antibodies (1F11, IgG1, against keyhole limpet haemocyanin, dotted line) or antibodies against IL-3Rα (7G3, IgG2a). HUVEC were either unstimulated, activated with TNF-α 100U/ml for 4h, or activated with TNF-α 100U/ml for 24h. Single experiment, representative of 4 experiments, 10,000 cells counted for each histogram. Raw histogram data was smoothed for graphing, as described in materials and methods.

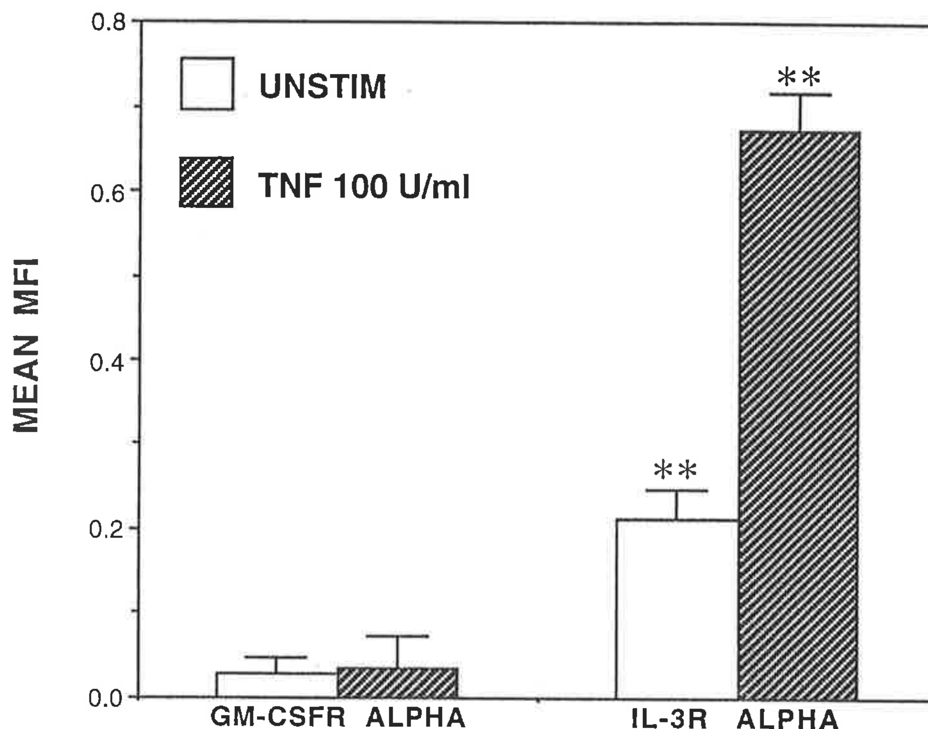


Fig. 8.2 Demonstration of IL-3Rα chain on EC by flow cytometry- pooled data

Averaged MFI values of multiple flow cytometry experiments on HUVEC, unstimulated or activated by TNF-α 100U/ml for 24h, stained with antibodies against the IL-3Rα, or 8G6, an antibody against the GM-CSFRα. The average MFI of HUVEC stained with negative control antibodies or no primary antibody is subtracted from each column. Columns represent mean ± SEM of four experiments (GM-CSFRα) or six experiments (IL-3Rα). The MFI for the GM-CSFR α-chain antibody stained cells were not significantly different to negative control antibody; ** $p<0{\cdot}001$, significance of difference from negative control antibody; also, the values for IL-3Rα staining were significantly different in unstimulated *vs*. TNF-α stimulated HUVEC, $p<0{\cdot}001$ (by unpaired Student *t*-test).

Fig. 8.3 Visualisation of IL-3Rα chains on HUVEC by laser scanning immunofluorescence microscopy

HUVEC were cultured on glass slides, and either unstimulated (*a* and *c*) or stimulated with TNF-α 100U/ml for 24h (*b*, *d*), then incubated with MAb to the GM-CSFRα chain, 8G6 (*a*, *b*) or MAb to the IL-3Rα chain (7G3 and 9F5)(*c*, *d*) at 4°C before fixation/permeabilisation, to selectively stain the cell surface. Monolayers were then fixed in methanol/acetone and incubated with FITC labelled rabbit anti-mouse. The EC were then visualised by confocal laser scanning microscopy (MRC 600), and images recorded. Contrast and brightness were enhanced equally for each panel.

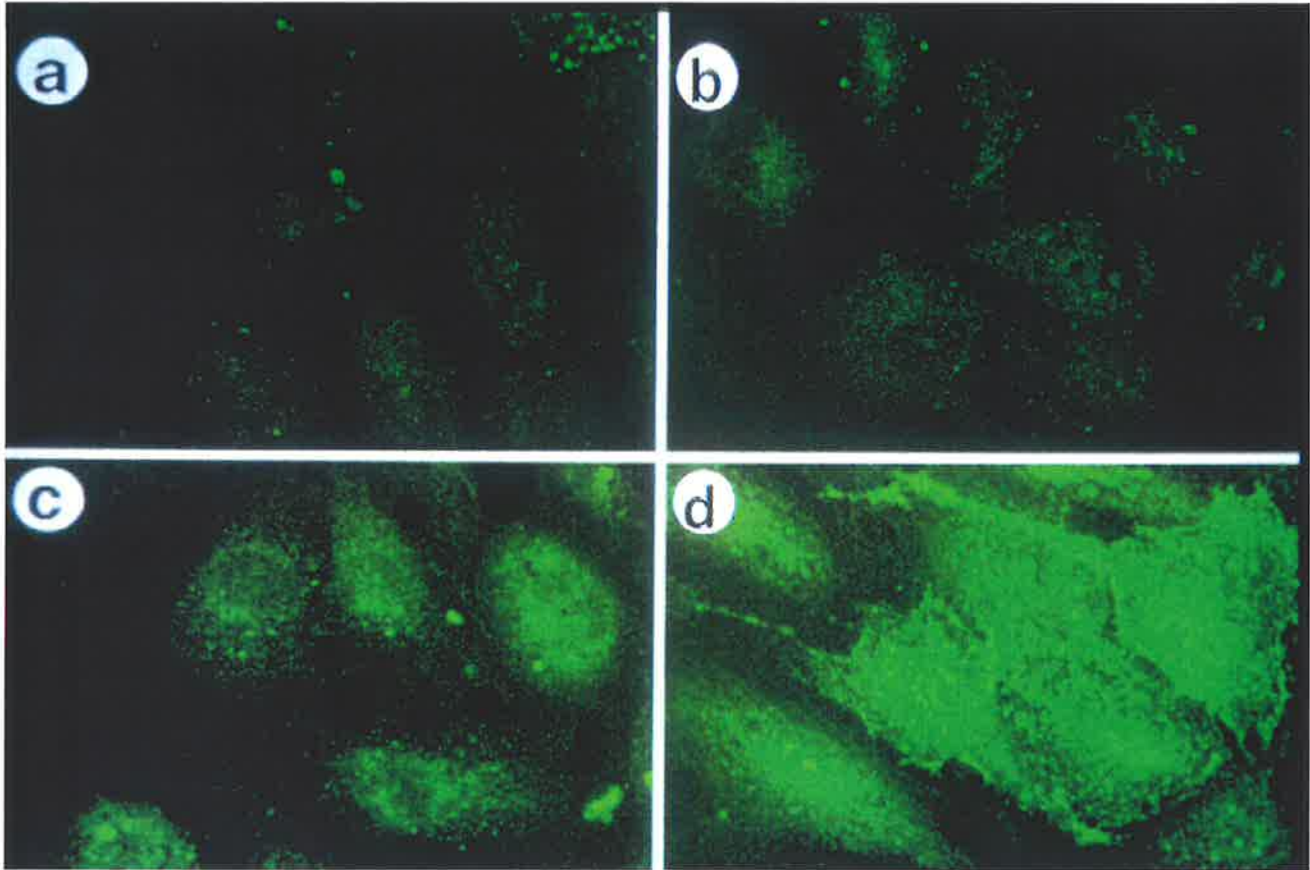
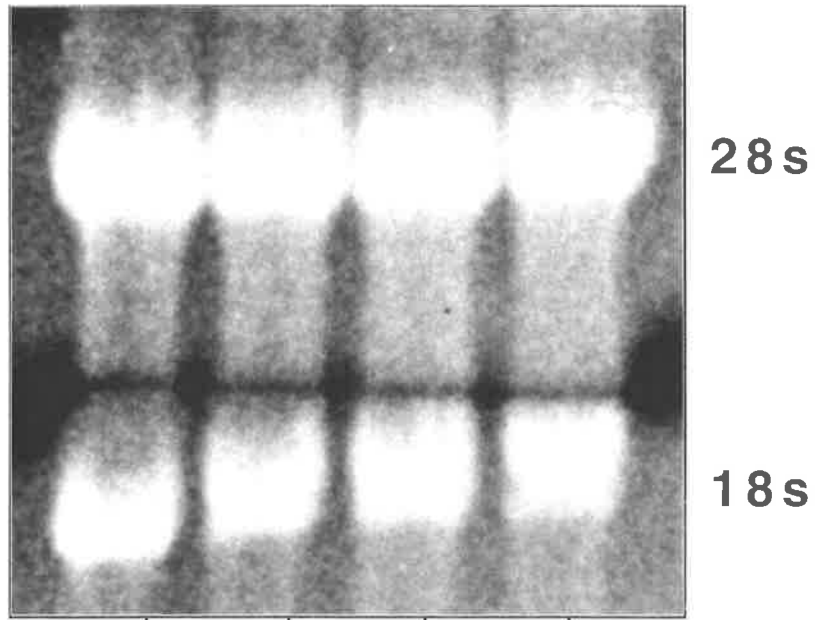

a
b
c
d

Fig. 8.4 Northern blot of RNA from HUVEC treated with TNF-α or IL-3

HUVEC cultured in 4 × 75cm^2 flasks were stimulated with medium alone, IL-3 30ng/ml for 6h, TNF-α 100U/ml for 24h, or both. RNA was extracted, separated by electrophoresis, and stained with ethidium bromide for UV photography (*top panel*). RNA was then transferred to nitrocellulose and probed with a ^{32}P-labelled IL-8 cDNA; the hybridisation signal is shown by a print of the phosphorimager scan (*lower panel*).


28s
18s

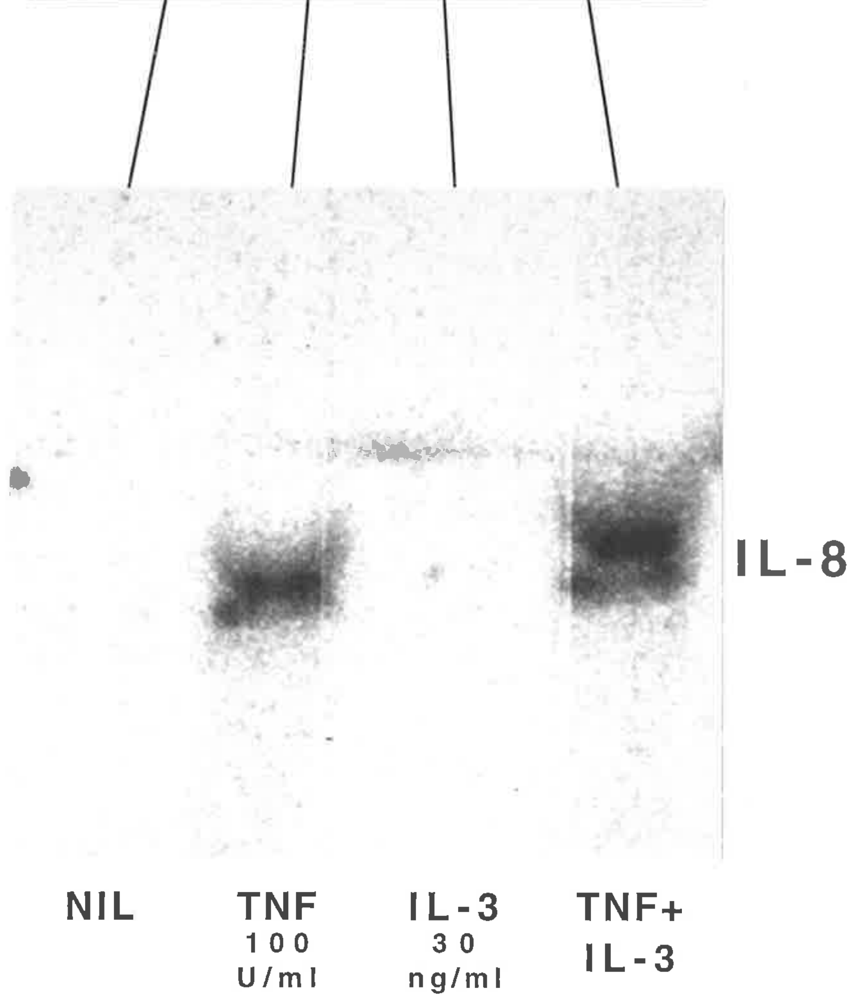
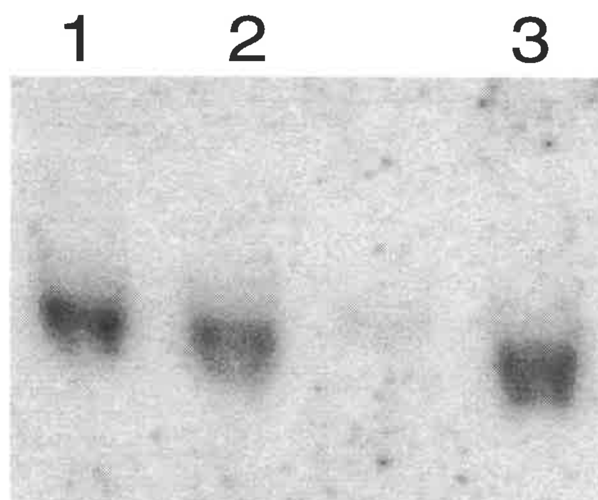
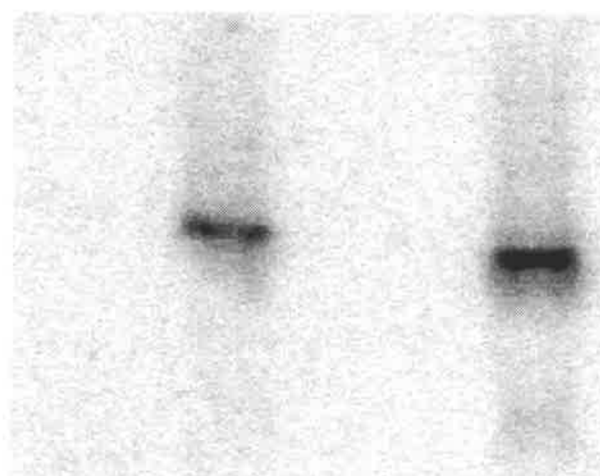

IL-8
NIL
TNF
100
U/ml
IL-3
30
ng/ml
TNF+
IL-3

Fig. 8.5 Northern blot of HUVEC RNA probed with GAP-DH and IL-8

HUVEC cultured in 3 × 75cm^2 flasks were stimulated with medium alone, TNF-α 100U/ml for 24h, or TNF-α + IL-3 30ng/ml for 6h. RNA was extracted, separated by electrophoresis, transferred to nitrocellulose and probed with a ^{32}P-labelled IL-8 cDNA; the filter was then stripped and probed with cDNA for GAP-DH. Hybridisation signal is shown by prints of the phosphorimager scans. The intensity of the bands was quantified as described in chapter 2, and ratios of the intensity of IL-8 to GAP-DH are shown in the column graph.

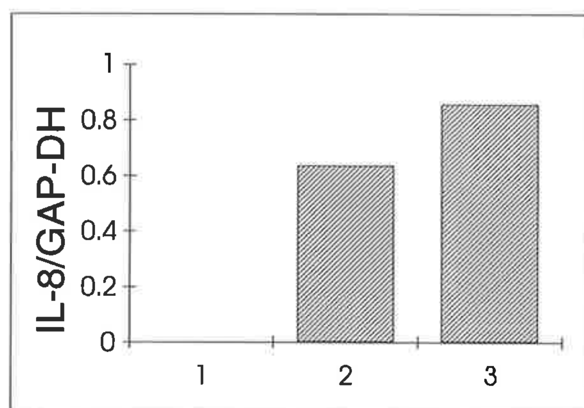


GAP-DH



IL-8

NIL TNF 100 U/ml TNF+ IL-3 30ng/ml



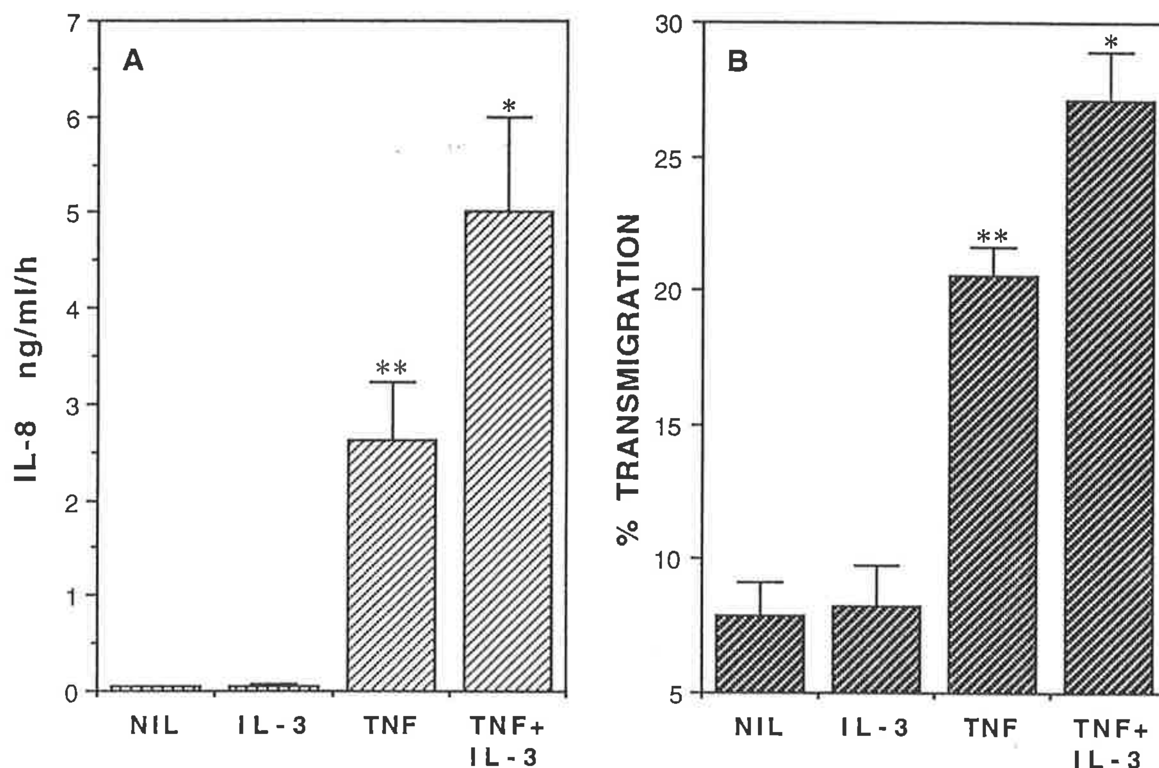


Fig. 8.6 IL-8 production and induction of neutrophil transmigration by HUVEC activated by TNF-α and IL-3

HUVEC monolayers were cultured in 24 well cluster dishes (A) or transwells (B) and either unstimulated, treated with TNF-α 100U/ml for 24h, IL-3 30ng/ml for 6h, or both. A- medium was changed and collected after 1h, IL-8 was quantitated by ELISA as described in chapter 2. The columns represent the mean ± SEM of four different experiments; ** p=0·006 compared to unstimulated cells, * p<0·01 compared to stimulation with TNF-α alone, by paired Student's t-test. B- Monolayers were washed, and the percentage transmigration of 10^6 neutrophils was quantitated as described in chapter 2. Columns represent the mean ± SEM of 8 experimental values, *ie*. 4 experiments in duplicate. ** p<0·001 compared to unstimulated HUVEC, * p<0·005 compared to stimulation with TNF-α alone, by independent Student's t-test.

DISCUSSION

The finding of receptors for IL-3 on endothelial cells in our laboratory was the first indication that this cytokine might act on cells outside of the myeloid lineage, and we were naturally interested to determine the nature of its effects on EC and the functional significance that this might imply. A pro-inflammatory role had already been suggested because of the effects of IL-3 on such mature myeloid cells as monocytes, eosinophils and basophil/mast cells, with the latter two activities furthermore implying a role in inflammation of allergic origin. IL-3 is made by T cells (T_H1 and T_H2), which initiate and coordinate inflammation of immunological origin, and by mast cells activated by cross linking of surface IgE, which initiate allergic inflammation, such as occurs in asthma.

The pro-inflammatory role of IL-3 can now be extended by the demonstration of its effects on TNF-α activated EC. IL-3 does not appear to act on its own on EC (at least not for the functions tested) but augments the activation of TNF-α treated cells, on which IL-3 receptors have been up-regulated. Mast cells produce TNF-α as well as IL-3, and so could up-regulate the IL-3 receptor as well as providing its ligand.

Increases in mRNA levels for IL-8 were found in TNF-α treated EC after stimulation with IL-3 (although the level of increase was modest), which may be a result either of higher levels of transcription, or reduced mRNA breakdown. This question has not been addressed, but would require nuclear run on studies (to determine rates of transcription) or mRNA stability studies. The production of immunoreactive IL-8 by EC activated by the cytokines TNF-α and IL-1 was characterised by ELISA in chapter 5. TNF-α stimulated IL-8 output by EC which increased up to the maximum concentration used, 100U/ml (Fig. 5.1). Time courses indicated that production was high at 4 hours after stimulation, and was maintained after 24 hours of stimulation (Fig. 5.2). Preliminary experiments indicated that combining IL-3 with TNF-α for 4 or 6 hours only did not result in a significant increase in IL-8 output compared to TNF-α alone. However when IL-3 was added to EC activated by TNF-α 18 hours earlier, *ie.* after up-regulation of IL-3 receptors, a significant augmentation occurred.

A theme developed in earlier chapters is the role of IL-8 in neutrophil transmigration through activated EC. Given this, we expected transmigration to be increased in IL-3 + TNF-α activated EC. However, IL-8 production is already quite high in EC activated by 100U/ml of TNF-α (Fig. 5.1); levels varied with different EC lines in different experiments, but were frequently around 1nM. Referring to Fig. 3.6, it can be seen that this is at the peak of chemokinetic IL-8 stimulated transmigration, which is the form that occurs in the usual static transmigration assay (Ch. 5), so that increases in IL-8 production caused by IL-3 may not result in increases in transmigration, and indeed this is what was initially observed. When the assay was modified to allow a gradient of IL-8 to be produced by the EC (which is probably closer to what occurs *in vivo*), then a significant increase in neutrophil transmigration was observed when EC were activated by both TNF-α and IL-3, compared to TNF-α alone. Although the increases in all three of these EC functions were modest, they were statistically significant, and correlate reasonably well quantitatively; a 36% increase in mRNA for IL-8 was accompanied by a 90% increase in IL-8 production, and a 50% increase in transmigration, *ie*. similar orders of magnitude.

The question of the mechanism by which IL-3 acts on cytokine activated endothelium is worthy of discussion. Interestingly, we observed no effect of IL-3 on resting HUVEC despite the presence of receptors demonstrated by flow cytometry, and confirmed by the presence of small amounts of receptor mRNA(249). Work in progress by others in our laboratory indicates that using different functional readouts, resting EC can indeed be stimulated by IL-3 (Khew-Goodall *et al.*, ms in preparation). This may imply that a threshold number of receptors need to be present to mediate IL-8 production. However this also does not appear to be the case, since EC activated by IFN-γ, which also show up-regulated IL-3R expression but do not produce IL-8, cannot be stimulated by IL-3 to produce IL-8. This leaves the likely mechanism that signalling via the IL-3R acts as a cofactor for TNF-α signalling via post-receptor mechanisms. Receptor up-regulation is important however, since IL-3 did not consistently increase IL-8 production in EC activated by TNF-α for 4-6h, when IL-8 production is maximal (Fig. 5.2) but receptors are not yet up-regulated.

The effects examined in this chapter were production of IL-8 by EC, and induction of neutrophil transendothelial migration, both likely to be significant for acute neutrophilic inflammation. This may appear to contrast with previous suggestions of roles for IL-3 in immunologic or allergic inflammation, and indeed IL-3 is not thought likely to be present at simple acute inflammatory responses. However, it should be noted that allergic inflammation, *eg.* the late phase inflammatory response of allergic asthma, includes significant numbers of neutrophils. We are also investigating the possibility that IL-3 may stimulate additional EC functions, resulting in the capture of leukocytes other than neutrophils, which might have more relevance in chronic or allergic inflammation.

SUMMARY

Endothelial cells act as regulators of inflammation, by controlling the traffic of leukocytes into tissues. This is accomplished by an increase in EC adhesiveness, and by the production of chemotactic cytokines, such as IL-8, which as shown in previous chapters, leads to the transmigration of neutrophils. EC assume this activated state when stimulated by cytokines, of which the prototypes are TNF-α and IL-1.

The finding of receptors for IL-3 on EC suggested the possibility that this cytokine may also influence EC function. The first indication that EC might express IL-3R arose when IL-3Rα mRNA was detected in these cells. Binding studies confirmed specific binding of IL-3 to activated EC. As reported in this chapter, receptors were detected by flow cytometry and immunofluorescence. A clear increase in receptor expression was seen by all modalities when EC were activated by TNF-α, although a low level of expression was also seen on unactivated cells.

A functional role for these receptors is shown by the ability of IL-3 to increase levels of mRNA for IL-8 in EC activated by TNF-α, and also to increase the production of immunoreactive IL-8 above that seen after stimulation with TNF-α alone. This augmentation of IL-8 output was shown to enhance the transmigration of neutrophils through endothelial monolayers when present as a gradient. These findings indicate the potential of a wider role for IL-3 in the regulation of the inflammatory response.

CHAPTER 9:

TGF-β AND IL-4 INHIBIT PRODUCTION OF IL-8 BY CYTOKINE ACTIVATED ENDOTHELIAL CELLS AND NEUTROPHIL TRANSENDOTHELIAL MIGRATION

INTRODUCTION

TGF-β is a pleiotropic cytokine, which has roles in the regulation of many biological processes from embryogenesis to wound healing. Of great importance are its anti-inflammatory effects. We have previously shown that TGF-β reduces the adhesiveness of EC for neutrophils[(355)] and lymphocytes[(356)], by inhibiting the induction of E-selectin on the EC surface[(357)]. Expression of other EC adhesion molecules, ICAM-1 and VCAM was unaffected, indicating that TGF-β does not depress EC activation in general. IL-4 is a central regulator of the immune response which has anti-inflammatory effects, particularly well investigated on monocytes[(358,359,360,361)], and also inhibited E-selectin expression by activated EC[(357)], although this factor enhanced expression of VCAM[(352,362,363)].

In the inflammatory reaction, leukocytes first roll and then adhere to the vessel wall, functions mediated by the selectins[(313)]. Subsequently, to enter the tissues at the inflammatory site, leukocytes must migrate across the endothelium of the vessel wall. This latter function, known as transmigration or diapedesis, is mediated by a separate set of adhesion molecules and chemotactic factors. IL-8, a small peptide of the chemokine family, is produced by EC activated by inflammatory cytokines(Chapter 5, and(304)) and has been demonstrated to have a major role in the transendothelial migration of neutrophils[(277)], and is also chemotactic for T lymphocytes[(176)].

I have therefore investigated the effect of TGF-β and IL-4 on the production of IL-8 by EC activated by pro-inflammatory cytokines. TGF-β reduced IL-8 mRNA levels and both TGF-β and IL-4 inhibited IL-8 production by EC stimulated by TNF-α or IL-1, in a parallel fashion to their inhibition of E-selectin expression. TGF-β was also found to inhibit neutrophil transmigration across EC monolayers *in vitro*, consistent with the role of IL-8 in this process. Therefore, taken together with previous work from this laboratory, this chapter shows that TGF-β is able to inhibit both arms (*ie*. adhesion and transmigration) of the EC contribution to neutrophil (and potentially lymphocyte) accumulation at inflammatory sites.

RESULTS

9.1 TGF-β reduces IL-8 mRNA levels in EC activated by TNF-α

RNA was prepared from primary HUVEC, pre-treated with medium or TGF-β 0·2ng/ml for 24 hours, then stimulated (or not) with TNF-α 1U/ml for 4h. RNA from several lines was pooled, electrophoresed and transferred to nitrocellulose. The filter was probed with a ^{32}P-labelled cDNA for IL-8, then washed and probed with a ^{32}P-labelled cDNA for glyceraldehyde phosphate dehydrogenase (GAP-DH). The filters were then exposed to a phosphor screen (ImageQuant) and scanned. Similar amounts of RNA were present in each lane, as seen by UV visualisation of 18s and 28s rRNA (data not shown), and by the intensity of the bands for GAP-DH RNA (Fig. 9.1). TNF-α stimulation is clearly shown to up-regulated the intensity of the band for IL-8, (Fig. 9.1). TGF-β pre-treatment of the HUVEC however reduces the intensity of the IL-8 bands in both resting and TNF-α activated HUVEC. The intensity of all bands was quantitated by area integration of the pixel intensity, and IL-8/GAP-DH intensity ratios calculated. The ratios were- nil 23·1, TGF-β 8·0, TNF-α 58·7, and TNF-α + TGF-β 27·7, indicating a 65% and 53% reduction of IL-8 mRNA in unstimulated and TNF-α activated HUVEC respectively.

9.2 Pre-treatment of HUVEC with TGF-β or IL-4 inhibits production of IL-8

Immunoreactive IL-8 was measured in supernatants of cultured HUVEC in ng/ml/h by ELISA. Increasing concentrations of TNF-α, added to the cultures 4h prior to the measurement, led to increasing production of IL-8. Addition of TGF-β 0·2ng/ml to EC 24h prior to the measurement inhibited the ability of TNF-α to induce IL-8 (Fig. 9.2). This inhibition was most effective at low concentrations of TNF-α; inhibition at 0·1U/ml was 76%, at 1U/ml 65%, at 10U/ml it was 53%, and at 100U/ml of TNF-α, inhibition was reduced to 45%. There was a small production of IL-8 in unstimulated HUVEC, seen in primary cultures but not in passaged lines, which was also inhibited by TGF-β (81%). The optimal concentration of TGF-β for inhibition of IL-8 production was tested by pre-treating HUVEC with varying concentrations of TGF-β for 24h, then stimulating with

TNF-α 1U/ml for 4h (Fig. 9.3). As was found to be the case for inhibition of induction of E-selectin[357], 0·2-2ng/ml of TGF-β gave maximal inhibition, with either lower or higher concentrations inhibiting less. This "bell-shaped" concentration/response curve is typical of the effects of TGF-β on many cell types. To determine whether TGF-β delayed the production of IL-8 by EC rather than inhibiting it, a time course experiment was carried out, using different times of TNF-α stimulation (1U/ml) on HUVEC treated with TGF-β for 24h (Fig. 9.4). This shows that IL-8 production was reduced at all time points examined. Interestingly, this also showed that IL-8 production by HUVEC not treated by TGF-β had diminished almost to baseline by 24h; comparison with Fig. 5.2 shows that higher concentrations of TNF-α not only stimulate higher levels of IL-8 production but prolong production for >48h (this was confirmed by parallel studies of 1 and 100U/ml stimulation in the same experiment, data not shown).

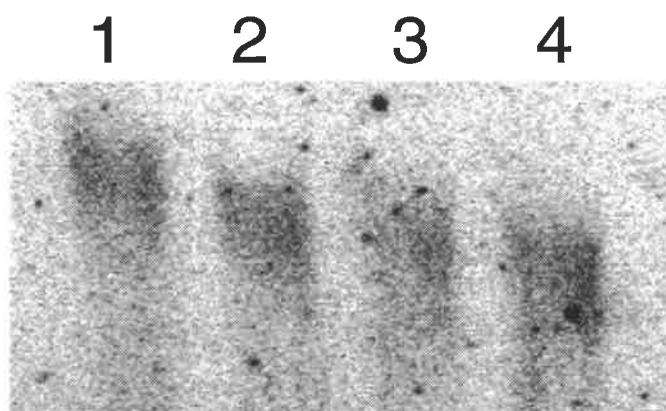
IL-4 preincubation of HUVEC also inhibited IL-8 production induced by TNF-α, and TGF-β together with IL-4 had additive inhibitory effects(Fig. 9.5). TGF-β also inhibited production of IL-8 by EC activated by IL-1 (data not shown).

9.3 Induction of neutrophil transmigration by TNF-α activated HUVEC is reduced by TGF-β pre-treatment of the HUVEC

HUVEC cultured on glass slides were pre-treated for 24h with TGF-β 0·2ng/ml, then treated for 4h with TNF-α 1 or 10U/ml. Monolayers were then washed and co-incubated with neutrophils for 20min, and adherent and transmigrated neutrophils were quantitated. TNF-α increased the number of both adherent and transmigrated neutrophils at this time point, although some lines did not increase significantly at 1U/ml, but did at 10U/ml. TGF-β inhibited the increase in transmigration by a mean of 83 ± 0·8% in 4 experiments (2 using 1U/ml of TNF-α, 2 using 10U/ml), $p<0{\cdot}01$ by Student's independent *t*-test. Inhibition of adhesion was variable; in the same experiments, it was inhibited by 50 ± 24%, (NS). Fig. 9.6 shows a representative experiment.

Fig. 9.1 TGF-β decreases IL-8 mRNA in resting and TNF-α stimulated HUVEC

Primary HUVEC lines were cultured in 6 well cluster dishes, and individual wells were treated with medium alone, TGF-β 0·2ng/ml for 24h, TNF-α 1U/ml for 4h, or both. RNA was then prepared from the cells as described in chapter 2, and RNA from 4 different EC lines was pooled to provide sufficient for analysis. The RNA was separated by electrophoresis, transferred to a nitrocellulose filter, and probed first with a ^{32}P-labelled IL-8 cDNA and after washing, then with a ^{32}P-labelled cDNA for GAP-DH. The filters were exposed to a phosphor storage screen and then scanned on a phosphor imager. The digitised images are shown. The ratio of intensity of the signal for IL-8 compared to the signal for GAP-DH is displayed in the column graph below.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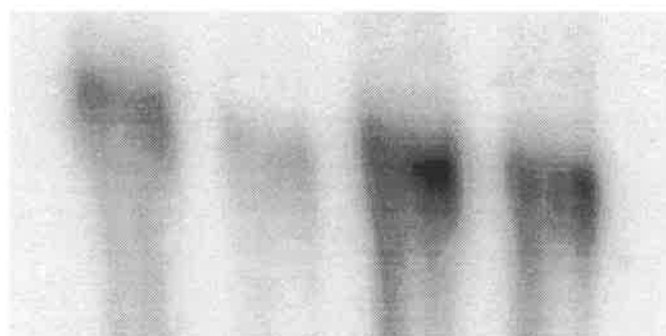
2
3
4
GAP-DH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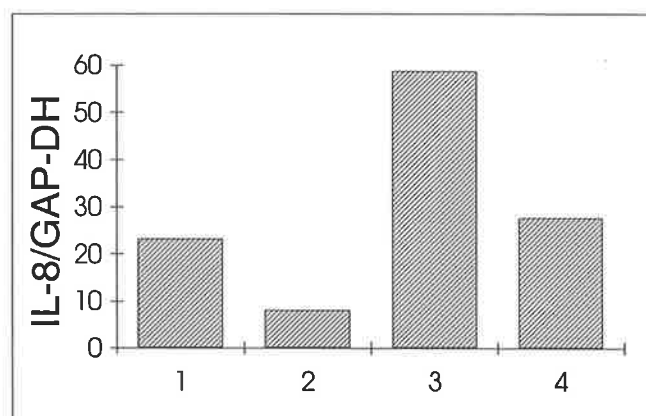
TGF
0.2
ng/ml
TNF
1U/ml
TNF+
TGF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50
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1
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4

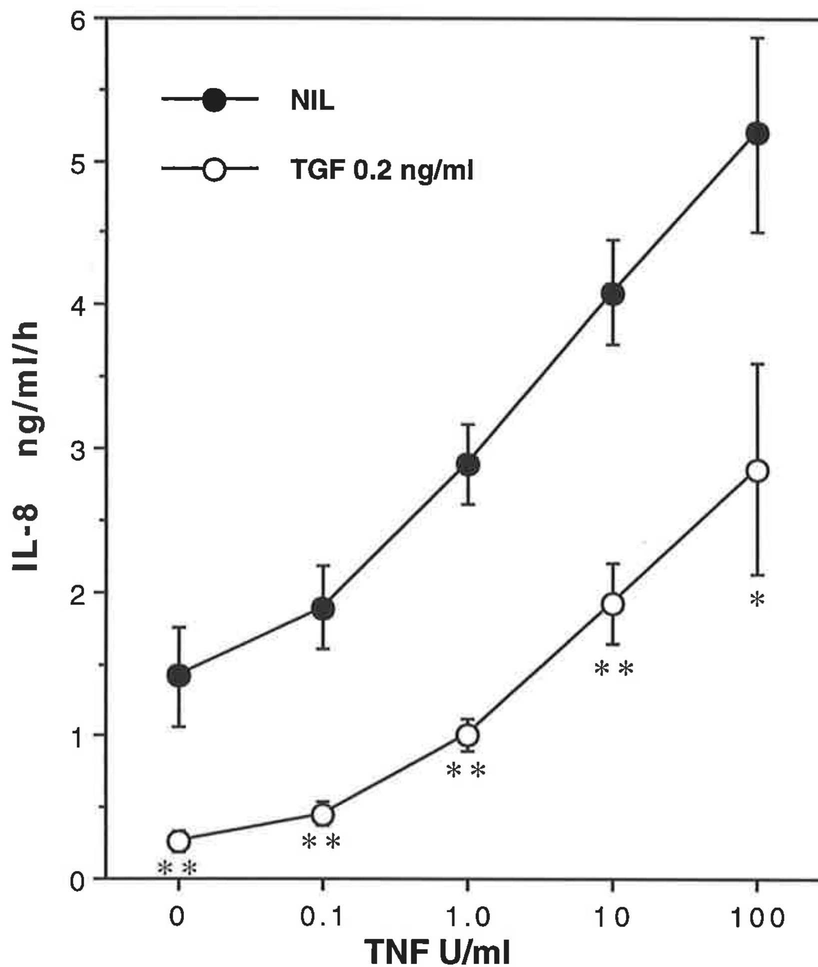


Fig. 9.2 TGF-β inhibition of IL-8 production by TNF-α activated HUVEC; TNF-α concentration/response curve.

HUVEC in 24 or 96 well culture dishes were pre-treated with medium alone or TGF-β 0·2ng/ml for 24h, then stimulated with different concentrations of TNF-α as indicated. After 2h, medium was exchanged, and after 1h was collected for measurement of IL-8 by ELISA. Each point represents the mean ± SEM of 8 experimental determinations, or 3 experiments, 2 in duplicate and one qintuplicate. * $p<0{\cdot}05$, * $p<0{\cdot}005$ for the difference between cells treated or not treated with TGF-β, by unpaired Student's *t*-test.

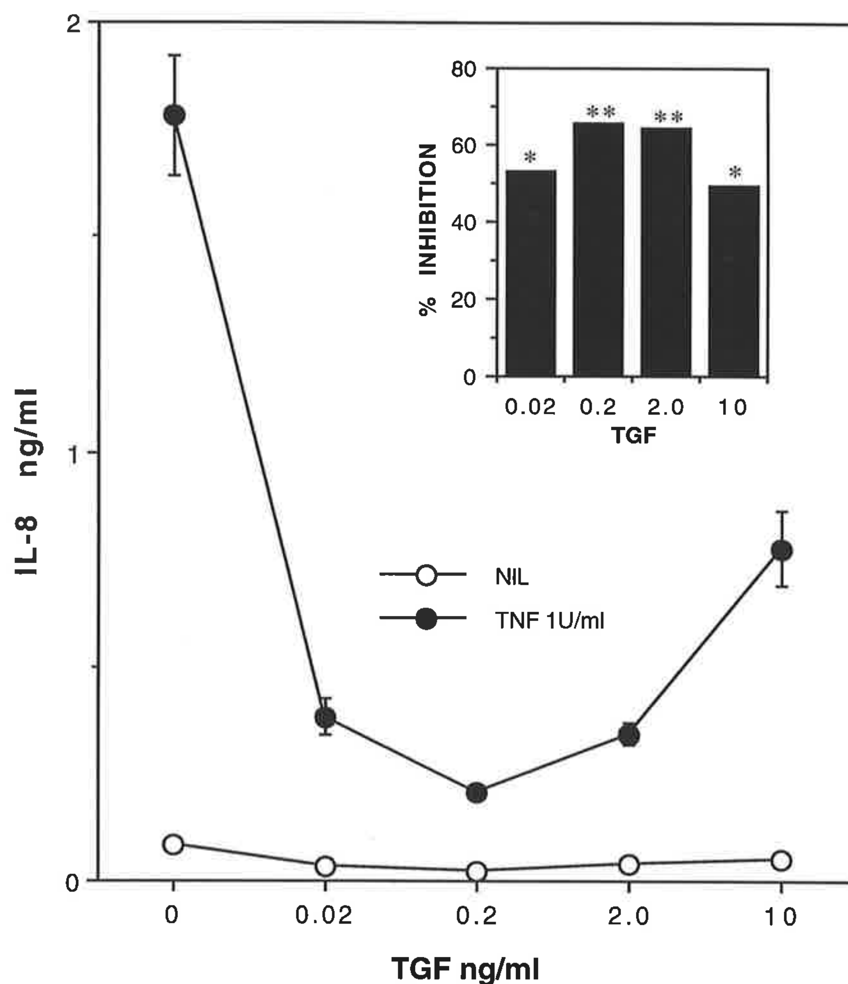


Fig. 9.3 TGF-β inhibition of IL-8 production by TNF-α activated HUVEC; TGF-β concentration/response curve.

HUVEC in 96 well culture dishes were pre-treated with medium alone or TGF-β at varying concentrations as indicated for 24h, then stimulated with TNF-α 1U/ml for 4h. Medium was changed, and then collected after 1h, and IL-8 quantitated by ELISA. Representative experiment, in quadruplicate, of 2 experiments. Each point is the mean ± SEM. The inset shows the percentage inhibition of IL-8 production calculated from pooled data of the 2 experiments. * $p<0{\cdot}05$, ** $p<0{\cdot}01$, compared to TNF-α alone, by Student's independent *t*-test.

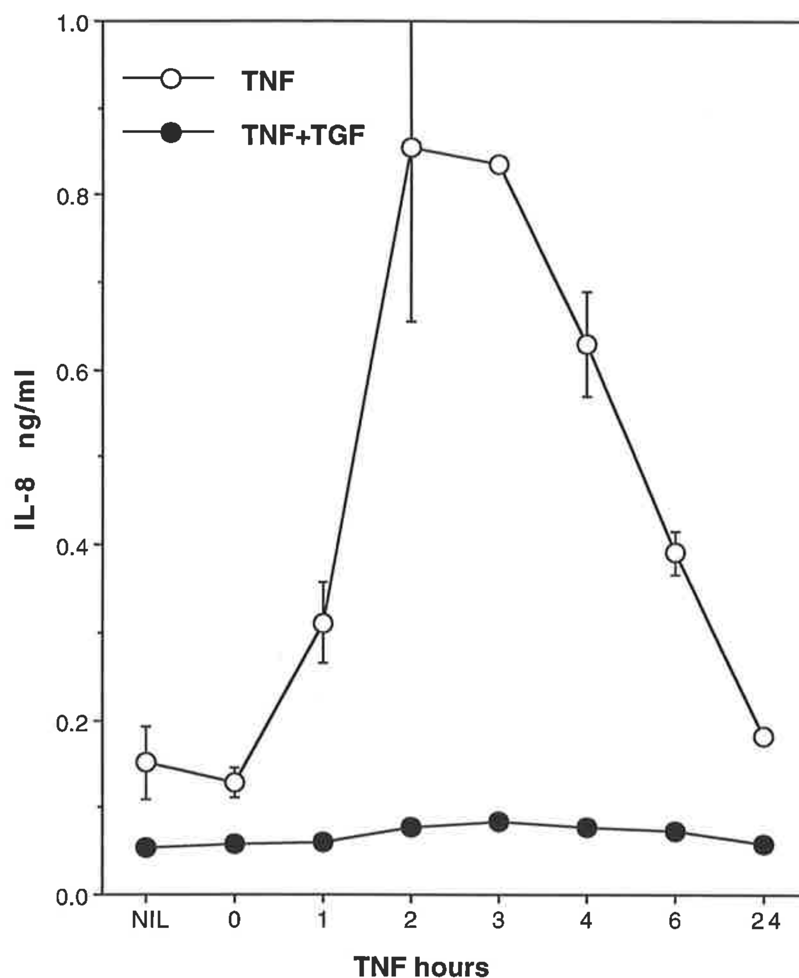


Fig. 9.4 Time course of IL-8 production by HUVEC treated with TGF-β and TNF-α

HUVEC cultured in 96 well cluster dishes were treated with TGF-β 0·2ng/ml for 24h, and during this period TNF-α 1U/ml was added either 24, 6, 4, 3, 2, 1 or 0h before the medium was changed. After 1h, medium was collected, and IL-8 quantitated by ELISA. Representative experiment, in duplicate, of 2. Each point is the mean ± SD.

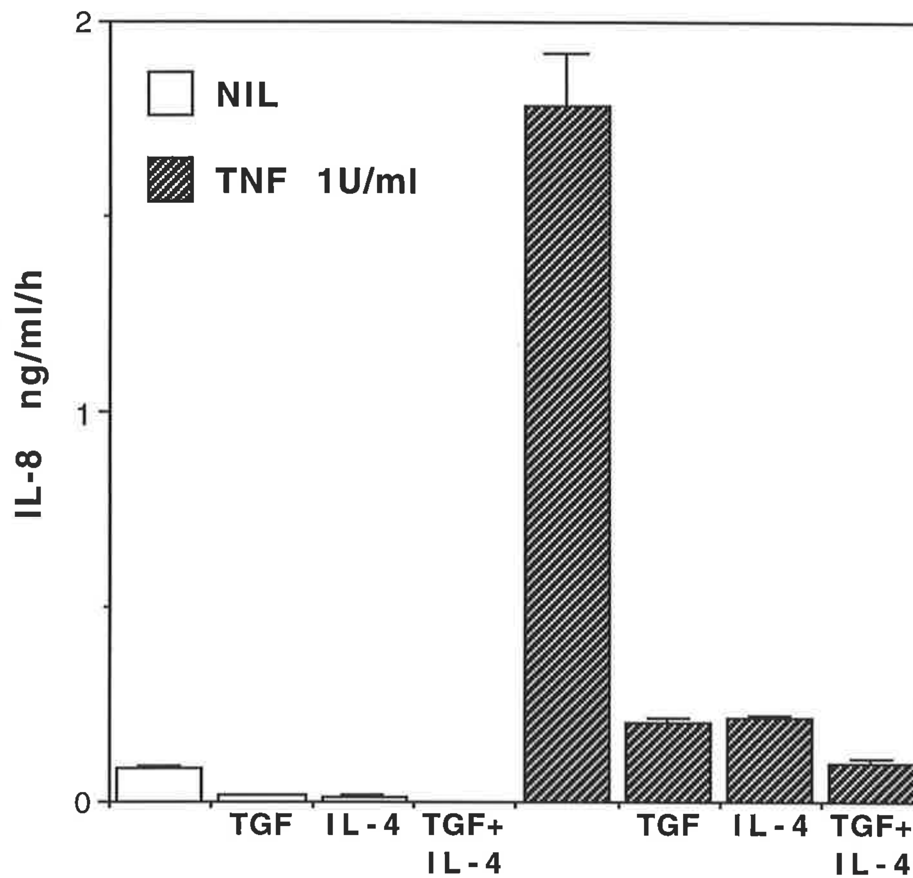


Fig. 9.5 IL-8 production by HUVEC treated with TNF-α, TGF-β and IL-4

HUVEC cultured in 96 well cluster dishes were treated with medium alone, TGF-β 0·2ng/ml, IL-4 10ng/ml or both for 24h, the stimulated with TNF-α 1U/ml for 4h. Medium was then changed and collected after 1h, and IL-8 quantitated by ELISA. Representative experiment of 2, in duplicate, columns show the mean ± SD.

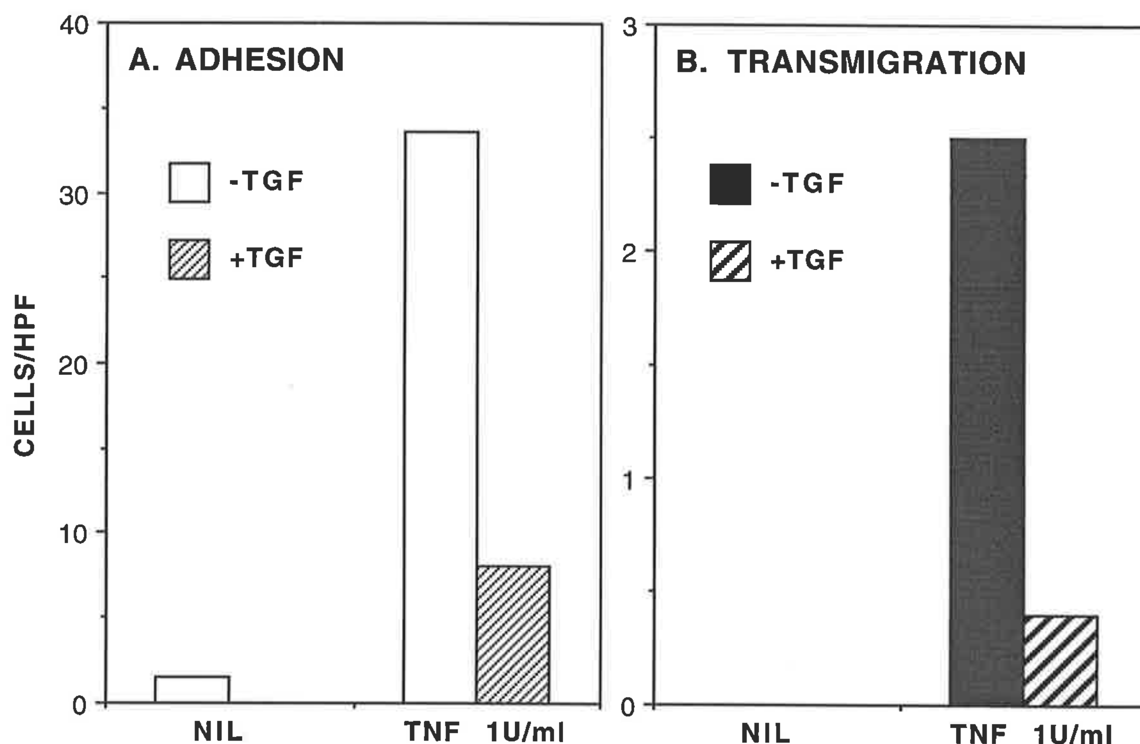


__Fig. 9.6 Transmigration of neutrophils through HUVEC on slides__

HUVEC were cultured to confluence on glass slides, treated with medium alone or TGF-β 0·2ng/ml for 24h and then activated with TNF-α 1 or 10ng/ml for 4h. After washing the monolayers were coincubated with 2×10^5 neutrophils for 20min, and then the nonadherent neutrophils were washed off. The monolayers with adherent and transmigrated neutrophils were then photographed on a phase contrast microscope, and the numbers of adherent and transmigrated cells were counted. Single experiment, representative of 4 experiments, the vertical axis shows the mean count of adherent (A) and transmigrated (B) cells from 6 photographs of each well. For examples of the slide assay, see Fig. 4.7.

DISCUSSION

The TGF-β are a highly conserved family of pleiotropic cytokines. TGF-β has both proliferative and anti-proliferative effects on different cell types, and increases extracellular matrix formation by cells. We have been interested for some time in the effects of TGF-β on endothelial cells. Three isoforms have been identified- TGF-β1 and 3 have similar effects on EC, while TGF-β2 is less active[364]. This may correlate with receptor binding, since while EC have both type I and type II receptors for TGF-β, they use endoglin, which binds only types 1 and 3, rather than β-glycan, which binds all three, as an accessory TGF-β binding molecule[365]. TGF-β is produced in a latent form, which is activated by EC surface proteases, and is then able to bind to cell-surface receptors[366]. It has been shown that TGF-β inhibits EC proliferation[367], and induces angiogenesis in permissive circumstances[368]. TGF-β also causes an increase in EC production of glycosamino-glycans[369] and collagen[370]. Our group have previously shown that EC adhesiveness for neutrophils[355] and lymphocytes[356] is inhibited by TGF-β, and that this is due to a decrease in transcription of the gene for E-selectin, resulting in a decrease in the induction of this molecule after EC activation by TNF-α[357]. Others have since reported that the adhesiveness of murine EC for tumour cells[371], and of rat HEV EC for lymphocytes[372], is reduced by TGF-β.

Other observations of the effects of TGF-β on inflammatory functions have given rise to some uncertainty regarding its true role. Most of its reported effects are clearly anti-inflammatory, *ie*. suppression of B- and T cell function, down-regulation of macrophages, and antagonism of TNF-α function[373], and *in vivo*, suppression of cardiac injury post ischaemia[374,375], and of arthritis in experimental models[376]. However, TGF-β has somewhat paradoxically been reported to be strongly chemotactic for monocytes, lymphocytes[118] and neutrophils[117], and to induce infiltration of neutrophils into rat synovial tissues after intra-articular injection[116], all the more surprising in view of the presence of active TGF-β in normal vessel walls[377], unassociated with leukocyte infiltration!

IL-4 is an important immunoregulatory cytokine, in particular for its roles in T cell phenotype and regulation of B-cell function. An anti-inflammatory role has also been established, particularly in relation to monocytes, where IL-4 has been shown to inhibit adhesion(378) and suppress the induction of many pro-inflammatory cytokines, including IL-8(358,359), IL-1, TNF-α, GM-CSF(359,360,379), and MIP-1α(361) (IL-10(360) and possibly IL-13(380) may have similar roles), and to induce IL-1 receptor antagonist(359). The effect of IL-4 on endothelium has been extensively investigated. IL-4 does not increase EC expression of E-selectin or ICAM-1, and inhibits the up-regulation of these adhesion molecules by TNF-α and IL-1(352). However, IL-4 specifically up-regulates VCAM-1 on EC(362,363), and synergises with TNF-α and IL-1 in this effect(352,381). IL-4 induces the production of monocyte chemotactic factor-1 (MCP-1) by EC and synergises in this with IL-1(353). These changes suggest that IL-4 is not suppressive of EC activation, but induces a qualitatively different form of activation. The induction of VCAM-1 would be expected to result in adhesion of leukocytes bearing VLA-4, and indeed, IL-4 activation of EC results in increased adhesiveness for monocytes(363), eosinophils(362) and lymphocytes(382) but not neutrophils.

The inflammatory role of endothelium extends beyond adhesiveness for leukocytes, as demonstrated in previous chapters. In this chapter, I have investigated the hypothesis that TGF-β or IL-4 may also inhibit the step subsequent to neutrophil adhesion, that of transendothelial migration. I have therefore investigated the effect of TGF-β and IL-4 on the production of IL-8 by EC. Pre-treatment of primary HUVEC with TGF-β inhibited the TNF-α mediated up-regulation of mRNA for IL-8 in 2 experiments (Fig. 9.1). Production of immunoreactive IL-8 in response to TNF-α was inhibited to an even greater extent than mRNA. Concentration/response experiments indicate that TGF-β inhibits IL-8 output more effectively at low levels of TNF-α. The concentration of TGF-β required was similar to that previously reported for inhibition of E-selectin(357). IL-4 at 10ng/ml also inhibited IL-8 production after TNF-α activation of HUVEC. IL-4 was reported not to reduce IL-8 mRNA expression in HUVEC stimulated by IL-1 or TNF-α(383), although the concentration of the latter was not indicated. I did not study the effect of IL-4 on IL-8 mRNA, however I

did note a variability in degree of inhibition of immunoreactive IL-8 by IL-4 in different HUVEC lines.

Inhibition of IL-8 by TGF-β was accompanied by a reduction in the number of neutrophils transmigrating through TNF-α activated endothelium (Fig. 9.6) This was not attributable solely to reduction in E-selectin expression, since the decrease in transmigration was greater that the decrease in adhesion, and also, since E-selectin is considered to have little role in transmigration in-vitro under static conditions(Chapter 5, (271)). Inhibition of IL-8 by neutralising antibodies only inhibits transmigration by approximately 50% (Ch. 5, (277)). IL-8 production by HUVEC activated by 1U/ml TNF-α is inhibited by 65%, whilst transmigration is inhibited by 83%, suggesting that TGF-β may also be inhibiting other, non-IL-8 dependent mechanisms of neutrophil transmigration.

The mechanism of the inhibitory effects of TGF-β and IL-4 on EC pro-inflammatory function is not known. TGF-β does not depress EC functions in general, since it increases expression of some genes[369,370,384] including its own[385] Also, TGF-β does not reduce the overall response of EC to TNF-α, for example by down regulating TNF-α receptors, since the induction of VCAM and up-regulation of ICAM-1 in response to TNF-α are unaffected[356]. Whilst IL-4 has been reported to increase expression of the inhibitory IL-1R2 on neutrophils[386], it does not do so on EC[237], and indeed can enhance some effects of IL-1[353].

It is interesting that induction of E-selectin and IL-8 are specifically inhibited by TGF-β and IL-4, since both of these molecules are involved particularly in neutrophil transmigration, suggesting a degree of coordinate gene regulation, and a programmed response by EC for neutrophilic inflammation. Since VCAM is not inhibited by TGF-β, the adhesion of monocytes, eosinophils and lymphocytes may still occur (although reduced, since these leukocytes also rely partially on E-selectin for adhesion). IL-4 specifically activates EC for adhesion of leukocytes other than neutrophils, and also results in the transendothelial migration of eosinophils but not neutrophils[290], although the involvement of an eosinophil chemokine in this process has not been established. TGF-β therefore acts on EC as a general inhibitor of leukocyte adhesion and neutrophil (and potentially memory

T lymphocyte) transmigration, whilst IL-4 inhibits the neutrophilic infiltration of acute inflammation, but activates EC to induce an allergic or chronic inflammatory type of cellular infiltrate.

The relevance of these observations is indicated by the demonstration that active TGF-β is found in the perivascular space of normal blood vessels *in-vivo*, suggesting a continuous activity on resting endothelium[(377)]. The origin of this active TGF-β is likely to be EC themselves in conjunction with perivascular smooth muscle cells, since these cells in co-culture produce the active form of TGF-β[(387,388)]. Cell-cell contact is required for activation, so that disruption of the intercellular cooperativity *ie.* in vascular injury may decrease the presence of active TGF-β, allowing unhindered inflammatory reactions.

The effects of TGF-β on the inflammatory response are of intense interest because of the phenotype of the TGF-β-null mouse. Animals which lack the gene for TGF-β were generated by homologous recombination in ES cells[(373)]. These mice appear to develop normally, and are healthy for the first 2-3 weeks of life. Subsequently however they develop spontaneous multifocal inflammatory disease, characterised by leukocyte infiltration into many tissues, and excess production of pro-inflammatory cytokines. Several explanations have been put forward for this phenotype, including the negative influence of TGF-β on the specific immune system through effects on B and T cells, and an interesting recent report of a negative effect of TGF-β on expression of MHC genes[(389)]. The observations reported here are also likely to be significant.

A general model for the function of TGF-β which emerges from these observations is that of an homeostatic regulator. Our observations suggest that TGF-β prevents the response of EC to small amounts of pro-inflammatory factors, thus guarding against an over-response to minor stimuli, but allows an endothelial response to strong stimuli. The emergence of spontaneous inflammation in the TGF-β-null mouse suggests that TGF-β normally functions to suppress inflammation, which is consistent with its expression in normal vessel walls (and highlights the significance of the endothelium in regulating inflammation). The control of inflammation in the normal animal may therefore depend not so much on the de-novo appearance of inflammatory factors in disease, and their

absence in health, but on a balance at all times between low levels of positive and negative regulatory factors, with pathology resulting from a decrease of negative or an increase in positive regulators of inflammation.

SUMMARY

It has previously been shown that TGF-β can inhibit several endothelial cell pro-inflammatory functions, including induction of E-selectin and adhesiveness for neutrophils and lymphocytes. Furthermore, TGF-β and IL-4 are both known to have a range of immunomodulatory and anti-inflammatory effects on a wide variety of cell types, and mice lacking in TGF-β develop spontaneous widespread inflammatory disease.

Endothelial cells activated by pro-inflammatory cytokines regulate inflammation not only by their adhesiveness for leukocytes, but also by producing chemokines which direct leukocyte transendothelial migration. In this chapter, I show that TGF-β, as well as IL-4, inhibits IL-8 production by EC activated by the pro-inflammatory cytokines TNF-α and IL-1. The effect of TGF-β was demonstrated at both the mRNA (by northern blotting) and protein (by ELISA) levels. Inhibition was maximal at a concentration of TGF-β previously demonstrated to be optimal for E-selectin down-regulation. Inhibition of IL-8 production by TNF-α activated EC was most effective at lower concentrations of TNF-α. Reduced production of IL-8 was accompanied by decreases in neutrophil transmigration through TNF-α activated endothelial monolayers.

This finding adds to the range of anti-inflammatory activities that can be ascribed to TGF-β and IL-4. The greater effectiveness of TGF-β at lower TNF-α concentrations is consistent with an homeostatic role for this cytokine, which is known to be present in the wall of normal vessels. This suggests a dynamic balance between pro- and anti-inflammatory factors in normal biology, and may explain the spontaneous inflammation in mice which lack this vital balancing factor.

CHAPTER 10

DISCUSSION

DISCUSSION

Historical background

It has long been recognised that extravasation of leukocytes constitutes one of the characteristic features of inflammation. Observations of leukocyte emigration from vessels by intravital microscopy were initially made more than a century ago. In the 1880's, Metchnikoff proposed that chemical signals emanating from sites of invading micro-organisms attracted leukocytes[390]; at the same time, Conheim suggested that "molecular alterations in the ... vessel walls" were responsible for leukocyte extravasation[391]. This conceptual duality has persisted, and we are now aware that both mechanisms are important. Over the ensuing years, leukocyte chemotaxis has been well studied, with *in vitro* work facilitated by the Boyden chamber, developed in 1962. The molecular basis of cellular adhesion has begun to be understood over the last 20 years. However, despite the many *in vivo* observations, relatively few *in vitro* studies specifically addressing the mechanisms of leukocyte transendothelial migration had been carried out before the last 5 years. Reports of the active role of the endothelium in regulating leukocyte transmigration first appeared in 1988-89, as did the first reports of the purification and cloning of IL-8. These two findings have provided the central themes for this thesis.

As already alluded to, recent work has indicated that leukocyte extravasation can be considered to occur by at least 3 steps: 1) rolling, 2) firm adhesion and 3) transmigration. Two mechanisms of neutrophil transmigration have been identified; chemotactic transmigration, and transmigration induced by activated endothelium. Whilst these ideas can be recognised in those originally formulated by Metchnikoff and Cohnheim, we now have a detailed, if as yet incomplete, understanding of these processes at a molecular level. In this chapter, I shall describe how the findings of my research fit into the current concepts of leukocyte transmigration.

Table 10.1 Summary of transmigration mechanisms

Steps of transmigration-	Chemotactic transmigration	Activated endothelium
	Mediated by-	Mediated by-
1. Rolling	Neutrophil L-selectin, selectin ligands	
	Endothelial P-selectin	Endothelial P-, E-selectin
2. Firm adhesion	Neutrophil β_2 integrins, activated by the chemoattractant	Neutrophil β_2 integrins, activated by IL-8, E-selectin, PECAM, ?PAF
	Endothelial ICAM-1, ?ICAM-2	Endothelial ICAM-1 (up-regulated), ?ICAM-2
3. Transmigration	Gradient of chemoattractant	IL-8 chemotaxis, chemokinesis, ?PECAM, ?PAF

10.1 Mechanisms of transmigration 1- Chemotactic transmigration

The ability of macrophages and many other tissue cells to produce IL-8 when subjected to inflammatory stimuli suggested that a gradient might be formed between the tissues and the bloodstream, *ie*. across the endothelial monolayer. I have demonstrated, as reported in chapter 3, that such a gradient is a powerful stimulus for neutrophil transendothelial migration. It is also shown that IL-8 activated the leukocyte integrin Mac-1 (CD18/CD11a), although the adhesion that resulted was weaker than that produced by other neutrophil stimulants, such as TNF-α and f-MLP. This work and that of others indicates that transendothelial chemotactic gradients can induce neutrophil transmigration. This mechanism undoubtedly accounts for significant amounts of transmigration *in vivo*, since the second mechanism, endothelial activation (discussed below) takes 2-4h to develop, and transmigration *in vivo* often commences within minutes. Chemotactic factors injected subcutaneously induce rapid neutrophil extravasation which is likely to be independent of endothelial activation. However, the simple concept of chemotaxis alone is

not sufficient to describe chemotactic transmigration. The model can be further refined, and considered in the terms of the steps involved in leukocyte extravasation (Table 10.1).

Neutrophil contact with the endothelium

Neutrophils in solution *ie.* circulating in the blood are not able to directionally respond to chemotactic factors, but must be adherent to do so(392). A neutrophil exposed to chemotactic activation in the blood may become hyperadhesive, but may contact the endothelium and adhere at a site downstream some distance from the focus of inflammation. Indeed, mechanisms exist to neutralise chemotactic factors released into the bloodstream(208,220), and to prevent the hyperadhesiveness of circulating neutrophils(393). In fact, if neutrophil CD18 was activated on the circulating cell, it may not result in adhesion of the neutrophil to resting endothelium, since integrin-mediated adhesion cannot occur in the presence of the shear force of flowing blood(64,307). These reservations may not apply in an established inflammatory site when the post-capillary venules are dilated and flow is slowed, but in this situation the endothelium is likely to be activated in any case.

In normal tissues in the acute situation, a mechanism must exist to bring the neutrophil into contact with the endothelial cell and slow its movement before integrin mediated adhesion and consequent chemotactic transmigration can occur. This mechanism is leukocyte rolling, mediated by the selectins. Whether rolling occurs in completely normal undisturbed vessels remains surprisingly controversial; although rolling is always seen in venules in intravital microscopy studies, there are concerns about the disturbance of tissues in these experiments, and the tissue and the animal are not in a completely resting state. Nevertheless, rolling was seen in the skin vessels of the mouse ear in a minimally disturbed preparation(6). P-selectin is expressed basally by resting EC *in vitro*, and is known to mediate rolling which facilitates integrin mediated adhesion(64). Therefore, it is likely that even in simple chemotactic migration, EC expression of P-selectin (and neutrophil L-selectin) are a necessary prerequisite. The delay of onset of inflammation in P-selectin gene deletion mice supports this contention(66).

Leukocyte integrins in chemotactic transmigration

The role of the neutrophil β_2 integrins is of central importance in chemotactic transmigration, but requires a more detailed explanation. They are not capable of mediating initial adhesion under conditions of flow, but when neutrophils are slowed by selectin interaction, the integrins when activated adhere to ICAM on the EC[(64)], resulting in a firm shear-resistant adhesion which is stronger than that of the selectins alone. It is possible that integrin-ICAM interactions continue during the neutrophil traversal across the EC surface and through the interendothelial junction. EM of transmigrating lymphocytes shows ICAM-1 in the regions of membrane contact between the leukocyte and the EC[(394)]. De-adhesion or transient adhesion is also important since the leukocyte must detach itself from adhesion points at the uropod in order to move on; the relatively weak and transient nature of adhesion induced by IL-8 (Ch. 3) may improve its ability to mediate migration. Whether required for the second step (after rolling) of firm adhesion, or for transmigration itself, or both, β_2 integrin function is unequivocally essential. Integrins require activation for adhesion to their ligands, which is probably provided by the chemotactic stimulant itself, at the endothelial surface, by "inside out" signalling (Ch. 3).

Role of the endothelium in chemotactic transmigration

In chemotactic transmigration, the endothelium has been considered to be passive, allowing the neutrophil to migrate through its junctions. As I have noted, except at high rates of leukocyte flux the endothelial barrier to macromolecular solutes is maintained during chemotactic transmigration, and EM studies show close membrane apposition between the migrating neutrophil and the endothelial cell junction. This argues against the destruction of interendothelial cell contacts, *eg.* by neutrophil proteases, to allow neutrophil passage, since this might be expected to result in a damaged junction after the neutrophil had passed, until the endothelium could repair itself. It is conceivable that the lamellipodium of the transmigrating neutrophil, projecting into the junction, provides alternative ligands for each of the interendothelial cell adhesion molecules, thus displacing their binding from each other onto the neutrophil membrane ligands, maintaining a seal between the cells while allowing neutrophil passage. However, neutrophil contact during chemotactic transmigration leads to an intracellular calcium flux in the EC, suggesting communication

between the neutrophil and the endothelium, and this calcium flux has been linked with the opening of the endothelial junction[283]. Whilst it is possible that both displacement and calcium-mediated junctional opening operate during transmigration, the clear implication is that the endothelium, whilst not in this situation controlling transmigration directly, is an active participant in the process.

The presence of an endothelial monolayer improves the efficiency of a chemotactic gradient across a polycarbonate filter at inducing neutrophil migration (Fig. 3.5). It has been suggested that chemoattractants might operate by altering the endothelium, such that it becomes more adhesive for neutrophils[395], but two pieces of evidence argue against this. Firstly, treating EC with IL-8 and then washing did not increase neutrophil adhesion (Ch. 3), and secondly, neutrophils could be specifically desensitised to the chemotactic factors IL-8 and f-MLP which blocked their chemotactic transmigration to the homologous factor, but left them still able to transmigrate to a gradient of the other (Fig. 4.2). This implies that the neutrophil responds directly via specific receptors to the chemoattractant in chemotactic transmigration and not to an altered endothelium. An alternative explanation for the greater efficiency of chemotactic transmigration across endothelium compared with chemotaxis across the filter is that the endothelium presents in impediment to the free diffusion of the chemotactic factor from the bottom into the top compartment, thus preserving a steeper concentration gradient. Chemoattractant diffusing from the endothelial paracellular gaps may attract the neutrophil towards the junctional region (Fig. 10.1).

10.2 Mechanisms of transmigration 2- Activation of endothelium

Endothelium incubated *in vitro* for >2h with the cytokines TNF-α or IL-1, and then washed, induces the migration of unstimulated neutrophils placed onto the monolayer, despite the lack of any exogenous chemoattractant. This is not due to any increase in the monolayer permeability, nor is it due to residual TNF-α or IL-1 in the system, since TNF-α tends if anything to decrease neutrophil motility[310], whilst IL-1 has no effect on neutrophil motility[265]. Transmigration due to endothelial activation has also been shown to occur *in*

vivo, since injection of IL-1 causes neutrophil infiltration that is delayed in onset, and prevented by protein synthesis inhibitors(396,397) (whereas that produced by injection of direct chemotactic agents is early in onset and not inhibited). TNF-α activates EC mainly through the p55 TNF receptor, although binding to the p75 receptor enhances its potency (Ch. 7)

Candidate mediators of EC induction of transmigration

The mechanism by which activated endothelium induces neutrophil transmigration has been of great interest. The known alterations that occur after endothelial activation have been considered for their potential roles in transmigration. Activation of EC by cytokines induces the expression of E-selectin, an adhesion molecule for most leukocyte types, and increases the expression of ICAM-1, a ligand for LFA-1 and Mac-1. In addition, activation induces the production and secretion of IL-8, and possibly other soluble mediators. EC activated by TNF-α produces and displays membrane surface PAF. Additional neutrophil chemotaxins produced by activated EC have been proposed as mediators of transmigration(277), and candidates include the chemokine MGSA, and the haematopoietic growth factors G-CSF and GM-CSF.

Adhesion molecules as mediators of transmigration

As is the case for chemotactic transmigration, β_2 integrins on the neutrophil are essential for transmigration induced by cytokine activated endothelium. The endothelium itself presumably activates their adhesive function- mediators of this are discussed later. One of the endothelial β_2 ligands, ICAM-1, is up-regulated on activated EC, but whether this up-regulation has any important effect on transmigration is not known. ICAM-2, a ligand for LFA-1, is not up-regulated, and its role in transmigration has not yet been established. E-selectin is not essential for transmigration, since E-selectin antibodies which inhibit adhesion of neutrophils to activated EC do not inhibit their transmigration, in static assays (Ch. 5, (271)). This suggests that the main purpose of selectin mediated adhesion (or rolling) is to slow the leukocyte sufficiently, under conditions of flow, for β_2 integrin mediated adhesion to occur, a function which is clearly not necessary in a static assay, but is crucial *in vivo*. Although P-selectin presumably fulfils this function at a basal level in

resting EC, the induction of E-selectin would be expected to enhance it considerably, since it is induced to much higher density than P-selectin, and due to the higher density of its ligand on neutrophils, mediates a tighter adhesion with slower rolling(65). Adhesion of neutrophils to E-selectin has been shown to stimulate Mac-1 activation(272) and may be one of the mechanisms by which activated EC cause β_2- ICAM adhesion prior to transmigration, but it is clearly not an essential mediator of this, as shown by the E-selectin blocking studies. This suggests redundancy in the mediators used by activated EC to activate β_2 integrins. E-selectin mediated activation of β_2 integrins may be more important at lower levels of EC activation(271). Although E-selectin was shown to be chemotactic for neutrophils in a Boyden chamber(272), there is at present no evidence to support the suggestion of a haptotactic gradient of E-selectin on the EC surface or in the junction.

IL-8 and integrin activation by activated EC

The production of IL-8 by activated EC is the most significant known factor in the induction of neutrophil transmigration. Its first role may be in activation of adhesion. IL-8 secreted from the apical surface of the endothelium into the circulation will be inactivated by adsorption to blood erythrocytes, potentially neutralised by endogenous IL-8 antibodies, and diluted and washed away by plasma flow. Nevertheless, the continual production of IL-8 by the EC means that immediately adjacent to the endothelium will be a layer of IL-8 in the active state, and there may also be IL-8 bound to the EC surface(398). Given that neutrophils have been brought into close proximity with the endothelial surface by rolling or adhesion on E-selectin, the neutrophil could then be stimulated by this IL-8, and the β_2 integrins become able to adhere to ICAM-1, leading to firm adhesion. *In vitro*, however, this mechanism for stimulation of neutrophil β_2 integrins also appears not to be completely necessary, since addition of neutralising antiserum to the upper compartment only of a transwell, which would be expected to neutralise IL-8 at the apical endothelial surface, did not inhibit and tended to increase the transmigration induced by activated EC (Fig. 5.5). A combination of blocking of E-selectin and IL-8 at the endothelial surface might abrogate β_2 activation and thereby inhibit transmigration, but this was not tested.

IL-8 and neutrophil motility in transmigration

The more important role of EC IL-8 in induction of transmigration is likely to be to induce neutrophil locomotion. As shown in Fig. 3.5, a gradient of IL-8 across the monolayer is an extremely effective means of inducing transmigration, whilst IL-8 present on both sides is somewhat less effective, but still increases it significantly. The importance of IL-8 is clearly demonstrated by the reduction in transmigration through activated endothelium that occurs when it is neutralised- 40% (Fig. 5.5) to 71%[277]. It was found that the secretion of IL-8 by EC in transwells is not polarised (Fig. 5.3), indicating that in this system it acts to induce transmigration by the less efficient mechanism of chemokinesis. It is of interest that neutralisation of IL-8 in only the lower compartment had the effect of reducing transmigration even more than neutralisation on both sides. This indicates that exposure of neutrophils to IL-8 in the upper compartment, which activates their β_2 integrins and enhances chemokinetic motility, is not enough to promote transmigration unless they are also able to move towards soluble IL-8, or alternatively stated, a negative gradient of IL-8 keeps them from migrating. The equivalent, adding IL-8 to only the upper compartment with an unstimulated monolayer, also does not cause transmigration (data not shown). Neutralisation of IL-8 in the upper compartment tended to increase transmigration in some experiments, probably due to increasing the effective IL-8 concentration differential between the upper and lower compartments. As outlined in chapter 5, a similar mechanism may occur *in vivo*, since IL-8 secreted into the intravascular space will be diluted and neutralised, thereby ensuring that the more efficient chemotactic mechanism will be used for transmigration. A modification of the transwell experiments, used in chapter 8, achieved a similar result, by replacing the medium in the upper compartment immediately prior to the addition of neutrophils, but leaving the medium in which IL-8 had been allowed to accumulate in the lower compartment, ensuring that the IL-8 produced by the endothelium acted by forming a chemotactic gradient.

***In vitro*, EC derived IL-8 acts in solution**

Although IL-8 has been postulated to act by an haptotactic mechanism, *ie*. by binding to the EC surface and acting as a fixed chemoattractant, this binding was seen only on post-capillary venules *in vivo*, and not with HUVEC[398]. Therefore, whilst binding of IL-8 to

the EC surface may activate neutrophil β_2 integrins and mediate haptotaxis *in vivo*, transmigration can still occur *in vitro* without it, indicating that surface binding of IL-8 is not a necessary part of the mechanism. I have incubated resting endothelium in IL-8 in an attempt to reproduce this binding, but after washing away soluble IL-8, no increase in transmigration (or adhesion) was seen. Therefore, it is likely that IL-8 produced by activated EC, at least *in vitro*, is acting in solution. Although in the transwell assay, the endothelium is washed prior to the addition of neutrophils, significant amounts of IL-8 continue to be produced during the 45-60min of the assay. It is not absolutely clear how the IL-8 is distributed and how neutrophils sense it. IL-8 is able to diffuse across endothelium (data not shown), suggesting that when placed in the lower compartment only, IL-8 will diffuse into the upper compartment during the assay, most likely by the paracellular pathway. Therefore, soluble IL-8 may be emanating from the EC junction region, with a concentration inversely proportional to the distance from this region (Fig. 10.1). Neutrophils adherent to surfaces migrate in the direction of a soluble chemotactic gradient, and so this may act to draw neutrophils towards the junction, and the same effect may cause them to migrate towards the higher concentration of IL-8 through the junction and on to the other side. When the concentration is equal on both sides of the monolayer, neutrophil motility is increased by chemokinesis which is directionally random. In this situation, another factor may be required to guide the neutrophil towards and through the junction- this may be PECAM (see below).

Non- IL-8 mediators of transmigration

Inhibition of transmigration by IL-8 neutralising antiserum is incomplete (Fig. 5.5), despite the effectiveness of this antiserum in neutralising IL-8 in solution (Fig. 5.4). It is possible that the antibody did not reach IL-8 sequestered in the interendothelial junction, or in the space between the endothelium and the subendothelial basement membrane. Alternatively, there may be additional, non-IL-8 mediators of transmigration produced by endothelium. Candidate mediators include the CSF, G-CSF and GM-CSF. These factors were reported to be chemotactic for leukocytes, and are produced by activated EC. However, as I have reported in chapter 6, their motility properties are not the same as those of IL-8, since they

promote migration across filters (and across endothelium) against a concentration gradient as well as with it. The CSF are poor stimulators of transendothelial migration compared to IL-8, and CSF of endothelial origin are unlikely to play a role in the induction of transmigration by activated EC, since neutralising anti-GM-CSF antibodies did not affect transmigration (Fig. 6.4). Another putative mediator of transmigration is PAF. It has been reported that EC activated by TNF-α and IL-1 express cell surface PAF which is a mediator of adhesion(280) and transmigration(278). However, the role of this cell surface PAF in adhesion has been called into question(399), and I have shown that transmigration in response to TNF-α activated endothelium occurs despite inhibition of PAF (Fig. 5.8). MGSA is a C-X-C chemokine closely related to IL-8, which has also been suggested as a possible mediator(277). This is feasible, since MGSA mRNA was upregulated in cytokine activated EC(185), and MGSA has neutrophil chemotactic activity only marginally less than that of IL-8(192). The IL-8 antiserum does not cross react with MGSA (Ch. 2), but neutrophils desensitised to IL-8 are also desensitised to MGSA, since both use a common receptor on neutrophils. Therefore, the hypothetical role of MGSA remains to be tested.

Neutrophil responsiveness to activated EC

Neutrophils do not require any prior stimulation to respond to activated EC; in fact, it appears that neutrophils pretreated with chemotactic factors are impaired in their transmigration response (Ch. 4). F-MLP pretreatment of neutrophils reduced their transmigration through cytokine activated endothelium partially, whereas IL-8 pretreatment reduced it completely. At least part of the effect of IL-8 is likely to have been due to receptor desensitisation, rendering the neutrophil unresponsive to IL-8 secreted by the endothelium. However, the f-MLP effect is not likely to have been due to receptor desensitisation. Importantly, chemotactic factor pretreatment does not impair the ability of the neutrophil to transmigrate in response to chemotactic gradients (although it effectively desensitises them to the chemotactic effects of gradients of the homologous factor (Fig. 4.2)). Therefore, these neutrophils are altered in such a way as to be unable to transmigrate in response to activated endothelium specifically, suggesting a qualitatively different mechanism of neutrophil motility is required. Luskinscas *et al.* made a similar

observation of inhibition of transmigration of chemotactic factor pretreated neutrophils through IL-1 activated EC, and suggested that this inhibition correlated with the ability of the factors to induce a cytoskeletal reorganisation, with prolonged actin filament polymerisation[332]. This may impair neutrophil motility such that the activated EC is unable to induce these neutrophils to transmigrate, yet a gradient of chemoattractant can overcome this impairment. A possible explanation for this might be that whilst pre-stimulated neutrophils can respond to chemotactic gradients, they may not be able to migrate by chemokinesis, which as we have subsequently shown, may be the means by which EC derived IL-8 promotes transmigration *in vitro*. This could be resolved by testing the transmigration response of pre-stimulated neutrophils to exogenous IL-8 at equal concentrations on both sides of the monolayer.

PECAM in transmigration

The role of PECAM (CD31) in transmigration has recently been reported[56]. This molecule of the interendothelial junction links EC by homophilic interaction with PECAM molecules on the adjacent cell, but also has an heterophilic ligand, a glycosaminoglycan, which is not yet fully characterised[50]. PECAM is also present on leukocytes including neutrophils, and although it has not been demonstrated to play a direct role in neutrophil-endothelial adhesion, the possibility of an indirect role is suggested by the connection between binding of PECAM and activation of the adhesion of β_1[54] and β_2[55] integrins on lymphocytes. Therefore several hypothetical functions for PECAM in transmigration can be envisaged. The first is that PECAM on the EC, binding to PECAM on the neutrophil, may be responsible for the activation of β_2 integrins leading to firm adhesion through integrin-ICAM interaction. In fact this may occur preferentially at or near the cell junction, since PECAM is concentrated there, which leads to another potential function- that of guidance of the adherent neutrophil towards the junction by haptotaxis, in preparation for paracellular migration. Furthermore, since PECAM appears to be richly expressed in the interdigitating folds which form the EC junction, a concentration gradient of PECAM may guide the neutrophil through the junction, suggesting that PECAM may be responsible for transmigration itself (Fig. 10.2).

Monoclonal antibodies to PECAM (FAb_2) were reported to inhibit the spontaneous transmigration of monocytes, as well as that occurring after endothelial activation. There was also a 40% inhibition of neutrophil transmigration in response to cytokine activated endothelium, however rabbit polyclonal antiserum blocked neutrophil transmigration by up to 80%. These findings have been replicated in our own laboratory (C. Bernard, ms. in preparation). It was observed that monocytes inhibited from transmigration by anti-PECAM antibody remain tightly adherent over the endothelial cell junction. This suggests that integrin activation had occurred despite the blockade of PECAM, and that also the cells had ben able to migrate to the junction region, which indicates that PECAM might not be necessary for these functions. These experiments have separated the steps of tight adhesion and transmigration, and indicate that even in the presence of endothelial derived IL-8, PECAM is necessary for transmigration. Binding of the leukocyte PECAM to PECAM or another ligand on the EC must therefore be necessary for traversal of the junction. One possible construction of this is that by displacing the homotypic-homophilic binding of PECAM on EC to each other, by homophilic-heterotypic binding, *ie*. PECAM on the neutrophil to PECAM on the EC, the neutrophil is able to "unzip" the EC junction, and that this is necessary for transmigration (Fig. 10.3B).

Homophilic binding by PECAM has been detected only in adherent cells, while heterophilic binding was detected using cells in suspension, and it has been inferred that this may be a generalised phenomenon[50]. It was recently shown that modifications (truncations) of the cytoplasmic domain of PECAM could convert the predominantly heterophilic binding of PECAM-transfected L-cells (in suspension) to homophilic binding, suggesting that the binding characteristics of PECAM are under the control of the cytoplasmic domain[400]. Interactions between the cytoskeleton and the cytoplasmic domain of PECAM may lead to homophilic binding in adherent EC. Neutrophils migrating across EC, which form pseudopods rich in polymerised actin[120] may therefore modify their PECAM via the cytoplasmic domain to become capable of binding to PECAM at the EC junction (Fig. 10.3).

PECAM does not appear to be altered by cytokine activation

One difficulty with the role of PECAM in neutrophil transmigration through cytokine activated EC is that PECAM itself does not appear to be altered, either quantitatively or qualitatively, by cytokine stimulation. *In vitro*, the distribution of PECAM on EC activated by TNF-α appears to be equal to that on resting EC (unpublished observations), but it has been reported that in organ culture of explanted skin, PECAM redistributes from the lumenal surface to the intercellular junctions(401). This may have significance for transmigration *in vivo*, but clearly is not absolutely necessary, since transmigration still occurs without it *in vitro*. The most important qualitative alteration of PECAM described to date is the phosphorylation, on serine and threonine, of the cytoplasmic domain, which alters its relationship with the cytoskeleton(53). However, a change in phosphorylation was not detected after activation of EC with TNF-α for 4h. This suggests that although PECAM is necessary for transmigration through cytokine activated EC, it does not itself regulate transmigration, since it is also present on resting EC. It is possible that an as yet undiscovered qualitative alteration in PECAM occurs after cytokine activation, which allows it to regulate transmigration. However, it is more likely that it acts in cooperation with IL-8 and other factors which direct transmigration, and is necessary but not sufficient for induction of transmigration by activated EC.

PECAM and the EC junction in transmigration

PECAM has a putative role in maintenance of the interendothelial cell junction(47). Histamine and thrombin, which increase endothelial permeability by releasing junctional adhesion, act via a calcium flux in the EC(283). These factors have also been shown to result in the phosphorylation of PECAM(51). Intracellular calcium flux is linked to the activation of PKC, which is a serine-threonine kinase. It has also been shown that the contact of a neutrophil with the endothelial surface prior to transmigration (either chemotactic or endothelium-driven) induces a calcium flux in the EC(283), through an uncharacterised pathway. This would therefore be expected to result in activation of PKC, for which PECAM is a potential substrate. It has not yet been shown that phosphorylation of PECAM is responsible for opening of endothelial junctions, and other molecules may also be phosphorylated or altered in parallel, but it is reasonable to hypothesise that

neutrophil contact, through PECAM phosphorylation, opens the interendothelial junction to allow transmigration. Neutrophil PECAM may then bind to EC PECAM to maintain a seal in the junction as the neutrophil migrates through (Fig. 10.3).

Summary- the mechanism of neutrophil transmigration

The information presented here allows a synthesis of how neutrophil transmigration is likely to occur *in vivo*. The first step, which is a necessary prerequisite, is neutrophil rolling, mediated by L-selectin and the selectin ligands on the neutrophil, P-selectin on the resting endothelium, and E-selectin on activated endothelium. The next step is activation of the neutrophil β_2 integrins leading to firm adhesion to endothelial ICAM. This may be mediated by a chemotactic factor being produced in the tissues, which diffuses into the local microvasculature and activates a neutrophil which is rolling on the non-activated endothelium, or it may be mediated by a factor from cytokine activated endothelium, which acts upon neutrophils rolling on E-selectin. This factor may be IL-8 produced by the activated EC, E-selectin itself, PECAM, or PAF, although there is evidence that no one of these is alone responsible. After this the neutrophil must move across the endothelial surface to the EC junction, and may be guided by soluble chemotactic substances emanating from the EC junction, or by haptotactic gradients on the EC surface, of PECAM or other surface bound molecules. Migration through the interendothelial cell junction is the final step being considered here, although the neutrophil still has subsequently to penetrate the basement membrane and travel through the tissues. Transmigration itself requires either an exogenous gradient of a chemotactic factor, or is at least partly dependent on the production of IL-8 by activated endothelium. In the latter case, PECAM is also necessary for transmigration, and may also play a role in endothelial junction separation and the maintenance of barrier seal during transmigration.

10.3 The regulation of neutrophil transmigration

Whilst the mechanism of transmigration is only partially understood, as discussed above, it is clear that the ability of EC to direct transmigration can be regulated by various factors. Even if IL-8 is only a part of the mechanism which EC use to direct neutrophil

transmigration, the control of its production correlates well with the control of endothelial induction of transmigration, and exceptions to this correlation have yet to be found. Therefore, factors which act upon EC to induce the production of IL-8, to increase this production or to suppress it, would be expected to have similar effects on neutrophil transmigration. However, it should be borne in mind that the induction of neutrophil transmigration by EC is only one mechanism by which neutrophil transmigration can occur; chemotactic factors produced within the tissues will also have significant effects, which may occur independently of or interact with the activation of EC.

IL-3 regulation of transmigration

In chapter 8, it was reported that IL-3 is also able to regulate the pro-inflammatory functions of EC. An increase in EC production of IL-8, stimulated by IL-3, was matched by an increase in the EC induction of neutrophil transmigration. For these functions, IL-3 appears not to be an independent regulator of EC, but is active only on EC which have already been treated with TNF-α. This may be in part due to the ability of TNF-α to increase EC expression of IL-3 receptors (Fig. 8.1-8.3). However, increased expression of receptors by EC is not sufficient to confer IL-3 responsiveness in terms of IL-8 production, since EC stimulated by IFN-γ, which increases IL-3R expression but does not itself induce IL-8, do not produce IL-8 when treated with IL-3 (E. Korpelainen *et al.*, ms. in preparation). This suggests that the signal generated by the binding of IL-3 to its receptor on EC cannot activate expression of the IL-8 gene, but enhances the signal generated by the TNFR. This ability of IL-3 to augment the effect of TNF-α on EC induction of neutrophil transmigration suggests a novel role for IL-3 in neutrophilic infiltration, with which it had not previously been associated. Whether IL-3 also influences the ability of EC to induce transmigration of other leukocyte types, particularly eosinophils, in line with previous concepts of IL-3 as a mediator of allergic inflammation, is currently under investigation.

Inhibition of transmigration- TGF-β

Prevention of neutrophil transmigration, either as an homeostatic control of inflammation in the normal organism, or in the resolution of an inflammatory response, is an area that has received little attention. Yet failure of a normal anti-inflammatory control is just as

likely to result in pathology and disease as is an aberrant pro-inflammatory stimulus. TGF-β has previously been demonstrated by our group to antagonise the induction of E-selectin on EC and thereby inhibit neutrophil adhesion[355,357]; I have now shown that TGF-β also inhibits the production of IL-8 by EC activated by TNF-α and IL-1, and in parallel inhibited the consequent neutrophil transmigration (Ch. 9). Other EC activation phenomena, such as the increase in ICAM-1 expression and induction of VCAM-1, were previously shown not to be inhibited[357], suggesting the possibility of a specific antagonism of neutrophilic inflammation. Although both IL-8 expression and transmigration were reduced, the magnitude of transmigration reduction was perhaps greater than that of inhibition of IL-8; while the relationship between the two may of course not be linear, this suggests the possibility at least that other non-IL-8 mechanisms by which activated EC induce neutrophil transmigration are also inhibited by TGF-β.

The ability of TGF-β to reduce the activation of EC for neutrophil transmigration, together with the important finding that mice lacking TGF-β (through gene deletion) suffer from spontaneous inflammatory disease[373], and the presence of TGF-β in the subendothelium of normal vessels[377], suggests a role for TGF-β as an homeostatic negative regulator of inflammation. This fits well with the ability of TGF-β to block IL-8 induction by low concentrations of TNF-α, but not as effectively at high TNF-α concentrations, since this would operate to dampen minor inflammatory perturbations in the system, but allow inflammation to occur where the stimulus is strong. The mouse which dies through lack of TGF-β may be the victim of a minor and normal inflammatory event which is not suppressed but instead can be amplified, by well known inflammatory cytokine cascades, leading to widespread inflammatory disease.

IL-4 also inhibited EC IL-8 production. While this factor has been shown to have several inhibitory effects on acute inflammatory functions[358,359,360,361], it activates EC production of other adhesion molecules and chemokines with activity for eosinophils and monocytes[353,362,363], suggesting that it switches EC from directing neutrophil migration to directing the migration of other leukocyte types, which might occur in the change from acute to chronic inflammation, or the induction of allergic migration.

IL-8 as a therapeutic target

Production of IL-8 by EC as well as by many different types of tissue cells and leukocytes is clearly of major importance in neutrophil transmigration, both through transendothelial chemotactic gradients and in the context of endothelial activation. In addition to the cytokines mentioned above which regulate endothelial IL-8 production and hence endothelium-driven neutrophil transmigration, there may be many other factors which influence IL-8 production by EC and other cell types. Inhibition of IL-8 production may potentially be an important therapeutic tool in the prevention or control of acute inflammation. Current studies of the promoter region of the IL-8 gene are defining the important regulatory elements. There is evidence that many genes encoding for pro-inflammatory cytokines and adhesion proteins have common regulatory elements which, if inhibited, could disrupt inflammation from many angles. Another approach would be to antagonise the effects of IL-8, and although neutralising antibodies may be one possibility, another more promising one is the development of IL-8 analogues with antagonist activity[402], or preferably small peptide derivatives thereof, and ultimately non-peptide mimetic antagonists.

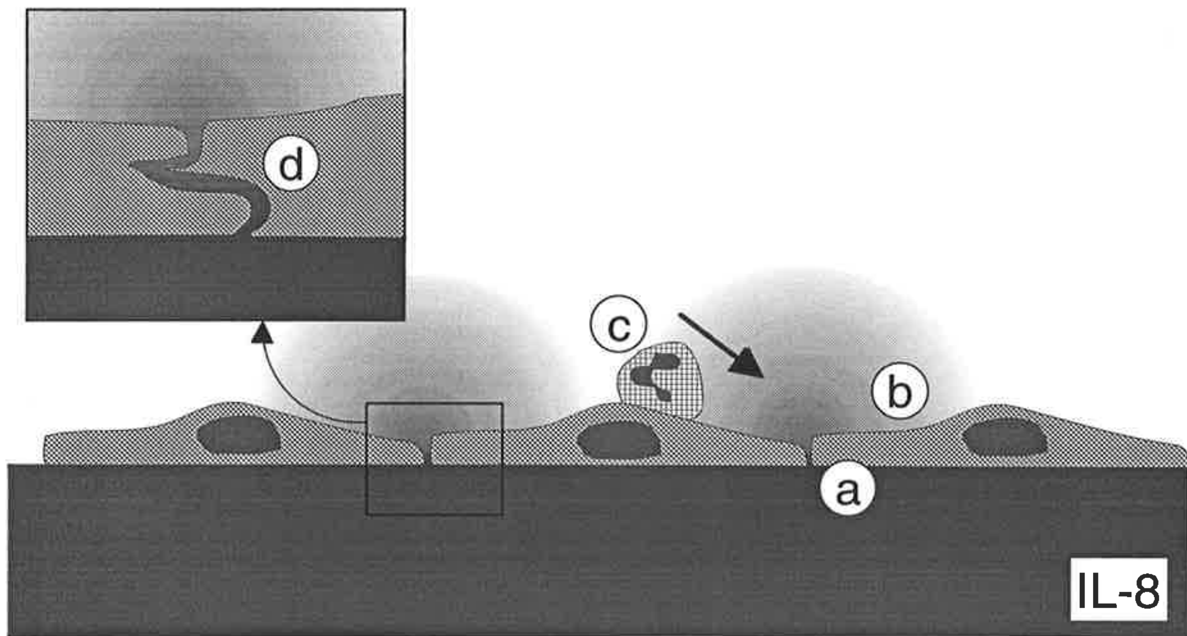


Fig. 10.1 Neutrophil transmigration in response to an IL-8 chemotactic gradient

A uniform high concentration of IL-8 is present below the monolayer. This diffuses through the interendothelial junction (a), and emanates from the apical opening of the junction, creating a soluble concentration gradient leading towards the junction (b). Neutrophils adherent to the apical endothelial surface are induced to move towards the junction by this soluble gradient. inset shows the interendothelial junction in more detail, which usually consists of overlapping folds. A concentration gradient of IL-8 would be established along the length of the junction from base to apex (c).

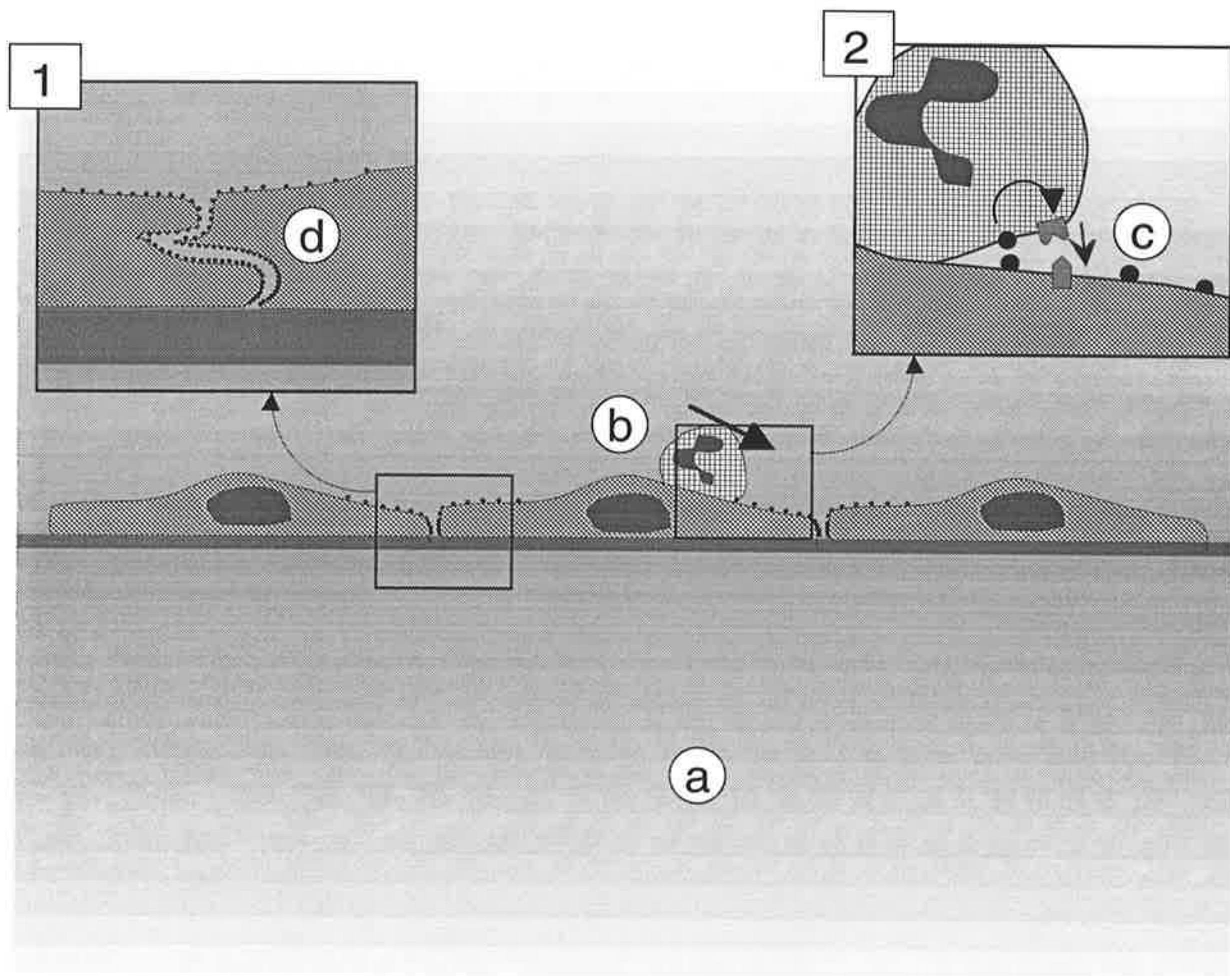
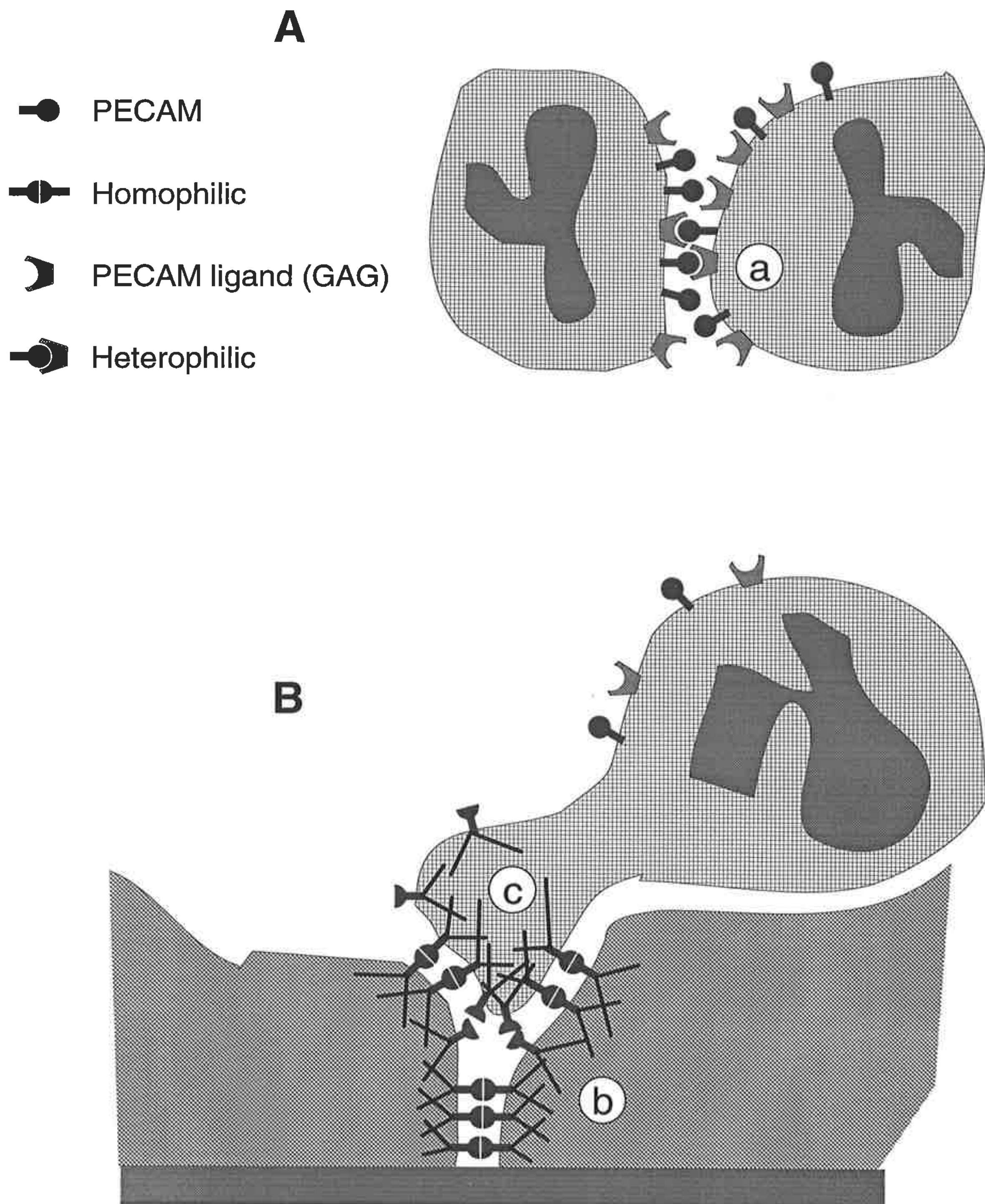


Fig. 10.2 Hypothetical roles of PECAM in neutrophil transmigration

PECAM is expressed preferentially at the cell junctions. In this example, where IL-8 is present on both sides of the monolayer (a), a haptotactic gradient of PECAM may attract neutrophils towards the junctional region. This may be direct (b) or mediated by the activation of β2 integrins by signalling from PECAM on the neutrophil (c). inset 1 shows the convoluted junctional region; it is possible that a gradient of PECAM density exists in this region which guides the neutrophil through the monolayer.

<u>Fig. 10.3 Neutrophil PECAM may "unzip" the endothelial junction</u>

PECAM on cells in suspension normally uses heterophilic binding, the ligand being a glycosaminoglycan. This is depicted in (A), although the PECAM ligand has not been confirmed to be present on neutrophils (*a*). Adherent cells including endothelial cells are believed to bind through PECAM-PECAM homophilic interactions, and the specificity of this appears to be controlled by the cytoplasmic domain, possibly under the control of the cytoskeleton (*b*). Neutrophils which extend pseudopods containing filamentous actin may thereby alter the specificity of the membrane PECAM locally, to favour homophilic binding (*c*), and thereby interact with endothelial PECAM and displace the endothelial homotypic adhesion, to separate the EC junctions and allow transmigration.


A
PECAM
Homophilic
PECAM ligand (GAG)
Heterophilic
a
B
c
b

10.4 Future directions

There are several areas of major interest which could be pursued in further research. Many details of the transmigration process remain unclear. One group of adhesion molecules whose roles are under explored are the ICAM; questions remain as to the role of ICAM-2 in transmigration and whether it can act as a ligand for Mac-1. Even more interesting is the possibility that these molecules may have a signalling role in EC, which is a novel suggestion, but is one implication of the finding that neutrophil binding induces a calcium flux in the EC. Clearly communication between the cells is occurring, but the pathway remains unknown.

I have discussed the recent implication of PECAM in transmigration, which has necessitated some revision of the models of the induction of transmigration by activated EC. We are currently investigating whether chemotactic transmigration has a similar requirement for PECAM. Several intriguing hypotheses have been raised: PECAM activates neutrophil β_2 integrins during transmigration; haptotaxis on PECAM surface gradients guides the neutrophil towards and through the EC junction; PECAM is phosphorylated as a result of the EC calcium flux that occurs on neutrophil binding, and phosphorylation is related to release of homophilic adhesion and opening of the junctions; PECAM on neutrophil pseudopods becomes capable of homophilic binding as a result of cytoskeletal association, and "unzips" the EC junction; and PECAM has a role in maintaining the seal around the neutrophil as it transmigrates. Exploration of these hypotheses and a clarification of the role of PECAM is the current major challenge in the understanding of the mechanism of transmigration.

The function of IL-8 as a specific chemoattractant for neutrophils, with a demonstrated role in acute inflammation *in vivo*, has created a new paradigm in the control of cellular migration in inflammation. It has led to the concept that combinations of adhesion molecules and chemokines with specificity for particular leukocyte types may be the mechanism by which different types of inflammatory reactions contain particular combinations of neutrophils, eosinophils, monocytes and lymphocytes(313). It is intriguing

that certain members of the C-C chemokine group even have specificity for lymphocyte subpopulations[177,205], suggesting a profound ability to regulate immunological reactions. Clarification of the roles of each of these factors in transmigration of different leukocyte types should provide a useful library of information which can be applied to inflammatory disease *in vivo*. Simple experiments such as the application of mixed populations of leukocytes into transwells and monitoring whether selected subgroups transmigrate in response to particular factors may be highly informative. Studies on the control of the production of other members of the chemokine family, in combination with the display of particular adhesion molecules, by endothelium, will reveal whether and how endothelial cells determine which kind of inflammation occurs in response to various stimuli.

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