MONOCYTE:ENDOTHELIUM INTERACTIONS AND THE CONTROL OF INFLAMMATORY GENE EXPRESSION

BY

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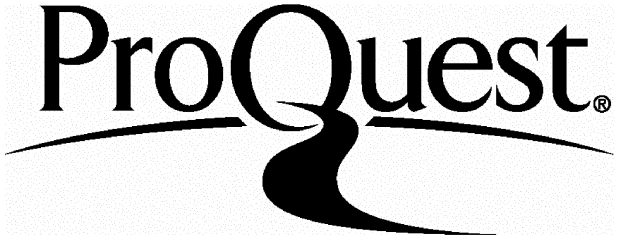

This thesis is dedicated to the memory of my Father for the love, support, guidance and encouragement that he gave to me.

Abstract

These studies have focused on the ability of monocytes to influence endothelial cell function in the context of immune and inflammatory responses. Monocytes when cocultured with endothelial cells upregulated E-selectin mRNA and protein expression, with initial appearance on endothelial cells at 3 hours and sustained expression at 21 hours. In contrast, IL-1 induced a transient upregulation. Cell:cell contact was important for monocyte induction of E-selectin expression, an effect which was partially mediated by TNF. Monocyte induction of E-selectin gene expression was NFκB dependent.

Addition of exogenous IL-10 to endothelial cell/monocyte cocultures inhibited E-selectin expression at 4 and 21 hours whilst having no effect on E-selectin induction by IL-1 or TNF. In addition, coculture with endothelial cells induced monocyte expression of IL-10 mRNA with maximal levels at 30 hours.

Monocytes influenced endothelial cell survival and expression of the bcl-2 homologue, A1, an anti-apoptosis gene. The level of A1 mRNA in serum starved endothelial cells decreased with time, however, addition of monocytes to serum starved endothelial cells increased A1 gene expression for up to 21 hours compared to the transient induction by IL-1. Coculture of monocytes with serum starved endothelial cells reduced endothelial cell death at 21 hours. Cell:cell contact was required for maximal A1 mRNA induction.

The role of calcium-dependent proteases in leukocyte transmigration was investigated using calpain inhibitors. Addition of calpain inhibitors reduced the migration of monocytes and neutrophils through cytokine stimulated endothelial monolayers as well as chemokine stimulated migration across unactivated endothelium. Adhesion of leukocytes to the endothelium was not affected by the presence of calpain inhibitors.

These studies have demonstrated the ability of peripheral blood monocytes to influence the function and survival of endothelial cells, and have also investigated the molecular mechanisms responsible for such interactions.

CONTENTS

List of Tables

List of Figures

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Abbreviations

Abbreviation	Definition
APAAP	Alkaline phosphatase anti-alkaline phosphatase
ARE	Adenylate/uridylate-rich element
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular calcium
cAMP	Adenosine 3', 5'-cyclic phosphate
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cells
CI	Calpain inhibitor I
CII	Calpain inhibitor II
CO_2	Carbon dioxide
CCR2	C-C chemokine receptor-2
$CuSO_4.5H_2O$	Copper sulphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5' triphosphates
DTT	Dithiothreitol
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid-dipotassium salt

EGF	Epidermal growth factor
ELISA	Enzyme linked immunoabsorbant assay
ESL-1	E-selectin ligand-1
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GAG	Glycosaminoglycan
G-CSF	Granulocyte colony stimulating factor
GITC	Guanidine thiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	Glycosyl-phosphatidylinositol
HBSS	Hanks buffered saline solution
HDMEC	Human dermal microvascular endothelial cells
HMG	High mobility group
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HS	Heparin sulphate
H_2SO_4	Sulphuric acid
HUAEC	Human umbilical aortic endothelial cells
HUVEC	Human umbilical vein endothelial cells
IAA	Isoamylalcohol
ICAM-1	Intercellular cell adhesion molecule-1
IFN-γ	Interferon -gamma
Ig	Immunoglobulin
IκB-α/β	Inhibitor kappa B-alpha/beta
IL-	Interleukin-

IL-1RI/II	Interleukin 1 receptor type I/II
IL-1Ra	Interleukin 1 receptor antagonist
icIL-1Ra I/II	Intracellular IL-1 receptor antagonist type I/II
sIL-1Ra	Soluble interleukin 1 receptor antagonist
IMDM	Iscoves Modified Dulbeccos medium
ITS	Insulin-transferrin-sodium selenite
JNK	c-Jun amino terminal kinase
kDa	Kilodalton
LAD I/II	Leukocyte adhesion deficiency I/II
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
Lyso-PC	Lysophosphatidylcholine
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
$MgCl_2$	Magnesium chloride
MGG	May-Grunwald and Giemsa stain
MHC	Major histocompatability complex
MLCK	Myosin light chain kinase
MMLV	Moloney murine leukaemia virus
MO	Monocyte
mRNA	Messenger ribonucleic acid
mTNF	Membrane bound tumour necrosis factor
NAC	N-acetylcysteine
$NaCO_3$	Sodium carbonate
NaOH	Sodium hydroxide

NF-κB	Nuclear factor-kappa B transcription factor
NK cells	Natural killer cells
NP-40	Nonidet P-40
O.D.	Optical density
OPD	O-phenylenediamine
OxLDL	Oxidised low density lipoproteins
PAI-I/II	Plasminogen activator inhibitor type 1/2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Positive regulatory domains
PDGF	Platelet derived growth factor
PDTC	Pyrrolidine dithiocarbamate
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGI_2	Prostaglandin I_2
PI	Propidium iodide
PKC	Protein kinase C
PNH	Paroxysmal nocturnal hemoglobinuria
PSGL-I	P-selectin glycoprotein ligand-I
RNA	Ribonucleic acid
RT	Reverse transcription
SCR	Short concensus repeat units
SDS-PAGE	Sodium-dodecyl-sulphate - polyacrylamide gel electrophoresis
sLe^a	Sialyl Lewis^a
sLe^x	Sialyl Lewis^x

SMC	Smooth muscle cell
sTNF	Soluble tumour necrosis factor
sTNF-R	Soluble tumour necrosis factor receptor
TBS	Tris buffered saline
TGF-β	Transforming growth factor-beta
THP-1	Monocytic cell line
TNF-α/β	Tumour necrosis factor-alpha/beta
TNF-R	Tumour necrosis factor-receptor
TPCK	n-Tosyl phe-chloromethylketone
uPA	Urokinase
uPAR	Urokinase receptor
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VMAP-1	Vascular monocyte adhesion molecule-associated protein-1

Chapter One

Introduction

1 Introduction

Atherosclerosis is responsible for nearly one half of deaths in the Western societies (Poston and Tidey, 1996) and is a multifactorial process that starts during childhood and becomes clinically manifest later in life. Although atherosclerotic lesions can be observed throughout the body, certain areas of the arterial tree are particularly prone to the development of lesions, such as the aortic arch, the branching sites of the larger vessels and the carotid and coronary arteries.

Histologically atherosclerotic lesions are characterised by the proliferation of intimal cells of the arterial wall, the accumulation of lipids and the deposition of extracellular matrix components. The general scientific concensus is that the precursor stage of atherosclerotic plaques are the fatty streaks which are macroscopically distinguishable, cushion like, whitish, subendothelial intimal areas that mainly comprise foam cells. Fatty streaks may either regress or progress to more severe stages of the disease (Wick et al,1995).

Today there are two main hypotheses regarding the pathogenesis of atherosclerosis: the so called 'response to injury' hypothesis, and the 'modified low density lipoprotein (LDL)' hypothesis. In the former, primary injury of the arterial endothelium occurs in response to a variety of factors such as hypercholesterolemia, mechanical stress caused by high blood pressure, smoking, immune reactions, toxins and viruses. Injured endothelial cells produce and secrete chemotactic factors inducing the local accumulation of platelets and monocytes. Monocytes migrate into the subendothelial areas of the intima and form fatty streaks by lipid uptake and transform into foam cells (Ross, 1993). The second theory postulates that modified (oxidised) LDL is taken up by endothelial cells, macrophages and smooth muscle cells, all of

which possess the so called LDL-scavenger receptor that exclusively binds modified LDL. LDL can be modified either in the serum or during the passage through the endothelium. In the subendothelial space, modified LDL can act as a chemoattractant for monocytes that adhere to the endothelium, migrate into the subendothelial space and become macrophages that transform into foam cells by uptake of altered LDL (Steinberg and Witztum, 1990).

1.1 Pathogenesis of atherosclerosis

Plaques which comprise mainly lipid and connective tissue matrix proteins begin to form in early life and by the third decade, advanced plaques are almost ubiquitious in Western populations (Davies and Woolf, 1993). The onset of clinical symptoms in a hitherto symptomless patient is often related to the development of thrombotic complications in association with plaques.

The first atherosclerotic lesions that appear are fatty dots or streaks barely raised above the intimal surface. Each lesion is made up of a focal collection of lipid filled foam cells within the intima. Progression beyond the fatty streak stage is associated with a sequence of changes starting with the appearance of extracellular lipid which begins to form a core to a lesion that is becoming more elevated. Recruitment of monocytes into these lesion susceptible sites is mediated by the production of chemotactic factors in these areas. Intimal monocytes recruited into the vessel wall are rapidly transformed into lipid laden foam cells by the unregulated uptake of modified lipoproteins via the scavenger and oxidised LDL receptor pathways (Gerrity and Antonov, 1997). Their increase in size as they accumulate lipid is the major contributor to increased lesion volume during the development of fatty lesions. Smooth muscle cells migrate into and proliferate within the plaque in response to mitogens released by foam cells/macrophages and by platelets adherent to the sites of endothelial

damage and form a layer over the luminal side of the lipid core. PDGF, which is chemotactic for smooth muscle cells is released by macrophages and may also take part in this latter process. These smooth muscle cells are responsible for the production and deposition of collagen, leading to increase in plaque size (Massy and Keane, 1996). This process culminates in what is known as an advanced plaque (Figure 1.1).

Endothelial cell damage occurs at sites where foam cells migrate or are in close proximity to the endothelium, and such sites are a foci for platelet adherence. Endothelial cell damage at these sites may be associated with excessive stretching of the endothelium by plaque growth, enhanced membrane fragility due to altered lipid composition, release of lytic enzymes by macrophages, or toxicity due to oxidation of intimal lipoproteins by the macrophages themselves (Gerrity and Antonov, 1997). The plaques have a core of extracellular lipid separated from the lumen by a thick cap of collagen rich fibrous tissue containing smooth muscle cells. Surrounding the lipid core are lipid filled foam cells. Much of the core lipid is thought to be derived from the death of lipid containing macrophage foam cells and the release of their intracytoplasmic contents. Once an advanced plaque is established endothelial denudation injury occurs, with exposure of subendothelial matrix and localised adhesion of platelets. As the plaque protrudes into the lumen, together with increased fragility of endothelial cell membranes there is episodic plaque rupture and/or endothelial denudation resulting in focal fibrin-platelet thrombi which may become incorporated into the plaque volume (Davies and Woolf, 1993).

This subsequent fibrous plaque growth combined with rupture and thrombosis ultimately results in clinically significant occlusion.

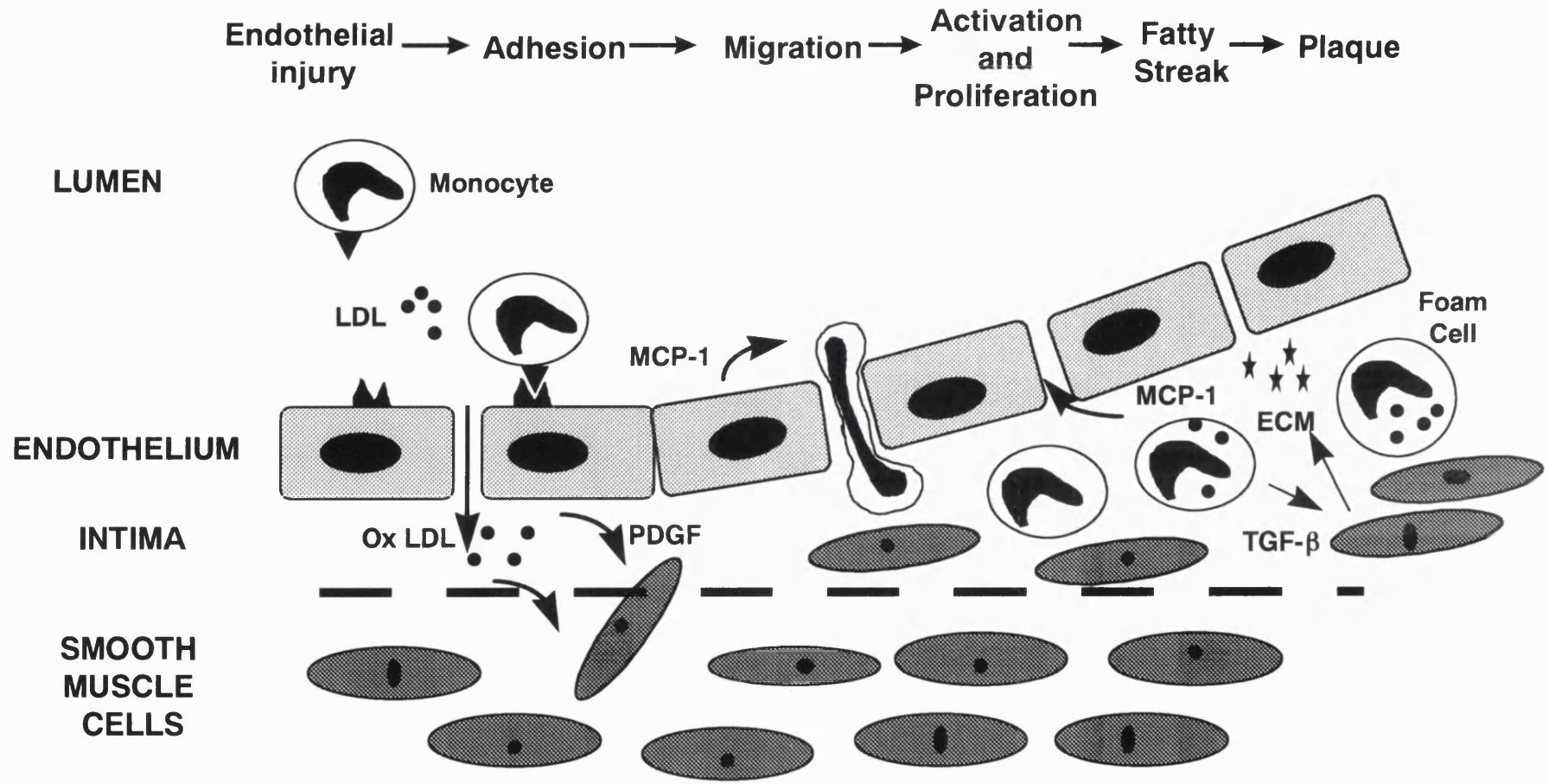

Figure 1.1 Schematic stages of development of atherosclerosis. Abbreviations: LDL=low density lipoproteins; Ox=oxidised; MCP-1=monocyte chemoattractant protein-1; PDGF=platelet derived growth factor; TGF-β=transforming growth factor-β; ECM=extracellular matrix proteins.

The early stages of atherosclerosis share many features with inflammatory disease. The atherosclerotic lesions contain an immune-mediated inflammatory reaction and consequently it has become important to understand the mechanisms that cause localisation of monocytes and T cells in the arterial wall, including most importantly the contribution of alterations in the function and behaviour of endothelial cells.

1.2 Vascular Endothelium

The unique position of the endothelium at the interface between the blood and the vessel wall confers upon it multiple functions. It is involved in the regulation of coagulation and fibrinolysis, leukocyte recruitment, vessel tone and vascular smooth muscle cell growth and also acts as a barrier to transvascular flux of liquids and solutes. Far from being a passive participant in these events it is a dynamic tissue, secreting and modifying vasoactive substances, influencing the behaviour of other cell types and regulating extracellular matrix production and composition (Stary et al, 1992). During the past twenty years the endothelium has been the focus of intense research, resulting in the evolution of a new appreciation of its potential role in vascular disease.

In 1628 William Harvey presented the first description of circulating blood, and studies by Malphigi soon after identified the existance of a network of vessels showing the physical separation between blood and tissues (Fishman et al, 1982). The idea that vessels were merely tunnels bored through tissues was quashed in the 1800s by von Reckingausen who established that vessels were lined by cells. In the late 1800s work by Starling who proposed his law of capillary exchange seemed to confirm the belief that the endothelium was principally a selective but static physical barrier, despite Heidenhahn's description in 1891 of the endothelium as an active secretory cell system

(Fishman et al, 1982). Electron microscopic studies of the vessel wall by Palade in 1953 and physiological studies by Gowans in 1959 describing the interaction between lymphocytes and endothelium of post capillary venules stimulated numerous subsequent studies that led to the current view of the endothelium as a dynamic, heterogenous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions (Fishman et al, 1982). Detailed studies of endothelial function first became feasible with the development in the 1970s of techniques to culture endothelial cells *in vitro* (Jaffe et al, 1973). Limitations of this approach have become apparent recently with the realization that cell culture perturbs endothelial cells from their quiescent *in vivo* state (0.1% replications per day) to an activated phenotype (1% to 10% replications per day) with loss of specialised functions associated with diverse vessels and organ systems (Cines et al, 1998). Most *in vitro* studies however are performed using endothelial cells originating from various tissue sources and grown up in culture.

The endothelial cell surface in an adult human is composed of approximately 1-6 x 10^{13} cells, weighs approximately 1 kg, and covers a surface area of approximately 1 to 7 m^2 (Augustin et al, 1994). Endothelial cells grow as a monolayer in normal vasculature and have a slow turnover rate. Endothelial cells line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules, and the blood cells themselves. This gate keeping role of endothelium is effected through the presence of membrane-bound receptors for numerous molecules including proteins, lipid transport particles, metabolites, and hormones, as well as through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions. The endothelium also plays an active role in the regulation

of vascular tone, platelet adhesion and aggregation, local coagulation events, vascular growth and immune processes (Glasser et al, 1996).

The endothelium also plays a pivotal role in regulating blood flow. In part, this results from the capacity of quiesent endothelial cells to generate an active antithrombotic surface that facilitates transit of plasma and cellular constituents throughout the vasculature. Perturbations such as that may occur at sites of inflammation or high hydrodynamic shear stress disrupt these activities and induce endothelial cells to create a prothrombotic and anti fibrinolytic microenvironment. Blood flow is also regulated in part through the secretion and uptake of vasoactive substances by the endothelium that act in a paracrine manner to constrict and dilate specific vascular beds in response to stimuli such as endotoxin (Shah, 1992). The current belief is that it is important that the endothelium remains in a resting or unperturbed state to optimise expression of anticoagulant activities which prevent thrombus formation. Normal endothelium functions in an inhibitory mode; it inhibits smooth muscle contraction, platelet aggregation, vascular smooth muscle growth, thrombosis, and leukocyte adhesion (Glasser et al, 1996).

It is now clear that the endothelium helps to coordinate functions of differentiated tissues in a way that meets the requirements of the organism as a whole. In part this is accomplished by the location of the endothelium at the interface with the blood and the capacity of these specialised cells to receive and transmit biochemical and physical information bidirectionally. Information sensed on the luminal surface of the endothelium can be transmitted either by direct permeation or active transport of soluble mediators through the capillaries to deeper tissue or indirectly through the capacity of endothelial cells to modulate the behaviour of smooth muscle cells and other components of the

vessel wall. In turn physiologic and pathophysiologic events in tissue alter endothelial cell interactions with soluble and cellular blood components.

The endothelium as with all cell types displays an immediate and prototypic response to diverse agonists that is modulated in complex ways by subsequent events. In the case of the endothelium this first response appears designed to prevent physical disruption of the vessel wall by trauma, microbial organisms, toxins or other threats to the maintanance of intravascular volume and oxygen delivery. This protective response is accomplished by the rapid transformation of the endothelium to a procoagulant, vasoconstrictive and proinflammatory state that has multiple effects on its structure and behaviour. An extensive experimental literature has emerged supporting the notion that several common human vascular diseases are in part a consequence of the same responses of the endothelium to stress i.e. that prolonged or exaggerated endothelial activation leads to dysfunction that is an early and often preclinical component of vascular disease. However since it is near impossible to collect tissue directly from the area the endothelial cell contributing to disease development can only be inferred.

1.3 Endothelial cells and Atherosclerosis

Our understanding of the role of endothelial cell dysfunction in the pathophysiology of atherosclerosis continues to evolve. Several factors, such as hyperlipidemia, hypertension, diabetes mellitus, smoking, hyperfibrinogenemia, hyperhomocysteinemia, herpes virus infections or immune mechanisms may induce endothelial cell injury, producing alterations in behaviour and function which represent the earliest manifestation of endothelial cell dysfunction (Massy and Keane,1996). Impairment of endothelial cell function exists even before the histological findings of atherosclerosis. Injured

endothelial cells appear morphologically different from normal endothelial cells, for example, they are typically not aligned in the direction of blood flow, and they have fewer intercellular attachments, resulting in increased permeability (Consigny, 1995).

The concept that atherosclerosis arises in response to endothelial injury was first proposed more than 20 years ago, when it was appreciated that irregularities in endothelial cell organisation are often found overlying early fatty streaks, whereas overt endothelial denudation is seen only in the late stages of the disease. There is now extensive evidence that this morphologically abnormal endothelium is also dysfunctional and actually contributes to the propagation of lesions (Cines et al, 1998).

1.4 Monocytes

Monocytes appear in the foetal circulation at approximately the fifth month of gestation and increase progressively during the third trimester when the bone marrow becomes the prominent haematopoietic organ (Mills, 1983). Monocytes develop from monoblasts through promonocytes in the bone marrow, and then develop into circulating monocytes. In culture, the development of the monocytic lineage occurs when the cells are exposed to cytokines or colony stimulating factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) (Gabrilove, 1989). GM-CSF is produced by T cells, endothelial cells, and fibroblasts, whilst M-CSF is also produced by endothelial cells and fibroblasts as well as by monocytes.

The monocyte half-life in the circulation is estimated to be 24 hours which is relatively long compared to other leukocytes, such as neutrophils that survive for 6 hours. The circulating monocytes are 5-10μm in diameter, with a kidney

shaped or folded nucleus, and when stained with MGG stain, a grey-blue cytoplasm. A further feature is the presence of a fluoride sensitive non-specific esterase (Douglas and Musson, 1986). The function of the monocyte is to phagocytose particles, opsonised bacteria and other antibody, and complement-coated organisms.

Approximately 75 percent of the circulating monocyte pool marginates along blood vessels. The monocytes then randomly migrate into the tissues where they become antigen presenting cells or phagocytic macrophages of a variety of types. Tissues that accommodate macrophages include skin, liver, lung, brain and kidney in addition to bone marrow and lymphoid tissue. The phagocytic macrophages include alveolar macrophages of the lung and Kupffer cells of the liver. Langerhans cells of the skin are antigen presenting cells (Hoffbrand and Pettit, 1988). The macrophages retain the ability for chemotaxis and have full capacity for phagocytosis whilst the antigen presenting cells are unable to phagocytose. These monocyte derived macrophages are larger than monocytes, and release a variety of intercellular messengers or cytokines (van Furth et al, 1979).

Monocytes and their precursors have Fc and C3b receptors on their surface, and these mediate the phagocytosis of microorganisms and cells which have become coated with complement.

As well as being producers of energy, providing it in the form of adenosine triphosphate (ATP); being phagocytic and providing bacteriocidal activity and antigen presentation, monocytes/macrophages also synthesise and secrete certain macromolecules (West et al, 1968). These functions occur in response to either particulate or soluble stimuli and are often activated by the interaction of these cells with endotoxin. The synthesis and secretion of products such as

lysozyme and lipoprotein lipase occur constitutively and may be part of the metamorphosis of monocytes to macrophages (Gordon et al, 1974). The secreted macromolecules are involved in proteolysis, inflammation and immunomodulation, hematopoiesis, coagulation, and cell adhesion.

The protein and peptide factors secreted by monocytes/macrophages are called monokines and they act on other cells to produce effects. The monokines released include interleukins, interferons, tumour necrosis factor, colony stimulating factors, chemokines and transforming growth factors. Many of these are produced in response to the lipid A component of lipopolysaccharide, or to cytokines synthesised by other cells (Fibbe et al, 1986). The process is calcium dependent (Simon, 1984) and requires the synthesis and increased stability of specific mRNAs (Beutler et al, 1986), protein synthesis, processing and secretion (Burchett et al, 1988). These monokines have interrelated functions that affect many processes, such as endothelial cell function, cell growth, protein synthesis, cell movement, cell-cell interactions and secondary cytokine synthesis .

1.4.1 Tissue Factor

A prominant function of activated monocytes is their ability to promote blood clot formation by virtue of the expression of tissue factor (TF) on cell surface. TF is a transmembrane glycoprotein (m.wt 47,000) and it is a high affinity receptor for coagulation factors VII and VIIa (Edgington et al, 1991). The resulting VIIa-TF complex rapidly catalyzes the conversion of factor X to Xa and IX to IXa, leading to formation of thrombin. Monocyte-derived TF can facilitate both intrinsic and extrinsic coagulation pathways, and it plays a central role in the initiation of coagulation protease cascades (Nemerson et al, 1988). Quiescent monocytes and endothelial cells are not endowed with TF activity,

however, various stimuli, including bacterial LPS, phorbol esters, C5a, and cytokines increase monocyte TF generation (Furie and Furie, 1992), and LPS, thrombin, IL-1β and TNF increase endothelial TF expression. TF-producing cells have been identified in atherosclerotic plaques, thus suggesting a role for TF in the prothrombotic state associated to atherosclerotic plaques (Wilcox et al, 1989). Monocyte-endothelial cell interactions play a key role in the inflammatory response, thrombosis and development of atherosclerotic lesions. Such a close apposition might be predicted to result in modulation of several biological functions by both cell types. In fact, monocytes adherent to cytokine-activated endothelial cells are stimulated to express increased TF, partially through Le^x binding, and the induction is rapid, peaking at 30 minutes and persisting up to 4 hours (Lo et al, 1995). Also, monocytes can upregulate endothelial cell expression of TF and the cytokines, IL-1β and TNF-α, are the mediators responsible for the activity (Napoleone et al, 1997). Furthermore, direct cell-cell contact between monocytes and endothelial cells can directly enhance plasminogen activator inhibitor (PAI-1) release by endothelial cells. The monocyte mediated increase was first detected at 12 hours post coculture and lasted for at least 48 hours (Hakkert et al. 1990). PAI-1 is an important physiological inhibitor of the fibrinolytic system, and protects the subcellular matrices from damage by cellular proteases released during inflammation, wound healing and thrombosis. However, monocytes caused PAI-1 release from the lumenal and subendothelial side of the endothelium, and thus monocytes accumulated underneath endothelial cell monolayers during the development of atherosclerosis may contribute to the local deposition of fibrin through enhancing PAI-1 release (Hakkert et al, 1990).

1.5 Role of Monocytes in Atherosclerosis

Adhesion of moncytes to endothelium is a crucial event in inflammatory processes and in the pathophysiology of atherosclerosis. Under normal conditions, monocytes constitute less than 10% of the circulating leukocytes. Turnover of circulating monocytes is considerable because the half life of monocytes in the circulation is estimated to be 24 hours (Kuijpers and Harlan, 1993). On the other hand, once monocytes have emigrated to the tissue these cells may develop into tissue macrophages with a life span of several months and the monocytes that have emigrated into tissue retain the capacity to divide (Kuijpers and Harlan, 1993).

Monocyte adhesion and migration into the arterial wall followed by differentiation into macrophages are important early events in atherogenesis and follows the accumulation of lipid within the artery wall (Watanabe et al, 1985). This recruitment of monocytes is mediated by selective processes since neutrophils which are initially the predominant leukocyte at an area of inflammation have not been found to be present in the atherosclerotic lesions. Monocytes first adhere to the endothelial cell surface, followed by migration across the endothelium. Endothelial activation increases expression of several adhesion molecules, which mediate leukocyte adhesion to the endothelium. Once adhered to the endothelium monocytes then stretch out, and migrate over the surface of the endothelial monolayer. Recent studies have identified an array of endothelial adhesion molecules which are actively involved in the initial adhesion of monocytes and other leukocytes to the endothelial wall (Faruqi and DiCorleto, 1993; Butcher, 1991).

1.6 Role of Lymphocytes in Atherosclerosis

Lymphocytes also accumulate in the developing atheroma and it is thought that their role is not merely one of being a bystander (Xu et al, 1990). The ongoing

inflammation within atheroma includes an immune component, as the T cells appear chronically activated. The antigens that may stimulate T cells are unknown but possibilities include modified lipoproteins and heat shock proteins (Emeson et al, 1988). These activated T cells release inflammatory cytokines such as gamma interferon, and additionally may interact with monocytes to stimulate the production of further proinflammatory cytokines, chemokines and tissue factor.

1.7 Role of Platelets in Atherosclerosis

Platelets do not directly contribute to plaque formation, however, platelet activation is a feature of atherosclerotic vascular disease. In acute plaque rupture, platelet activation is a key factor in acute vascular occlusion. Platelet microparticles, released from platelets by physiological agonists, contain proteins and lipids, which have pro- and anticoagulant properties. Platelet derived microparticles circulate and accumulate at sites of developing atherosclerosis, where they increase the likelihood of thrombosis and influence other aspects of cellular interactions with the vessel wall. Platelet derived microparticles increase the adhesive interactions between endothelial cells and monocytes via upregulation of monocyte CD11a and CD11b and endothelial cell ICAM-1 (Barry et al, 1998).

1.8 Leukocyte Recruitment at Inflammatory Sites

The recruitment of circulating leukocytes is considered to occur as an adhesion cascade of four sequential steps: tethering, triggering, strong adhesion and migration (Figure 1.2). Tethering causes the circulating cells to slow their flow and roll along the vessel wall. Because leukocytes do not have cilia, they cannot 'swim' to the vessel wall in response to extravascular chemotactic stimuli. Initial contact with the vessel wall then is in large part a random event,

perhaps enhanced by local alterations in flow. Rolling is a phenomenon that is observed only under conditions of flow, and is the result of shear forces acting on the individual leukocyte and an adhesive interaction between the leukocyte and the endothelium (Atherton and Born, 1993). Interestingly, a consistent *in vivo* observation has been that leukocyte rolling seldom occurs along the endothelium of arterioles. Although 39% of leukocytes have been calculated to roll along the endothelium of rat mesenteric venules, only 0.6% rolled along the endothelium of arterioles (Ley and Gaehtgens, 1991). Since atherosclerosis develops in the arteries the low level of rolling but the high levels of monocyte adherence indicates that other factors must exist that encourage this successful interaction between leukocytes and the endothelium in these areas of high flow rates. Triggering factors present on the vessel wall activate cell adhesion molecules such as integrins and this is a crucial step since integrins on circulating leukocytes do not bind well until they are activated. Activated integrins on rolling leukocytes bind to endothelial adhesion molecules, leading to strong adhesion, cessation of movement and flattening of the leukocyte. In the migration step, movement into tissue is directed by local promigratory factors, such as chemoattractants (Carlos and Harlan, 1994).

1.9 Adhesion Molecules

1.9.1 Selectins

The selectin family consists of three closely related cell surface molecules: L-selectin (MEL-14, LAM-1, CD62L), E-selectin (ELAM-1, CD62E), and P-selectin (PADGEM, GMP-140, CD62P). All of the selectins have an unique and characteristic extracellular region composed of an amino-terminal calcium-dependent lectin domain, an epidermal growth factor $(EGF)^2$-like domain, and

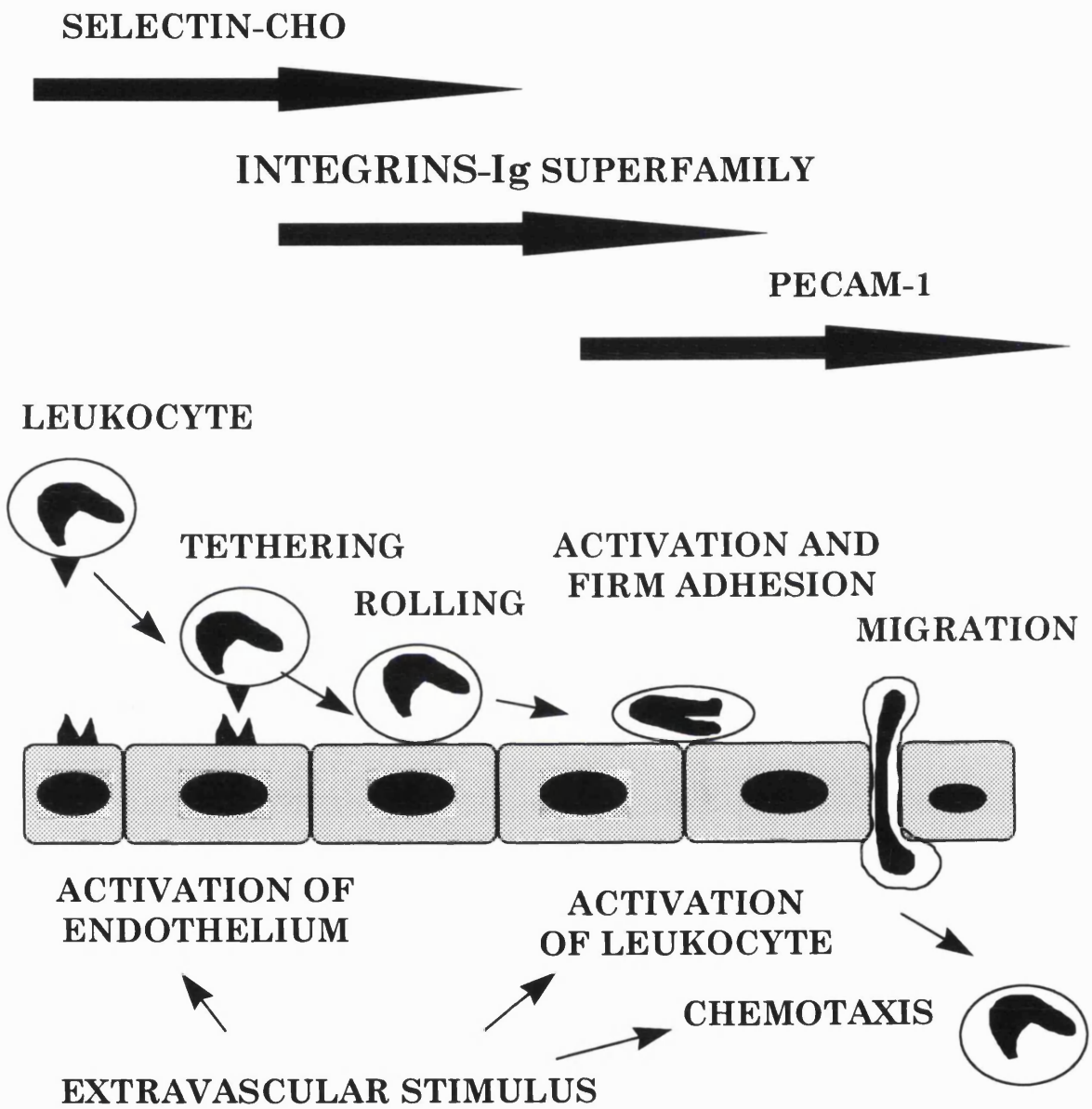

Figure 1.2 Mechanisms of leukocyte adherence to and transmigration across the endothelium

two to nine short concensus repeat (SCR) units homologous to domains found in complement binding proteins. The selectins are the only known example in which these three domains are found in immediate juxtaposition, which suggests that this spatial relationship is important for receptor function (Bevilacqua et al, 1993). Whilst the three domains are distinct modules with little apparent association it has been indicated that EGF and SCR domains may play important roles in cell adhesion because deletion of either domain reduces the receptors ability to mediate cell binding (Tedder et al, 1995). The binding of selectins to requisite ligands requires Ca^{2+}. Optimal binding of selectins to their carbohydrate ligands may require a conformation of the lectin domain that is determined by interactions with Ca^{2+} ions, the adjacent EGF domain, and one or more concensus repeats (McEver et al, 1992). The genes for the selectin family are closely linked on chromosome 1 (q21-24) (Watson et al, 1990).

L-selectin and ligands for P- or E-selectin are localised on the microvilli of leukocytes, where they are likely to first contact the vessel wall. Selectins and their ligands also cluster to enhance binding avidity, project the binding domains above the glycocalyx to faciliate rapid contact, and interact with the cytoskeleton to resist extraction from the membrane.

- **E-selectin**

E-selectin was first described as a 115 kDa antigen that was induced on cultured human umbilical vein endothelium after stimulation by IL-1 (Bevilacqua et al, 1987). Translation of E-selectin yields a core protein of 64 kDa with 11 potential N-glycosylation sites. The 32 amino acid cytoplasmic domain contains tyrosine residues that have been suggested to mediate the internalisation of other transmembrane proteins, and may account for the short half life of E-

selectin at the cell surface (von Asmuth et al, 1992). E-selectin protein production is strongly and rapidly induced by a variety of inflammatory mediators, including IL-1β, TNF-α, interferon-γ, substance P (Smith et al, 1993), and LPS. *In vitro*, E-selectin is present on the surface of human umbilical vein endothelial cells 4-6 hours after activation and declines to basal levels by 24 hours, while *in vivo* it is broadly expressed within the vasculature at sites of inflammation. It is found in arthritic joints, in heart and renal allografts undergoing rejection, and in cutaneous vessels of inflammed skin with psoriasis, contact dermatitus, and delayed type hypersensitivity reactions. A soluble form of E-selectin is found in serum which is elevated in patients with various inflammatory syndromes (Tedder et al, 1995). E-selectin expression requires *de novo* mRNA and protein synthesis. Levels of expression *in vivo* however do not seem to follow the same transient time course as is observed *in vitro*. Possible explanations for persistent expression *in vivo* range from a lack of synchronicity in endothelial cell activation to prolonged expression caused by the local presence of cytokines at the inflammatory site. E-selectin expression *in vitro* may also be in part stimulus dependent. TNF induces a marked initial increase in E-selectin expression on HUVECs but is followed by low level expression for at least 48-72 hours. Finally, the *in vivo* observations depend on immunohistochemical techniques that are not able readily to quantify levels of surface (versus intracellular) antigen expresion (Kuijpers and Harlan, 1993) .

- **P-selectin**

P-selectin is constitutively found in Weibel-Palade bodies of endothelial cells and in alpha granules of platelets (Bonfanti et al, 1989; Steinberg et al, 1985). Within minutes of activation by thrombogenic and inflammatory mediators such as thrombin, histamine, complement fragments, oxygen-derived free radicals,

and cytokines, P-selectin is mobilised to the cell surface (Springer, 1994). Cell surface expression of P-selectin is usually short lived (minutes), which makes it an ideal candidate for mediating early leukocyte-endothelial cell interactions. Initially P-selectin on HUVECs was found to be rapidly and transiently expressed for only 10 to 30 minutes after histamine or thrombin treatment, coinciding with the fusion of Weibel-Palade bodies with the plasma membrane (Hattori et al, 1989). A role for P selectin in later phases of leukocyte adherence, however, is suggested by the recent findings of continued expression of messenger RNA in activated endothelium in mice (Sanders et al, 1992). This increase after treatment with cytokines or LPS showed maximal P-selectin expression at 4 hours after stimulation The importance of P-selectin *in vivo* is supported by experiments in which P-selectin blockade produced prolonged protection against tissue injury induced by ischaemia-reperfusion and lung injury after massive complement activation (Winn et al, 1994; Mulligan et al, 1992). Prolonged expression of P-selectin caused by *de novo* synthesis has also been observed on TNF treated microvascular endothelioma cell lines (Hahne et al, 1993). On the other hand activation of HUVECs with TNF or IL-1 does not induce surface expression of P-selectin. therefore an alternative explanation for the *in vivo* results is that P-selectin still depends on local mediators like thrombin or histamine during inflammatory reactions but in addition, synthesis of new proteins and formation of new Weibel-Palade bodies occurs. The core protein has a predicted molecular weight of 86 kDa. There are twelve potential N-linked glycosylation sites which if fully used would yield a protein of 122kDa. A functionally active soluble form of P-selectin is also found in serum (Johnston et al, 1989).

- **L-selectin**

L-selectin is found only on leukocytes and most classes of leukocytes constitutively express L-selectin at some stage of differentiation. L-selectin is expressed continously throughout myeloid differentiation and is expressed by most circulating neutrophils, monocytes, and eosinophils. L-selectin is concentrated on the microvillus projections of unstimulated neutrophils, an optimal location to enable early contact with cognate endothelial ligand. Optimal L-selectin function involves a change in receptor affinity after cellular activation and requires an intact cytoplasmic domain. The sequence of human L-selectin encodes a core protein of 37 kDa that has eight possible sites for glycosylation. The molecular weight of L-selectin differs among lymphocytes (75kDa), neutrophils (95 to 105 kDa) and monocytes (110kDa) and there is abundant evidence that leukocyte activation leads to L-selectin shedding due to preteolytic cleavage near the membrane insertion (Carlos et al, 1994). Circulating L-selectin may modulate leukocyte adhesion to endothelium during inflammation since the soluble form may fill a certain percentage of the binding sites for L-selectin on endothelium (Gearing and Newman, 1993).

- **Counter-structures for selectins**

All 3 selectins can bind to sialylated, fucosylated glycoconjungates. E- and P-selectin can bind to multivalent forms of the tetrasaccharide, sialyl Lewis^x and its isomer sialyl Lewis^a. sLe^x is expressed on glycoproteins and glycolipids of myeloid cells. L-selectin, whose ligand is on endothelial cells, rather than on leukocytes, also binds to multivalent forms of sLe^x and sLe^a (Foxall et al, 1992). Furthermore, lymphocytes that express ligands for E- and P-selectin do not display epitopes recognised by antibodies to sLe^x and sLe^a. Thus while all 3 selectins bind to sialylated, fucosylated glycoconjugates, interaction with higher

affinity may require ligands with additional structural features or specific orientations. A particular selectin might bind to different preferred ligands, depending on the target cell. Modifications of a basic structure could also generate distinct ligand specificities for each selectin.

The higher affinity binding of selectins to specific glycoprotein ligands suggests that such recognition plays a critical role in at least some cell-cell interactions. L- and P-selectin binding may depend to a certain extent on sulfation of the oligosaccharides involved in cell adhesion. Fucosylation is essential for the formation of critical recognition epitopes as determined by the existence of a disease state (LADII) where a general defect in fucose metabolism leads to the absence of fucose containing oligosaccharide determinants. Leukocytes from these patients fail to bind to E-selectin on activated endothelial cells and a reduction in rolling occurs along inflamed vessels. Sulfation is also required for GlyCAM-1 and CD34 to bind L-selectin (Hemmerich et al, 1994).

The carbohydrate ligands for L- and P-selectins are *O*-linked to specific mucin-like molecules. Mucins are serine and threonine rich proteins that are heavily *O*-glycosylated and have an extended structure. The mucin-like P-selectin glycoprotein ligand, PSGL-1, isolated from human neutrophils has *O*-linked glycans extended with poly-*n*-acetyllactosamine, some of which terminate in sLe^x. These mucin-like glycoproteins are considered to act as scaffolds for clustering of oligosaccharides. The selectin binds with low affinity to the individual glycans but with high avidity to the clustered glycans. However, clustering of glycans is less likely to provide the basis for E-selectin binding to ESL-1, which has not been shown to have *O*-linked carbohydrate, and has only five sites for attachment of *N*-linked oligosaccharides on an extracellular domain. Alternatively, the protein may acquire unique glycosylation or other

post-translational modifications. P-selectin interacts with a specific region of PSGL-1 and this region must have features that distinguish it from other *O*-glycosylated portions of the protein and from other mucin like proteins on leukocytes that do not bind P-selectin. Some glycosyltransferases modify only a subset of optimally presented oligosaccharides. In addition, PSGL-1 is expressed on all lymphocytes, but only a few of these cells bind P-selectin, suggesting that the glycosylation of PSGL-1 is highly regulated. Furthermore PSGL-1 is recognised by E-selectin, thus one glycoprotein can be recognised by two selectins but in different ways (Li et al, 1996). In addition, several leukocyte surface structures modified by sLe^x, Le^x or related structures have been reported to bind to E-selectin, including L-selectin (Zollner et al, 1997), CD66 and β2 integrins. Finally, engagement of Lewis X antigen (CD15) by its natural ligands can result in the activation of a variety of macrophage responses such as induction of TNF-α release and increased IL-1β mRNA (Lo et al, 1997).

1.9.2 Integrins

Integrins are transmembrane cell surface proteins that bind to cytoskeletal proteins and communicate extracellular signals. Each integrin consists of a noncovalently linked, heterodimeric α and β chains. To date, 8 known β chain subunits have been molecularly cloned. Integrins have been arranged in subfamilies according to the β subunits and each β subunit may have from one to eight different α subunits associated with it (Smyth et al, 1993). As many as 21 different integrin combinations have been reported since individual α subunits may be associated with several different β subunits too (Smyth et al, 1993). Integrins are thought to be the most versatile of the adhesion molecules. Integrin adhesiveness can be rapidly regulated by the cells on which they are

expressed. The transient nature of activation of integrin adhesiveness provides a mechanism for de-adhesion and perhaps for retraction of the trailing edge of the leukocyte from the substrate during cell migration. Integrins rely on divalent cation-binding however divalent cation specificity differs among the various α chains, with CD11a/CD18 requiring magnesium whilst calcium is necessary for VLA-5 function. The β chain appears to determine tertiary structure of the molecule whilst the cytoplasmic domain of the β chain in concert with the α subunit is also necessary for avidity modulation. The I domain of leukocyte integrins is important in ligand binding and mutations in these domains block ligand binding function (Schwartz et al, 1995).

Five integrins are important in the interaction of leukocytes with endothelial cells (Table 1.1)(Springer, 1994).

- **β2 integrins**

β2 integrins share a common β chain (CD18) to which the three α subunits are non covantly associated with (Arnaout et al, 1990). The expression of the β2 integrins are restricted to leukocytes, but among the subtypes of leukocytes the distribution of CD11/CD18 differs (Table 1.1). Intracellular storage pools of CD11b/CD18 and CD11c/CD18 are present in neutrophils and monocytes but there is no storage pool of CD11a/CD18. Activation of leukocytes provokes rapid translocation of CD11b/CD18 and CD11c/CD18 from intracellular granules to the plasma membrane. However quantitative changes in leukocyte adhesion molecule expression are less important than qualitative alterations in function. Surface expression of CD11b/CD18 and CD11c/CD18 is increased by a variety of agonists: calcium ionophore, phorbol esters, FMLP, GM-CSF, C5a, and TNF-α (Carlos and Harlan, 1990). In addition activation of the β2 integrin receptors increases their avidity for their counterreceptors. Qualitative changes

in adhesion receptor avidity play a critical role in leukocyte adhesion to endothelium or matrix components. Integrin receptors on circulating leukocytes are normally in an inactive or low avidity state in that they do not bind, or bind only minimally, to their endothelial ligands. This ability of the integrin receptor to transform rapidly from a low avidity to a high avidity state allows leukocytes to circulate freely but then stick firmly at sites of inflammation. It is equally important for leukocytes to modulate integrin receptors from the high to the low avidity states since retaining receptors in a high avidity state prevents migration (Figdor et al, 1990). In the case of phagocytes, integrins are likely to be induced to the high avidity state on cellular activation by chemoattractants or cytokines, for example the adhesiveness of Mac-1 and LFA-1 on neutrophils and monocytes is activated by N-formylated peptide and IL-8. This increased adhesiveness is due to a conformational change in the integrins upon activation rather than due to the increased expression on the cell surface that does also happen.

The importance of the β2 integrin receptors in leukocyte emigration is evidenced by the failure of leukocytes to accumulate in inflammatory/infective foci in patients with LAD type I. LAD type 1 syndrome is characterised by heterogenous mutations in the β2 (CD18) subunit that results in deficient expression of all 3 heterodimers (Bowen et al, 1982; Kishimoto, 1987).

Ligands for the β2 integrins include proteins expressed by cells and soluble proteins such as fibrinogen, factor X and complement fragments (Table 1.1).

- **β1 integrins**

The β1 integrins share CD29 as their common β subunit (Hemler, 1990). This widely distributed family of integrins contains a series of cellular receptors for extracellular matrix proteins including fibronectin, collagen, laminin and

FAMILY	INTEGRIN	INTEGRIN NOMENCLATURE	CD NOMENCLATURE	EXPRESSED BY	ENDOTHELIAL CELL COUNTER RECEPTOR
β2	LFA-1	αLβ2	CD11a/CD18	All leukocytes	ICAM-1 ICAM-2
	Mac-1	αMβ2	CD11b/CD18	Neutrophils, Monocytes, Eosinophils	ICAM-1 Others?
	p150,95	αXβ2	CD11c/CD18	Neutrophils, Monocytes, Eosinophils	?
β1	VLA-4	α4β1	CD49d/CD29	Monocytes, Eosinophils, Lymphocytes	VCAM-1 Fibronectin Thrombospondin Others?
	VLA-5	α5β1	CD49e/CD29	Monocytes	Fibronectin
β7	L-PAM-1?	α4β7	CD49d/CD-	Lymphocyte subsets	VCAM-1 Fibronectin Others?

Table 1.1 Integrins involved in leukocyte:endothelial cell interactions

vitronectin. Monocytes differ importantly from neutrophils in that they express significant levels of β1 (VLA) integrin receptors including VLA-2, VLA-4, VLA-5 and VLA-6 (Hemler, 1990). VLA-4 mediated monocyte adherence to endothelial VCAM-1 may represent an alternative CD18-independent pathway for monocyte emigration.

• **Integrin Counterstructures**

VCAM-1 and ICAM-1 -2, are members of the immunoglobulin supergene family and are present on endothelial cells. ICAM-1 is also expressed on leukocytes, fibroblasts and epithelial cells. These transmembrane proteins interact with the β1 and β2 integrin family expressed on leukocytes. ICAM-2 is expressed constitutively by endothelial cells and therefore mediates binding to resting endothelium. It is not subject to upregulation by cytokines (TNF, IL-1 or IFN) or LPS (Nortamo et al, 1991; de Fougerolles et al, 1993). ICAM-1 is expressed at low levels constitutively but can be induced by cytokines and predominates in binding to inflamed endothelium. Endothelial expression of ICAM-1 *in vitro* peaks at 12 hours post stimulation, and the protein persists for at least 72 hours before returning to baseline levels. VCAM-1 expression occurs only after specific stimulation with proinflammatory cytokines and the expression peaks at 12 hours. The protein persists for up to 72 hours before the levels return to baseline. Both ICAM-1 and VCAM are induced by TNF-α, IL-1, thrombin and LPS. However there are both common and specific pathways of induction of endothelial adhesion proteins since ICAM-1 is also upregulated by phorbol esters, hypoxia/reoxygenation, oxygen radicals, TNF-β, and IFN-γ. These agonists do not induce VCAM-1 expression but IL-4 does. The induced expression of ICAM-1 and VCAM-1 is largely dependent on synthesis of new

mRNA and protein becuse there are no storage forms of these adhesion molecules.

1.9.3 CD14-A monocyte specific mechanism of adherence

A potential monocyte specific mechanism of adherence to endothelial cells has recently been suggested to occur via the CD14 antigen, a glycosylphosph-atidylinositol lipid-anchored glycoprotein highly expressed on monocytes and, to a much less extent on neutrophils (Beekhuizen et al, 1991). It functions as a receptor for LPS complexed with LPS-binding protein, an acute phase protein, However recent work has shown that by blocking CD14 with mAbs there has been inhibition of monocyte adherence to TNF, IFN-γ and IL-1 stimulated human umbilical artery-derived and vein-derived endothelial cells. This inhibition was specific to monocytes since neutrophil adhesion was unaffected (Beekhuizen et al, 1991). The nature of the induced endothelial counterstructure for CD14 is unknown. Cross linking of CD14 by mAbs has recently been shown to activate monocytes, resulting in CD11/CD18-dependent adherence (Beekhuizen et al, 1993). This observation raises the possibility that interaction of monocyte CD14 with its endothelial ligand triggers subsequent β2 integrin-dependent binding of monocytes to endothelial ICAM-1. Furthermore, blood from paroxysmal nocturnal hemoglobinuria (PNH) patients, whose leukocytes lack all glycosyl-phosphatidylinositol (GPI) anchored proteins including CD14, does not amplify the endothelial response to LPS, and do not enhance β2 integrin dependent binding of monocytes (Pugin et al, 1993).

1.9.4 uPAR

The leukocyte integrin CD11b/CD18 and the urokinase receptor (uPAR, CD87) mediate complementary functions in monocytic cells. The urokinase receptor (uPAR), a highly glycosylated 55 to 60 kDa protein, is anchored to the cell

surface via a glycosylphosphatidylinositol (GPI) moiety and is made up of three homologous domains. uPA, which binds to the amino terminal domain of uPAR, greatly potentiates plasmin generation and localises proteolytic activity to the cell surface, especially important in the setting of plasminogen activator inhibitor types 1 and 2 (PAI-1 and PAI-2) (Blasi, 1997). The uPA system also affects adhesion: cells expressing uPAR bind to, adhere and spread on vitronectin in an RGD-independent way, due to a direct interaction between uPAR and vitronectin. uPAR also changes the substrate specificity of cell adhesion by favouring adhesion to vitronectin and inhibiting cell binding to fibronectin or fibrinogen. This is due to the direct interaction of uPAR with cytoskeletal-engaged integrins (Simon et al, 1996; Sitrin et al, 1996). Thus, under conditions where relatively large amounts of fibrinolytic activity exist, CD11b/CD18 function appears to be downregulated. Conversely, under conditions, where relatively little plasmin is being generated by uPAR, the uPAR functions to promote CD11b/CD18 activity (Wei et al, 1996). In addition, β2 integrin-mediated leukocyte-endothelial cell interactions and recruitment to inflamed areas require the presence of uPAR (May et al, 1998). Finally, neutrophils from PNH patients deficient in uPAR, are impaired in their transendothelial migration *in vitro* (Pedersen et al, 1996).

1.9.5 Other Adhesion Molecules

More work is needed to identify the molecules required for monocyte recruitment associated with early lesion formation. A novel vascular monocyte adhesion molecule-associated protein, VMAP-1, that plays a role in adhesion of monocytes to activated endothelium has recently been identified (McEvoy et al, 1997). This molecule appears to be important in the binding of monocytes to aortic endothelial cells stimulated with minimally modified low density

lipoprotein, and is found focally constitutively expressed in aorta and other large vessels. VMAP-1 has a sustained superinduced expression compared to the other well known adhesion molecules such as P-selectin.

1.10 Cytokines and Atherosclerosis

Vascular cells are both a target for and a source of cytokines. These soluble polypeptide mediators serve to communicate with leukocytes as well as with other tissues and organs. Proinflammatory cytokines such as TNF-α and IL-1 are involved in the triggering of the immune response, induction of acute inflammatory events, and transition to, or persistence of, chronic inflammation. The action of proinflammatory cytokines is regulated by their receptor expression, downregulation and shedding, by specific inhibitors, and by inhibitory cytokines such as IL-10 and IL-4.

1.10.1 TNF

Tumour necrosis factor (TNF) is a pleiotropic cytokine that is primarily produced by activated macrophages and lymphocytes, but is also expressed in other cell types. TNF is a major mediator of inflammatory, immunological, and pathophysiological reactions. TNF is produced by macrophages, endothelial cells and smooth muscle cells in atheromatous vessels and participates in endothelial cell recruitment of monocytes by inducing surface adhesion molecule expression (Barath et al, 1990). TNF has been shown to stimulate angiogenesis and to alter the endothelial cell responsiveness. Also, TNF stimulates human vascular endothelial cells to release neutrophil chemotactic factors thus promoting the transendothelial neutrophil influx (Moser et al, 1988; Strieter et al, 1988). In addition TNF is a chemotaxin for lymphocytes and TNF released by comigrating monocytes promotes transendothelial migration of activated lymphocytes (Green et al,

1998). Furthermore, TNF increases the expression of class I major histocompatibility complex (MHC) antigens on the endothelium surface and thus increases its immunogenicity (Gamble et al, 1985; Pohlman et al, 1986). It also increases the production of procoagulants and decreases the production of thrombomodulin, thus converting vascular endothelium to a procoagulant surface (Stern and Nawaroth, 1986; Bevilacque et al, 1986).

Other factors produced in response to TNF include mitogens, growth factors and other inflammatory mediators, for example, platelet derived growth factor (Haijer et al, 1987), platelet activating factor (Camussi et al, 1987), prostacyclin (Kawakami et al, 1986) and haemotopoietic growth factors including G-CSF, and GM-CSF(Broudy et al, 1987; Munker et al, 1986). TNF also induces IL-1 synthesis by cells of the monocyte/macrophage lineage (Bachwich et al, 1986). TNF may also stimulate smooth muscle cell proliferation. In addition it is responsible for the development of neovasculasation within the plaques (Barath et al, 1990).

Two distinct species of the TNF molecule exist: the 26kDa membrane expressed form of TNF (mTNF), and the soluble 17 kDa cytokine (sTNF) which is derived from proteolytic cleavage of the 26 kDa membrane form (Grell et al, 1995).

TNF acts on target cells via the binding to two transmembrane receptors of molecular weight of 55-60 kDa (TNF-R_{60}) and 70-80 kDa (TNF-R_{80}) (Bradley et al, 1995). Most cell types and tissues express both TNF receptor types which transduce different signals. In addition, soluble TNF receptors (sTNF-R) exist as a result of TNF-R shedding, and are able to interfere with TNF activity by impairing its interaction with the target cell.

1.10.2 IL-1

IL-1 has been implicated as a regulatory protein in the development and clinical sequelae of atherosclerosis. IL-1 is secreted by endothelial cells and macrophages in atherosclerotic lesions (Galea et al, 1996). The predominant cells in the monkey atherosclerotic plaque expressing IL-1α and β mRNA are foam cells in the intima, and the microvascular endothelium. Adherent leukocytes and vascular smooth muscle cells also express IL-1α mRNA and those cells expressing IL-1 mRNA frequently express IL-1 protein (Moyer et al, 1991). In addition, serum IL-1β concentrations are raised in patients with ischaemic heart disease (Hasdai et al, 1996).

IL-1 is the term for two polypeptides (IL-1α and IL-1β) that possess a wide spectrum of immunologic and nonimmunologic activities and IL-1β is the prominent form of IL-1. IL-1 lacks a clear signal peptide, and thus a considerable amount of the IL-1 that is synthesised remains cell associated and is biologically active (Dinarello, 1989), however the amount that is secreted depends upon the cell type and the conditions of stimulation. The monocyte/macrophage appears to be the cell best equipped to secrete IL-1. Endothelial cells and smooth muscle cells secrete IL-1 after stimulation with various cytokines. In the absence of *in vitro* or *in vivo* stimulation, the IL-1 genes are not expressed. Diverse inducers including LPS, complement components, cytokines such as TNF, IFN-γ, GM-CSF and IL-1 itself promote gene transcription.

IL-1 acts on endothelial cells to promote the expression of procoagulant molecules such as tissue factor, and to inhibit fibrinolysis. IL-1 also increases production of PAI-1 and all of these events *in vivo* lead to activation of thrombin in the initiation of clotting, with a decrease in blood flow in vessels and increase

in accumulation of leukocytes and platelets. Furthermore, IL-1 activates endothelial cells to synthesise and release $PGI_{2,}$, PGE_2, and PAF. However, in monocytes, IL-1 production is inhibited by the addition of $PGE_{2,}$; this inhibition acts as a negative feedback limiting IL-1 production (Kunkel et al, 1986). IL-1 also induces the expression of adhesion molecules which promote monocyte recruitment such as ICAM-1 and E-selectin, VCAM-1 (Clinton et al, 1992). In addition, chemokines such as MCP-1 are produced in endothelial cells in response to IL-1 stimulation. IL-1 initiates the expression of many genes encoding growth factors, and cytokines such as M-CSF and IL-6 (Mantovani et al, 1992). The promotion of bFGF production results in stimulation of smooth muscle cell proliferation. Smooth muscle cells are also stimulated to produce prostaglandin and nitric oxide. IL-1 produced by macrophages acts on macrophages as well as lymphocytes inducing IL-2, IL-3, IL-6 and IFN-γ.

IL-1 exerts its effects through two IL-1 receptors (IL-1R), with most activities mediated exclusively via the IL-1RI, whereas IL-1RII has no signalling activity and inhibits IL-1 activities by acting as a decoy for IL-1 (Collotta et al, 1994). Endothelial cells exclusively express IL-1RI (Collotta et al, 1993) whilst monocytes express both types, but IL-1 effects are exerted via IL-1RI (Sims et al, 1993).

Finally, stimuli that induce IL-1 also cause production of IL-1 receptor antagonist (IL-1Ra), which may counterbalance the action of IL-1. Multiple forms of IL-1Ra exist including soluble IL-1Ra (sIL-1Ra) and intracellular IL-1Ra type I and II (icIL-1RaI/II). These receptors are expressed by mononuclear phagocytes and neutrophils (Haskill et al, 1991).

1.10.3 IFN-γ and IL-12

IFN-γ production is limited to T cells and NK cells. IFN-γ induces endothelial cell expression of MHC class II antigens, amplifies responses to TNF, slowly stimulates ICAM-1 expression and augments LPS-induced production of IL-1. IFN-γ is the predominant T cell cytokine in atherosclerosis and can activate cells to release proteases which can disrupt the overlying plaque capsule (Uyemura et al, 1996, Libby and Hansson, 1991).

IL-12 is detected in atherosclerotic lesions, and monocytes are the major source of this cytokine (Uyemura et al, 1996). Highly oxidised LDL induces production of IL-12 in a dose dependent manner. IL-12 released by monocytes is known to amplify immune responses by preferentially expanding T cells capable of producing IFN-γ. Evidence suggests that IL-12 does not act directly on endothelial cells, but rather via induction of IFN-γ. Thus the local production of IL-12 may potentiate the chronic inflammatory T cell and macrophage responses leading to tissue injury in atherosclerosis.

1.10.4 IL-10

IL-10, a 35-40kDa protein was first identified as a Th2 cytokine that inhibited proliferation of Th1 cells by down regulating IFN-γ and IL-2 production. Subsequent studies have shown that IL-10 is produced by various other cells, including B cells, mast cells and monocytes. IL-10 has been detected in atherosclerotic plaques and oxLDL induces IL-10 release from monocytes (Uyemura et al, 1996). Monocytes are the main source of IL-10. IL-10 synthesis in human peripheral blood monocytes is regulated by TNF with peak expression at 24 hours stimulation. IL-10 activities are pleiotropic and its role in the immune system is multi-faceted. IL-10 can induce proliferation of T and B cells and differentiation of cytotoxic T cells but can also act to inhibit cytokine synthesis in

various cell types. IL-10 inhibits production of IL-1β, IL-6, IL-8 and TNF in monocytes and macrophages, IL-4 and IL-5 in Th2 cells and IFN-γ and TNF-α in NK cells.

1.10.5 IL-4

IL-4 is actively secreted by Th_2 subset lymphocytes and IL-4 has the potential to modulate the functional properties of the newly infiltrated monocytes, differentiated macrophages, and lipid laden foam cells, as may be reflected by alterations in surface antigen expression. IL-4 selectively upregulates VCAM-1, but not E-selectin or ICAM-1, and also induces synthesis and secretion of MCP-1. IL-4 also has certain anti-inflammatory effects on both activated and resting monocytes, such as inhibition of adherence (Elliott et al, 1991) and of IL-1 and TNF-α transcription (Hart et al, 1989).

1.11 Chemokines and Atherosclerosis

Chemoattractants are important in activation of integrin adhesiveness and in directing the migration of leukocytes. In chemotaxis, cells move in the direction of increasing concentration of a chemoattractant, which is typically a soluble molecule that can diffuse away from the site of its production, where its concentration is highest (Devreotes et al, 1988).

A recently described family of chemoattractive cytokines, termed chemokines, are 70-80 residue polypeptides and have specificity for leukocyte subsets (Oppenheim et al, 1991; Miller and Krangel, 1992). Two subfamilies of chemokines have been defined and are distinguished according to the position of the first two cysteines, which are either adjacent (C-C), or separated by one amino acid (C-X-C). Table 1.2 lists some of the best characterised chemokines. The C-C family are primarily chemotactic for monocytes, whilst C-X-C proteins are mainly chemoattractive to neutrophils. The effects of chemokines on

leukocytes are mediated by heptahelical receptors coupled to GTP-binding proteins. The binding of a chemoattractant to its receptor results in the activation of the associated G protein (Bokoch, 1995). The most impressive effect is the shape change that is observed within seconds after addition of an attractant to a leukocyte suspension. Polymerisation and breakdown of actin leads to the formation and retraction of

lamellipodia, which function like arms and legs of the migrating cells (del Pozo et al, 1996). This cytoplasmic projection is termed a 'uropod' and represents a specialised structure with important functions for motility and adhesion. Uropod formation is thought to be a critical event for transmigration of leukocytes (Vaddi, 1992). Stimulation by chemokines also induces the upregulation and activation of integrins, which enable the leukocytes to adhere to the endothelial cells of the vessel wall before migrating into the tissues (Springer et al, 1994).

Most chemokines are produced under pathological conditions by tissue cells and infiltrating leukocytes. Expression of chemokines can be induced by or on a variety of immune and non immune cells such as fibroblasts, epithelial cells, endothelial cells and smooth muscle cells as well as mononuclear cells and granulocytes.

1.11.1 MIP-1α

MIP-1α is a C-C chemokine produced by mononuclear cells and acts on T-lymphocytes, monocytes and neutrophils. In addition, coculture of monocytes with endothelial cells can lead to elaboration of MIP-1α (Lukacs et al, 1994). Unstimulated monocytes in contact with unstimulated endothelial cells produced MIP-1α with peak production at 24 hours. Monoclonal antibodies to ICAM-1 reduced MIP-1α production with a parallel decrease in monocyte adhe-

C-X-C Chemokines

ORIGIN	CHEMOKINE	RESPONDING CELLS
Macrophages T lymphocytes Endothelial cells Fibroblasts	IL-8	Neutrophils T lymphocytes Smooth muscle cells Basophils
Monocytes Endothelial cells Fibroblasts	GRO-α	Neutrophils Fibroblasts

C-C Chemokines

ORIGIN	CHEMOKINE	RESPONDING CELLS
Monocytes Endothelial cells Fibroblasts T lymphocytes Smooth muscle cells	MCP-1, -3	Monocytes Basophils T lymphocytes
T lymphocytes Platelets	RANTES	Monocytes T lymphocyte subpopulation Eosinophils
Monocytes T lymphocytes Basophils	MIP-1 α and β	Monocytes Neutrophils T lymphocyte subpopulation Basophils Eosinophils

Table 1.2 Chemokines

sion while anti-VCAM-1 Ab was without effect (Lukacs et al, 1994). These results thus indicate that activation of monocytes and production of chemokines, during adhesive interactions with endothelium are an important mechanism in sustaining continuous recruitment of cells during inflammatory responses as would be the situation in a nascent atheroma.

1.11.2 MCP-1

MCP-1 is expressed by a variety of cells including endothelial cells, smooth muscle cells and macrophages (Strieter et al, 1989). TNF, IL-1, IFN-γ, oxidised LDL and endotoxin have all been shown to increase MCP-1 mRNA levels in endothelial cells and smooth muscle cells (Yoshimura et al, 1992). Atherosclerotic plaques exhibit increased MCP-1 mRNA, especially in macrophage rich inflammatory regions, and the majority of MCP-1 production is by macrophages in human atherosclerotic plaques (Yla-Herttuala et al, 1991; Nelken et al, 1991). There is also recent evidence that MCP-1 stimulates the expression of adhesion molecules by smooth muscle cells and monocytes (Ikeda et al, 1993). This would allow for the perpetuation of macrophage participation in atherosclerosis (Jiang et al, 1992).

Recent work has shown that where monocytes are in contact with endothelium there is an increased expression of MCP-1 and IL-8, that could be detected as early as 4 hours and peaking at 24 hours (Lukacs et al, 1995). Here, adhesion molecules, ICAM-1 and VCAM do not play a role in the elaboration of these chemokines and the cytokines, IL-1 and TNF also are not involved in this production. The effects could however, be inhibited through use of soluble matrix proteins if these were added before the interactions. Hence the interactions of infiltrating monocytes with subendothelial matrix components in atherosclerotic plaques could induce the production of chemokines that can

attract further recruitment of not only monocytes but also lymphocytes thus increasing the size of the plaque and the attendant risk of rupture (Lukacs et al, 1995).

The role of MCP-1 in atherogenesis was further investigated using low density lipoprotein (LDL) receptor-deficient mice, which were made genetically deficient for MCP-1 and fed on a high cholesterol diet. Whilst these double deficient mice had comparable levels of cholesterol in their serum with the LDL receptor alone deficient mice, these mice had 83% less lipid deposition through out their aortas. There was also fewer macrophages in the aortic wall. These results appear to suggest that MCP-1 plays a role in the initiation of atherosclerosis (Gu et al, 1998).Through generating mice that lack the MCP-1 receptor similar results were obtained. showing a decrease in lesion formation (Boring et al, 1998). Further recent work has concentrated on the upregulation of the MCP-1 receptor (CCR2). It has been shown that LDL is a positive regulator of CCR2 expression and that CCR2 expression on monocytes is dramatically increased in hypercholesterolemic patients compared with normals. In addition incubation of monocytes with LDL induces a rapid increase in CCR2 mRNA and protein. By 24 hours the number of cell surface receptors is doubled causing a 3 fold increase in the chemotactic response to MCP-1. This effect was only caused by native LDL, and this induction appears to be mediated by LDL derived cholesterol and reliant on LDL binding and internalisation (Han et al, 1998). Finally MCP-1 has been shown to induce tissue factor expression in monocytes and this induction might be an important link between hemostasis and inflammation, especially in atherosclerosis where prothrombotic conditions exist (Ernofsson and Siegbahn, 1996)

1.11.3 Other Chemokines

RANTES is a chemokine that selectively chemoattracts T lymphocytes, NK cells, monocytes and eosinophils. RANTES mRNA and protein have been detected in the lymphocytes, macrophages and endothelial cells of arteries undergoing accelerated atherosclerosis but not in normal coronary arteries suggesting a role for RANTES in mediating the cellular infiltrate that is characteristic in transplantation-associated accelerated atherosclerosis (Pattison et al, 1996)

IL-8 mRNA and protein have also been detected in macrophages incubated with oxidised LDL. The chemoattractant and/or mitogenic effects of IL-8 on neutrophils, T cells, smooth muscle, or vascular cells may contribute to the progression and complications of atherosclerosis (Tabas et al, 1996)

1.12 Proteoglycans

The surface of endothelium is covered by a dense layer of proteoglycans, the main functional components of which are glycosaminoglycans (GAGs). The most abundant endothelial GAG is the polyanionic species heparin sulphate (HS), which is structurally and biosynthetically related to heparin. Heparin is known to bind with high affinity to a range of growth factors and cytokines including the chemokines, IL-1, TNF-α and IFN-γ. It is considered also that heparin sulphate binds chemokines, such as IL-8 so that their effective local concentration is maintained and such immobolisation allows them to be presented to the marginating leukocytes (Webb et al, 1993).

Without such a mechanism, cytokines would lose biological activity rapidly by dilution into the blood or surrounding extracellular fluid (Douglas et al, 1997). Proteoglycans also play a role in the accumulation of lipoproteins following passage across the endothelium. LDL and proteoglycans co-localise within the

lesions, and oxidation of LDL enables LDL binding to proteoglycans (Saxena et al, 1994).

1.13 Multistep model of leukocyte recruitment

1.13.1 Tethering

Tethering is mediated by the selectins, each of which promotes leukocyte rolling under flow conditions. P-selectin is rapidly mobilised from cytoplasmic granules to the endothelial cell surface on activation, however E-selectin is expressed on endothelial cells only after several hours since *de novo* synthesis is required, thus it seems likely that E-selectin contributes to the later recruitment of leukocytes. However recent work has demonstrated through use of P- and E-selectin double deficient mice that P- and E-selectin together play a critical role in both the early and late stages of atherosclerotic lesion development. In cases where both selectins were absent the size of the fatty streaks were five times smaller compared to the wild type after a short time period (Dong et al, 1998). L-selectin is constitutively expressed on all circulating leukocytes.

Selectins are ideally suited to the tethering role since they have a long molecular structure that extends above the surrounding glycocalyx and allows them to capture passing leukocytes that express the appropiate receptor. Furthermore L-selectin has been found on the tips of leukocyte microvillus, which are the first points of contact with the endothelium. The selectins mediate a degree of adhesion that is strong enough to induce rolling along the vessel wall but not so strong as to stop leukocytes completely. The transient nature of this adhesive interaction is important since it allows leukocytes to sample the local endothelium for the presence of specific trigger factors that can activate leukocyte integrins and allow the cascade to continue. In the absence of such

factors, the transient nature of selectin binding allows leukocytes to disengage and move on.

1.13.2 Triggering/Integrin Activation

Strong adhesion of leucocytes is mediated by integrins. However because integrins on circulating leukocytes do not bind well unless they are activated, a triggering step is required to activate integrins and thereby promote strong adhesion that can stop the rolling leukocyte. The activation of leukocytes triggers an increase in avidity caused by a conformational change in the integrin heterodimer, resulting in greater affinity for ligands and/or to postreceptor events (Ginsberg et al, 1992). There are diverse mechanisms whereby leukocyte integrin avidity can be modulated. The binding of chemokines (MCP-1, MIP-1β), cytokines or chemoattractants to leukocytes expressing complementary receptors transduces signals that can augment β1 and β2 integrin-dependent adhesion. These activating agents may be derived from local tissue cells, infiltrating leukocytes, microorganisms, endothelium itself or as a result of the oxidation of LDL (Carlos and Harlan, 1994). Structural variation of protoglycans on endothelium may add additional specificity to the adhesion cascade by regulating which chemokines are retained. Thus site specific or activation specific endothelial proteoglycans may bind and present different proadhesive cytokines to particular subsets of leukocytes. In addition this method of immobilising proadhesive cytokines on the endothelial lumen would confine and concentrate triggering of leucocyte adhesion to the vessel wall at the inflammatory sites thus preventing cytokines from being swept away by blood flow and acting at sites distant from their source. Activation of leukocyte integrins can also occur after engagement of counter structures by other leukocyte surface receptors. Cross linking of certain surface proteins have

been shown to induce high avidity binding of leukocyte CD11a/ CD18 (Shimuzu et al, 1992). Moreover the binding of leukocytes to endothelial adhesion proteins may also transduce activating signals. It is important to note that some endogenous mediators may also function to reduce leukocyte-endothelial cell interactions. Under certain assay conditions IL-8, a neutrophil specific pro-adhesive protein can reduce neutrophil adherence to the endothelium (Gimbrone et al, 1989). TGF-β can also suppress inflammatory responses by inhibiting E-selectin expression on endothelial cells (Gamble et al, 1993). IL-4 can also reduce monocyte adherence to endothelium (Elliott et al, 1991). If proadhesive factors predominate, the net result of the activation process is to promote firm adhesion via integrin interactions with endothelial Ig-like ligands. Shear-sensitive, selectin-mediated tethering is replaced by shear resistant, activated integrin-mediated adhesion.

1.13.3 Strong Adhesion

Strong adhesion is mediated by leukocyte integrins that bind to counter receptors on endothelium. These counter receptors are often expressed *de novo*. Two important integrin subfamilies are the β1 integrins and the β2 integrins. At least five leukocyte integrins can mediate strong binding to endothelium. The most important of these for monocytes are β1 integrin VLA-4 (CD49d) and the β2 integrin Mac-1 (CD11b). β2 integrins bind to intercellular adhesion molecule1 (ICAM-1) and ICAM-2, which are expressed on the endothelium. ICAM-2 is constitutively expressed and therefore mediates binding to resting endothelium, whereas ICAM-1 expression is increased with activation and predominates in binding to inflamed endothelium. The β1 integrin VLA-4 mediates monocyte binding to the endothelial adhesion receptor vascular cell adhesion molecule (VCAM-1). Expression of VCAM-1 is also

induced on inflamed endothelium by proinflammatory cytokines. The pathways used by the leukocytes to bind at activated endothelium depend on the site and nature of the endothelial-activating stimulus.

1.13.4 Transmigration

Many of the leukocytes that are tightly bound to endothelium next crawl over the lumenal surface, a process that requires reversible adhesion (Stossel et al, 1993). Upon encountering an intercellular junction some of the migratory leukocytes then squeeze between endothelial cells to enter the extravascular tissue under the influence of promigratory factors. Migration across endothelial monolayers involves the adherence to endothelium, movement over the endothelial luminal surface, and often some component of migration through the subendothelial matrix in addition to penetration between endothelial cells. Adhesion molecules important in this stage are CD11/CD18/ICAM in conjunction with VLA-4/VCAM-1 and E-selectin. If all these adhesive pathways are inhibited through use of blocking antibodies then 68% of monocyte migration is inhibited (Hakkart et al, 1991).

Recently the leukocyte and endothelial cell adhesion molecule PECAM-1 has been demonstrated to be directly involved in the process of monocyte diapedesis between endothelial cells (Muller et al, 1993). PECAM-1 is an Ig superfamily cell adhesion molecule that is concentrated at the junctions between endothelial cells and is also expressed on platelets, monocytes, neutrophils and a subset of T cells. In the presence of anti-PECAM antibodies the monocytes remain bound to the endothelial surface over the intercellular junctions thus clearly defining the role of PECAM in diapedesis between endothelial cells. On the other hand, anti-PECAM-1 mAb has no effect on monocyte adhesion to endothelial cells. The addition of anti-PECAM-1 to either

the leukocytes or the endothelial cells inhibits transmigration suggesting that homophilic interaction between leukocyte and endothelial PECAM-1 exists (Muller et al, 1993). PECAM-1 also promotes heterophilic adhesion, via binding to glycosaminoglycans or $\alpha_v\beta_3$ integrin. PECAM-1 is also involved in the upregulation of other adhesion molecules by inside-out signalling. Engagement of PECAM-1 upregulates the avidity of integrins such as CD49d and CD11b/CD18 (Tanaka et al, 1997, Berman et al, 1995) and also induces TNF-α secretion from monocytes (Elias et al, 1998).

1.14 Leukocyte Recruitment in Atherosclerosis

In areas affected by atherosclerosis additional stimuli exist that can influence the adhesion molecules expressed and hence the leukocyte type recruited. Recent studies have shown that both ICAM-1 and VCAM-1 are found present in human atherosclerotic lesions (O'Brian et al, 1993; Poston et al, 1992). In animal studies it was found that VCAM-1 is selectively expressed in fatty streaks in rabbits fed a high cholesterol diet and also in Wantanabe heritable hyperlipidemic strain of rabbits which develop rampant atherosclerosis (Cybulsky and Gimbrone, 1991). The expression was restricted to atherosclerosis lesion areas. Other work by this group showed that VCAM-1 induction by cholesterol is a very early event (within one week of diet initiation) and precedes infiltration by monocytes. Monocytes and T lymphocytes are the only leukocyte subsets to express the counterreceptor for VCAM-1, that being VLA-4, thus these results seem to explain why monocytes and T lymphocytes are the predominant cell types in lesions (Li et al, 1993). *In vitro* studies have shown that lipoproteins, in native or oxidised forms, can induce adhesion molecule expression and monocyte adhesion. This has led to further experiments to identify the lipoprotein component that can mediate these

lipoprotein effects. Lysophosphatidylcholine (lyso-PC) is a major phospholipid component of atherogenic (oxidised) lipoproteins. Cultured rabbit aortic endothelial cells treated with lyso-PC showed increased mRNA and cell surface expression of VCAM-1 and ICAM-1, which was associated with increased adhesion of monocytes (Kume et al, 1992). In addition, increased monocyte recruitment and adherence has been found in human atherosclerotic plaques. The addition of LDL to human aortic endothelial cells and to smooth muscle cells resulted in induction of monocyte adhesion and transmigration (Navab et al, 1991). Antioxidants and anti-inflammatory agents blocked the LDL induced migration suggesting that the oxidative modification of LDL, or oxidative signals may be important for monocyte recruitment during atherogenesis (Navab et al, 1993). Lipoprotein not only alters adhesion molecule expression but can further promote monocyte adherence through the generation of chemoattractant molecules. The lipid product of LDL oxidation, lysophosphatidylcholine, has been demonstrated to be a selective chemoattractant for mononuclear cells (Kume et al, 1992). In addition incubation of LDL with artery wall cells results in a significant induction of monocyte chemoattractant protein 1 (MCP-1) mRNA and protein (Navab et al, 1991).

1.15 PhD Aims

The various factors which influence the recruitment of monocytes into atherosclerotic lesions are gradually being identified. Whilst monocyte recruitment and infiltration are critical factors in the pathogenesis and propagation of atherosclerosis, the adhesive interactions between monocytes and endothelial cells may also play a crucial role in determining the inflammatory functions of these cells. There is evidence from previous work to show that the close proximity of monocytes with endothelial cells modulates the

production of molecules such as prostacyclin, plasminogen activator inhibitor (Hakkert et al, 1990), von Willebrand factor (Hakkert et al, 1992), procoagulants, mitogens (Grandone et al, 1995), chemokines such MIP-1α (Lukacs et al, 1994) and MCP-1 (Lukacs et al, 1995) and tissue factor (Lo et al, 1995; Collins et al, 1995; Napoleone et al. 1997). The specific aims of this thesis were to :

- Determine the effect of monocyte/endothelial cell coculture on E-selectin expression in endothelial cells.
- Determine the effect of monocyte/endothelial cell coculture on A1 induction and the ability of monocytes to confer survival in serum starved endothelial cells.
- Identify the adhesive pathways that underlie gene induction in endothelial cells cocultured with monocytes.
- Explore the effect of immunomodulatory cytokines, such as IL-10 on E-selectin and A1 induction.
- Characterise the molecular mechanisms that mediate monocyte and neutrophil transmigration.

Chapter Two

Methods

2.1 Materials

All materials, plastics, antibodies, reagents (including compositions) and equipment are listed in the Appendix.

2.2 Cell Isolation and Culture

2.2.1 Isolation and Culture of Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells were isolated from umbilical cords by a modification of the method originally described by Jaffe et al (1973). Briefly umbilical cords were obtained within 24 hours of delivery and stored at room temperature in HBSS. The cords were checked for needle or clamp marks and any areas of damage or bruising removed. The ends of the cord were trimmed with a surgical blade and the vein of the cord at both ends cannulated with 14 gauge intravenous cannulae and secured in place using sterile ties. The vein was flushed out using HBSS and this was continued until the cord was free from blood and the effluent ran clear. After ensuring the complete removal of the HBSS, warm collagenase (670μg/ml or 0.067% in HBSS) was inserted into the cord and the cannulae ends closed with plugs. The cord was incubated for 20 minutes at 37°C. After incubation the cord vein was flushed out with warm HBSS/1% FCS, the contents collected and centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in complete culture medium (Iscove's Modified Dulbecco's Medium with 20% FCS, 50μg/ml ECGS and 20U/ml heparin). The cells were grown to confluence in fibronectin-coated 25 cm^2 Falcon tissue culture flasks precoated with fibronectin (100μg/ml) for 30 minutes at room temperature. Once the endothelial cells had reached confluence the growth media was removed and the cells washed gently in HBSS. Warm trypsin/EDTA was added to the cells, and following incubation at

37°C for 2-5 minutes, 5-10mls of HBSS/2% FCS were added and the detached cells collected, centrifuged for 5 minutes at 1000rpm and the cell pellet resuspended in complete culture medium. The cells were then further grown to confluence in 75cm^2 tissue culture flasks or used in experiments. Endothelial cells were seeded onto 96 well plates (2x10^{4}cells/100μl) for monocyte coculture assays, 48 well plates (5x10^4 cells/200μl) for adhesion assays, 6 well plates (2x10^5/2ml) for serum starvation and survival assays or onto Transwell filters (3x10^4/100μl) for transmigration assays. Only passage 2 to passage 5 cells were used in experiments. Endothelial cells were identified by their characteristic cobblestone morphology and by detection of E-selectin protein expression (Section 2.4. and Chapter 3).

2.2.2 Leukocyte Isolation

- ***Monocytes***

Monocytes were purified from venous blood taken into EDTA (3.2μg/ml of blood). 4 parts of EDTA blood were mixed with 1 part of Hespan and the red blood cells were allowed to sediment, at an angle of 45°, over 30 minutes at room temperature. The plasma layer, containing the leukocytes was removed after this time and 7 mls of plasma were layered over 4 mls of Nycoprep 1.068 in a polypropylene 15ml tube. Following centrifugation at 600g for 15 minutes the clear plasma was removed down to 3-4mm above the interface and the remainder of the plasma and all of the Nycoprep 1.068 solution harvested to just above the red cell pellet. The cell suspension was washed twice with 0.9% sodium chloride solution containing 0.13% (w/v) EDTA and 1% FCS by centrifugation for 5 minutes at 600g. To remove the contaminating platelets the cell pellet was resuspended in 1ml of HBSS and layered over 3 mls of autologous EDTA plasma, centrifuged at 50g for 20 minutes and the

supernatant containing the platelets removed. Finally the cells were washed twice at 1600rpm for 5 minutes with 5 mls of HBSS and the resulting cell pellet was resuspended in 1 ml of IMDM/20% FCS. Cells, diluted 1 in 10 in acetic acid, were counted on a haemocytometer. The monocytes obtained by this method were 80% pure as determined by morphological examination of cytospin preparations stained with MGG.

- ***Neutrophils***

Neutrophils were isolated from venous blood taken into heparin (10U/ml of blood) using double density centrifugation with Lymphoprep above Histopaque 1119 in 50ml polypropylene tubes. Following centrifugation at 2900rpm for 45 minutes the neutrophil rich interface was harvested and the cells washed twice in HBSS/2% FCS/5mM glucose (HBSS/Glucose) at 1600rpm for 5 minutes. The neutrophils were either used straight away in experiments or labelled with chromium for migration studies (section 2.5.). The purity of the neutrophils obtained with this method was >95% as determined by morphology, as detailed above, and >99% viable as assessed by trypan blue exclusion.

- ***Lymphocytes***

Lymphocytes were isolated from peripheral blood taken into heparin (10U/ml of blood) by density centrifugation, monocyte adherence and negative selection with Dynabeads. Briefly, the blood was diluted in equal volumes with HBSS and 3 parts blood layered onto 2 parts Lymphoprep and centrifuged at 2000rpm for 30 minutes. The mononuclear cell interface was harvested and the cells washed twice with 50 mls with HBSS, the first spin at 1500rpm and the second spin at 1200rpm. The cell pellet was resuspended in RPMI (+glutamine)/10% FCS, transfered to a 75cm^2 tissue culture flask and incubated for 1 hour at 37°C to allow monocytes in the preparation to adhere to the plastic. Following 1 hour,

the medium with non-adherent cells was removed and the flask washed out with RPMI/10% FCS and the cell suspension transfered to a universal tube. The cells were centrifuged twice for 10 minutes at 1200rpm and then a cell count performed. Appropriate volumes of antibodies (monocyte specific CD14, DRII) were added and the resuspended cells left for 15 minutes at room temperature. After this incubation the cells were made up to 20 mls with RPMI and spun for 10 minutes at 1200rpm. The supernatant was then removed, cells transfered to a FCS-coated 15ml tube, RPMI added and a further centrifugation performed. After removing the supernatant, washed Dynabeads and 2 mls of RPMI/1% FCS were added to the cells. These cells were centrifuged at 2000rpm for 5 minutes, for a total of 5 times with no removal of supernatant. The cells were just resuspended between washes. 5 mls of RPMI were added and the tube of cells placed in a magnet. The supernatant was poured off into a fresh FCS-coated tube containing more Dynabeads and the cells spun quickly at 2000rpm. This process was repeated three times. After the final wash the cells were counted and the complete removal of Dynabeads checked. The purity of the lymphocyte preparation was confirmed by FACS analysis with anti-CD45 and anti-CD14 and found to be >99%. The Dynabeads used in these experiments were goat anti-mouse Ig-coated.

2.2.3 Leukocyte Characterisation

- ***Cytospins***

50μl of a cell suspension at $2x10^6$ in IMDM/20% were added to the cytospin well, followed by 50μl of PBS and spun at 400rpm for 2 minutes. The slides were then airdried before staining. Staining was performed on an automated cell stainer using May-Grunwald and Giemsa stain. The slides were then examined under a light microscope and differential counts performed.

- ***Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP)***

APAAP is an immuno-enzymatic technique used to detect cytoplasmic, nuclear and surface antigens. This technique was employed to confirm the complete removal of monocytes by EDTA from endothelial cell cocultures. Cytospin preparations of endothelial cells which had had monocytes removed by incubation with 2mM EDTA were made as detailed overleaf. The cytospin slides were fixed in acetone for 10 minutes and then the fixative was washed off with Tris Buffered Saline (TBS), pH 7.6., solution. All excess TBS was shaken off and the slides were then incubated with diluted anti-CD14 monoclonal antibody or the negative control consisting of rabbit anti mouse Ig for 30 minutes in a moist chamber. The excess antibodies were then washed off with TBS, and the second antibody added. The slides were incubated with diluted rabbit anti mouse Ig for 30 minutes at room temperature in a moist chamber. The slides were then again washed with TBS and then the APAAP complex diluted in TBS added and the slides incubated for a further 30 minutes at room temperature. Following washing, the slides had 1ml of alkaline phosphatase substrate added and were incubated for 17 minutes in moist chambers. After another wash with TBS the slides were dipped into tap water prior to counterstaining in Harris's Haematoxylin for 5 minutes. Any excess stain was removed by washing with tap water and the slides were then air dried and mounted with aqueous Apathy's mountant. The slides were reported using a light microscope under a x100 oil lens and CD14 positive cells (monocytes) showed pink cytoplasmic and surface staining whilst negative cells (endothelial cells) were blue. At least 300 cells were counted per slide.

2.3 Leukocyte:Endothelial Cell Coculture

Endothelial cells were grown to confluence on 96, 48 and 6 well plates or on Transwell filters. The endothelial cell monolayers were then washed in IMDM/20% FCS and leukocyte suspensions in IMDM/20% FCS added at a ratio of 1:1 leukocyte:endothelial cells. At this stage any cytokines or blocking antibodies were added (details found in the legends). In experiments designed to prevent leukocyte:endothelial cell contact leukocytes were cultured on 0.45μm Transwell filters and inserted into wells containing confluent endothelial cells such that soluble proteins could diffuse but cell movement prevented. The cells were then incubated over a period of up to 21 hours at 37°C, 5% CO_2 in a humidified incubator. For each condition there were quadruplicate wells.

2.3.2 Serum Starvation of Endothelial cells

In those experiments designed to study the effect of monocytes on apoptosis of endothelial cells, endothelial cells were grown to confluence in 6 well plates ($1x10^5$/2mls), then washed with warm HBSS and incubated in RPMI/0.1%FCS/ITS for 5 hours at 37°C. Following this, leukocytes resuspended in the same medium ($5x10^5$cells/well), or cytokines such as IL-1 and TNF were added and incubated over a period of up to 21 hours.

2.4 Determination of Surface Antigen Expression on Endothelial Cells

- ***ELISA***

An enzyme immunoassay, modified after the method used by Smith et al (1988) was used to quantify surface adhesion molecule expression on endothelial cells in culture. Endothelial cells grown to confluence in 96 well plates were cocultured with leukocytes or stimulated with cytokines over a period of up to 24 hours. At the end of the experiment each well was fixed with 1% paraformaldehyde in PBS for 30 minutes at room temperature. Following three washes with large volumes of PBS, 100μl of 2% BSA/PBS were added to

each well for 30 minutes at room temperature. This was then removed and replaced with 40μl of monoclonal antibodies, diluted in 1% BSA/PBS to a concentration of 10μg/ml. The antibodies were incubated with the cells for 45-60 minutes at 37°C before being removed by three washes with PBS and the addition of 100μl of HRP-conjugated goat anti-mouse monoclonal antibody diluted to a concentration of 0.5μg/ml in 1% BSA/PBS. Following incubation for 45-60 minutes at 37°C there were three further washes with PBS prior to the addition of the substrate. 100μl of 0.67mg/ml of OPD in citrate phosphate buffer, pH 5, with 0.03% H_2O_2 were added to each well and the reaction was stopped by the addition of 50μl of 1M H_2SO_4. The optical density of each well was read at 405nm on a microplate reader.

- ***FACS Labelling***

Cells were placed in FACS tubes (5-10x10^4 in 50μl per tube) and 7μl of the primary antibody or isotype control were added to each tube. Samples were incubated on ice for 45 minutes, then washed three times with cold PBS and the second antibody, rabbit anti mouse fluorescein-conjugated $F(ab')_2$ fragment was added (1:40 diultion) for 30 minutes at 4°C. Two mls of PBS were then added and the cells centrifuged for 5 minutes at 1500rpm, After a final wash, 300μl of 2% paraformaldehyde were added to the tubes and the samples stored at 4°C in the dark until flow cytometry (Becton Dickinson).

2.5 Adhesion and Transmigration Assays

- ***Labelling of Leukocytes***

Purified monocytes and neutrophils in HBSS/2% FCS/5mM glucose at a concentration of 1x10^7 cells/ml were incubated with ^{51}Cr (2μCi/10^6cells) for 1 hour at 37°C with occasional gentle agitation. Following three washes with large

volumes of the above media the leukocytes were resuspended in IMDM/20% FCS to an appropriate cell concentration depending on the experiment.

- ***Neutrophil/ Monocyte Adhesion Assays***

Endothelial cells were seeded in a 48 well plate at $5x10^4$cells/well in 200μl of complete culture medium (200μl per well) and grown to confluence over two days as described above.

For experiments where endothelial activation was required, cytokines (TNF and IL-1) were added 4 hours prior to the experiment. Once the leukocytes were chromium labelled the confluent endothelial cells in 48 well plates were washed twice with IMDM/20% FCS (adhesion medium). 100μl of leukocytes resuspended in adhesion medium at a concentration of $3x10^6$ neutrophils/ml or $5x10^5$ monocytes/ml were then added to each well and the cells incubated at 37°C for 45 minutes. In some experiments cytokines or calpain inhibitors were added at this stage. Triplicate wells were used for each condition. After the incubation the nonadherent leukocytes were removed by three 500μl washes using HBSS/5%FCS. The integrity of the endothelial monolayer was confirmed between washes microscopically. After the final wash 0.5ml of 1% NP-40 were added to each well to lyse adherent cells as well as endothelial cells, and after 1 hour, the contents of each well were collected and associated radioactivity counted on an automated gamma counter. The percent adherence was calculated by dividing the counts per minute (cpm) of adherent fraction by cpm of the cell suspension initially added to each well.

- ***Neutrophil/ Monocyte Migration Assays***

Polycarbonate membrane Transwell filters (6.5 mm insert diameter, 3 micron pore size) fit in 24 well cluster plates and accomodate 100μl of media above the insert and a volume of 600μl below in the cluster plate. The Transwell filters

were soaked for two hours in HBSS and then coated with fibronectin at room temperature for 30 minutes. Endothelial cells following passage were counted and the cell concentration adjusted to $3x10^5$cells/ml in IMDM, 20% FCS, 100μg/ml ECGS and 20U/ml heparin. The endothelial cells were added to the microporous membrane in a volume of 100μl and the cells grown to confluence over 48 hours in the same conditions as described above. The formation of confluent layers was confirmed firstly by microscopic analysis, by exclusion of media when Transwell inserts were placed on new media in cluster plates and also by demonstration that gradients of soluble factors could be maintained across the monolayers. Briefly, ^{51}Cr-EDTA was placed in the top chamber and after 30 minutes the distribution of the radioactivity between the upper and lower chambers counted. Confluent endothelial cell layers will exclude diffusion of the radiolabelled EDTA and the percentage of radioactivity in the bottom chamber after 30 minutes will be less than 85% of the total added. If the endothelial cells are not confluent EDTA will distribute equally between the top and bottom chambers, resulting in 85% of the total added found in the bottom as 600μl of medium are in the bottom and 100μl in the top.

In experiments where cytokine stimulation of endothelial cells was required IL-1 or TNF were added to both the upper and lower chambers of the Transwells for 4 hours prior to the transmigration assay. After this time, the confluent endothelial monolayers were washed three times in IMDM/20%FCS and placed in a fresh 24 well plate with 600μl of IMDM/20%FCS in the wells. In some wells a chemoattractant such as FMLP or MCP-1 was placed in the lower chamber. 100μl of labelled leucocytes at a concentration of $3x10^6$ cells/ml were added to the upper chamber. In some experiments, calpain inhibitors were also added to the upper chamber of the Transwell at this stage. The plate was incubated for

1.5 hours for neutrophil migration and 4 hours for monocyte migration at 37°C in 5% CO_2 humidified incubator. Duplicate wells were used for each condition. After this time, the migrated cells were harvested; the lower surface of the filter was swabbed with a cotton wool bud and placed in a tube and the contents of the bottom chamber were aspirated and placed in the same tube. To recover any remaining cells adherent to the bottom of the well 1ml of 1% NP-40 was added to the bottom chamber for 1 hour, the contents collected and added to the harvested migrated cells and associated radioactivity counted on an automated gamma counter. Percent migration was calculated by dividing the cpm of migrated fraction by cpm of the cell suspension initially added to each filter.

2.6 Analysis of Gene Transcription

- ***Preparation of samples for RNA Isolation***

After harvesting by trypsin/EDTA the endothelial cells were resuspended in 1 ml of HBSS, transferred to a 1.5ml eppendorf and centrifuged at 10,000rpm for 10 seconds. The supernatant was removed and 500μl of ice cold GITC solution containing 7.2μl/ml of 1M β-mercaptoethanol added. The cell pellet was homogenised with a 21 gauge needle and the sample then stored at -70°C. In experiments where monocytes were cocultured with endothelial cells the monocytes were separated from the endothelial cells by incubation for 2 minutes with 2mM EDTA and then were treated in a similar manner to endothelial cells.

- ***RNA Isolation*** (all performed at 4°C)

Total RNA was isolated using the acid GITC-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). To cells in 500μl of ice cold GITC solution, 50μl of 2M sodium acetate, pH 5, were added and vortexed for 5

seconds. 500μl of phenol were then added followed by a 10 second vortex prior to the addition of 100 μl of chloroform/isoamylalcohol (IAA), (49:1), and a further vortex of 10 seconds. Following an incubation of 20 minutes on ice, the microcentrifuge tubes were spun for 20 minutes at 13,000rpm. The aqueous phase was removed into a clean eppendorf and 250μl of phenol and 250 μl of chloroform/IAA were added and the solutions vortexed for 10 seconds. After 5 minutes incubation on ice the tubes were centrifuged for 15 minutes at 13,000rpm. Again the aqueous phase was removed into a fresh eppendorf and 500μl of chloroform/IAA added, a 10 second vortex and a five minute incubation on ice undertaken. After the final centrifugation at 13,000rpm, 15 minutes, the aqueous phase was again taken into a fresh eppendorf and 1ml of absolute alcohol added. The tubes were incubated at -20°C overnight before another spin, 13,000rpm for 20 minutes. The supernatant was removed and the pellet was washed twice with 500μl of 70% ethanol diluted in DEPC-treated water. After the final spin the supernatant was removed and the pellet left to air dry at room temperature for 20 minutes. The pellet was then dissolved in 20μl of DEPC-treated water and stored at -70°C before reverse transcription.

- ***Quantification of RNA***

RNA was quantified using two methods: spectrophotometry and by electrophoresis of RNA on an agarose gel. First, 2μl of RNA were dissolved in 500μl of DEPC water and the optical densities of each sample measured at 260nm and 280nm on a spectrophotometer. The ratio of O.D. at 260nm: 280nm gave an indication of the purity of the RNA whilst the concentration of RNA (μg/ml) was calculated by multiplying the O.D. value at 260nm by 40 and by the dilution factor.

By running 1μl of RNA on a 1% agarose gel with ethidium bromide and viewing under U.V. light the amount of RNA isolated could be visualised and its integrity determined.

- ***Reverse Transcription (RT)***

Into a 500μl eppendorf was placed 1μg of RNA and sufficient distilled water to make the total volume 8μl. The RNA samples were heated to 70°C for 4 minutes and then cooled quickly before addition of 12μl of RT mix comprising of 5x RT buffer, 2.5mM dNTP's, 0.1M DTT, random hexamer primers, RNAsin and reverse transcriptase, Moloney murine leukaemia virus (MMLV). The samples were then incubated in a 37°C waterbath for 1.5 hours after which they were then heated to 80°C for 5 minutes and then stored at -70°C.

- ***Competitive Poiymerase Chain Reaction (PCR)***

Competitive RT PCR analysis (Siebert and Larrick, 1993) of cDNA preparations was carried out in 25μl reactions containing DEPC-treated water, 10x thermo buffer, 25mM $MgCl_2$, 25mM dNTP's, 500μg/ml of forward and reverse primers and Taq DNA polymerase. Each reaction also contained a fixed amount of the appropriate PCR mimic as an internal standard. Mimic templates for competitive RT PCR were constructed essentially as described (Siebert and Larrick, 1993) and consisted of luciferase gene sequences flanked by sequences complementary to the forward and reverse primers for the gene of interest. These templates were constructed by PCR amplification of a cloned luciferase gene (pGL2 vector) using tandem primers consisting of the luciferase forward and reverse primers linked to the 3' ends of the forward and reverse primers respectively.

The reaction was carried out for differing numbers of cycles depending on the transcript studied using various annealing temperatures (Table 2.1 and 2.2).

PCR products were fractionated by agarose gel electrophoresis and photographed under UV illumination. Band intensities were quantified by laser densitometric scanning and normalised with respect to the intensity of the mimic band obtained in each amplification reaction. The results were expressed as a ratio of the intensity of the specific band relative to the intensity of the actin band obtained by amplification of the same cDNA. For those PCR reactions where no mimic template had been constructed the reactions were carried out as detailed above with the addition of an extra 1μl of water to replace the mimic template in the 25μl reaction mixture, and the results calculated as a ratio of the specific gene band intensity relative to the intensity of the actin band only.

2.7 Western Blotting

- ***Isolation of Cytoplasmic Proteins***

Endothelial cells were harvested using trypsin/EDTA, washed once in HBSS/5% FCS and again in PBS. The cells were then transferred to a 1.5ml microcentrifuge eppendorf tube and centrifuged at 4-5000rpm for 3 minutes at 4°C. The supernatant was discarded and 100μl of Lysis Buffer with protease inhibitors were added to each sample and the cell suspension pipetted up and down several times, followed by a 15 minute incubation on ice. The cells were then centrifuged at 14000rpm for 5 minutes and the supernatant removed and retained. The lysates were stored at -70°C.

- ***Quantification of Protein in Lysates***

To quantify the protein in lysates a modified Lowry assay was used and this procedure measures as little as 1μg of protein in 800μl of solution. 2μl of sample prepared as detailed as above, were placed into a 1.5ml Eppendorf microcentrifuge tube and diluted to 800μl with water and then chilled for a few

Gene	Sequence 5' 3'	Cycle Number	Tm °C	Mimic Product size nt	cDNA Product size nt
A1 F	GAC TAT CTG CAG TGC GTC CTA CAG	34	56	470	286
R	GGG CAA TTT GCT GTC GTA GAA G				
Bcl-2 F	ACA ACA TCG CCC TGT GGA TGA C	30	56.1	555	409
R	ATA GCT GAT TCG ACG TTT TGC C				
Actin F	TGC TAT CCA GGC TGT GCT AT	30	58	555	485
R	GAT GGA GTT GAA GGT AGT TT				
Bcl-x F	GGA ATT CTT GGA CAA TGG ACT GGT TGA	30	56	555	L 778
R	CCC AAG CTT GTA GAG TGG ATG GTC AGT G				S 589
Bax F	GGA ATT CTG ACG GCA ACT TCA ACT GGG	28	59.2	555	227
R	GGA ATT CTT CCA GAT GGT GAG CGA GG				

Table 2.1 Primer sequences, cycle numbers, annealing temperatures and product sizes for those transcripts studied by competitive RT-PCR F=forward primer, R=reverse primer.

Gene		Sequence 5' 3'	Cycle Number	Tm °C	cDNA Product size nt
E-selectin	F	GCT GTG AGA TGC GAT GCT GTC C	26	62	596
	R	CCT CTA GTT CCC CAG ATG CAC C			
IL-10	F	CTG AGA ACC AAG ACC CAG ACA TCA AGG	36	58	352
	R	CAA TAA GGT TTC TCA AGG GGC TGG GTC			

Table 2.2 Primer sequences, cycle numbers, annealing temperatures and product sizes for those transcripts studied by RT-PCR

minutes on ice. 200μl of ice cold 50%(w/v) trichloroacetic acid were then added and the mixture allowed to sit for at least 30 minutes on ice. The sample was then centrifuged for 2 minutes at 14000rpm and the supernatant was poured away. 200μl of 0.5M NaOH were then added to the invisible pellet and the sample incubated overnight at room temperature. 1ml of 2% $NaCO_3$(w/v)/0.5% $CuSO_4.5H_2O$ (w/v)+1% Na^+-citrate (50:1) was added to the sample, vortexed, and allowed to stand for 10 minutes before addition of 100μl of phenol reagent (Folin-Ciocalteu) diluted 1:2 with water. Following a 30-120 minute incubation at room temperature the absorbance was read on a spectrophotometer at 660nm. A standard curve was also run with each batch of samples and this consisted of 0, 1, 2, 5, 10, 20 and 40μg/ml of BSA in water. Triplicates for each value and sample were used.

- ***Electrophoresis of Proteins***

10μl of x4 Laemmli sample buffer were added to 30μl of sample, boiled for 10 minutes and then 30-40μg/ml of protein run on a 10% SDS-PAGE at 125V. Molecular weight markers were also run.

- ***Transfer of Proteins to Nitrocellulose Membrane***

Nine filter papers were individually soaked in blotting buffer and separately placed on the blotting apparatus ensuring that no bubbles were trapped between the layers. On top of these papers was added a nitrocellulose membrane, also pre-soaked in blotting buffer. The gel was then placed on top of the membrane and a further nine filter papers positioned above. The amps/per hour used to transfer the proteins were determined by the area of the membrane (cm^2) x 0.8mA.

- ***Addition of Antibodies***

After blotting, the filter papers and gel were carefully removed and the membrane blocked in blocking solution and incubated at room temperature for 1-3 hours with continual shaking. Following this, the solution was poured away and 5mls of primary antibody, diluted in blocking buffer, added to the membrane. The membrane was incubated with the primary antibody overnight at 4°C, again with continual shaking. After two quick washes, one 20 minute wash and two 5 minute washes, 5 mls of the HRP linked secondary antibody, diluted in the blocking buffer, were added to the membrane for 2-3 hours at room temperature. Three quick washes, two 20 minute washes and five 5 minute washes then followed in washing buffer.

- ***ECL Development*** **(Enhanced chemiluminescence)**

Excess washing buffer was removed and 6mls of ECL reagents were added to the membrane for 1 minute. The membrane was then wrapped in clingfilm and exposed to X-ray film for a period of time. The film was then developed in an automated processing machine.

2.8 Determination of Endothelial Cell Survival

- ***Morphology***

Following serum starvation endothelial cells and monocytes were removed from the culture plates with the use of trypsin/EDTA and centrifuged at 1000rpm for 5 minutes. The resulting cell pellet was resuspended in 1ml of serum starvation medium and a cell count was performed and cell viability assessed using trypan blue exclusion. Cytospin preparations were made as described in Section 2.2.3 and stained with MGG. Apoptotic cells were identified by the presence of nuclear condensation and fragmentation. All slides were assessed by two observers in a blinded fashion and 200 cells counted.

- ***Annexin/PI Staining***

Cells harvested as above were washed twice in cold PBS and resuspended in 1x binding buffer from the Apoptosis Detection Kit (R&D) to give a final cell concentration of $1x10^6$cells/ml. To $1x10^5$cells, 10μl of fluorescein-conjugated annexin V and/or 10μl of propidium iodide were added and the cells incubated for 15 minutes at room temperature in the dark. 400μl of 1x binding buffer were then added to each tube and the samples were analysed on a flow cytometer. This technique is designed to quantitatively determine the percentage of cells undergoing apoptosis by virtue of their ability to bind annexin V and exclude propidium iodide. Those cells undergoing necrosis will take up both stains whilst live cells will not stain with either annexin V nor propidium iodide (Chapter 4, Figure 4.7).

2.9 Statistics

Statistical analyses were performed using unpaired 2-tailed Student's t-test, and the Graphpad Instat programme on the PC.

Statistically significant differences between results are labelled in the text as such, whilst differences that are not labelled are not statistically significant. In addition, the error bars on Figures and ±numbers in the text are SEM.

Chapter Three

The Influence of Monocytes on E-selectin Gene Expression In Endothelial Cells

Introduction

E-selectin was originally described as an adhesion molecule mediating the binding of neutrophils and monocytes (Leeuwenberg et al, 1992), but more recently it has been shown to participate also in the binding of memory T lymphocytes to activated endothelial cells, supporting the rolling and stable adhesion of leukocytes at sites of inflammation. E-selectin has also been shown to function as a signalling receptor. The lectin-like domains on the extracellular portion of E-selectin bind complex carbohydrate ligands on the surface of leukocytes during their adhesive interactions whilst leukocyte adhesion to activated cultured endothelial cells induces association of the cytoplasmic domains of E-selectin with cytoskeletal elements, suggesting that outside-in signalling may occur during E-selectin mediated adhesion (Yoshida et al, 1998). In addition, E-selectin, which is constitutively phosphorylated in cytokine-activated endothelial cells, undergoes an enzymatically regulated dephosphorylation following leukocyte adhesion, providing additional evidence for a transmembrane signalling function of this molecule during leukocyte-endothelial interactions (Yoshida et al, 1998). Furthermore, engagement of E-selectin with its ligands induces changes in endothelial [Ca^{2+}] and formation of stress fibres (Lorenzon et al, 1998).

The expression of E-selectin has been studied extensively on human umbilical vein endothelial cells (HUVEC). Although not constitutively expressed on HUVECs, E-selectin expression can be transiently induced by treatment with IL-1, TNF, and LPS, peaking after 4-6 hours of stimulation and returning to baseline by 24 hours (Bevilacqua et al, 1989). Levels of expression, *in vivo*, however, do not seem to follow the same transient time course as is observed *in vitro*. Cultured endothelial cells are also refractory to reinduction of E-selectin

by the same stimulus prior to a requisite recovery period of 12 hours (Sepp et al, 1994) suggesting that other factors *in vivo* must influence E-selectin expression.

Monocytes are implicated in the vascular pathology of atherosclerosis and cellular interactions between monocytes and endothelial cells can influence inflammatory events such as cytokine production. Monocyte-endothelial cell interaction induces monocytic tissue factor (Collins et al, 1996), monocyte chemoattractant protein-1 (MCP-1) (Lukacs et al, 1995), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Takahashi et al, 1996) in monocytes and endothelial cells suggesting that their interaction activates both cell types. Thus monocytes interacting with endothelial cells are likely to influence inflammatory responses of the endothelium, including the expression of surface adhesion molecules. The aims of this chapter were to investigate the ability of monocytes to regulate E-selectin expression on endothelial cells, and the mechanisms whereby such effects are achieved.

Results

Monocytes induce E-selectin expression on endothelial cells

Endothelial cells do not constitutively express E-selectin protein (Figure 3.1a), however induction of E-selectin protein expression on endothelial cells occurs following treatment with IL-1(10U/ml), (O.D. 0.56±0.12 on IL-1 treated endothelial cells compared to 0.02±0.005 on control endothelial cells at 4 hours, $p<0.001$, $n=6$, Figure 3.1a). After 21 hours of IL-1 treatment E-selectin protein on the surface of endothelial cells is still detectable (O.D. 0.3±0.07 on IL-1 treated endothelial cells compared to 0.02±0.005 on control endothelial cells, $p<0.005$, $n=6$, Figure 3.1a), but the levels are lower than those measured at 4 hours.

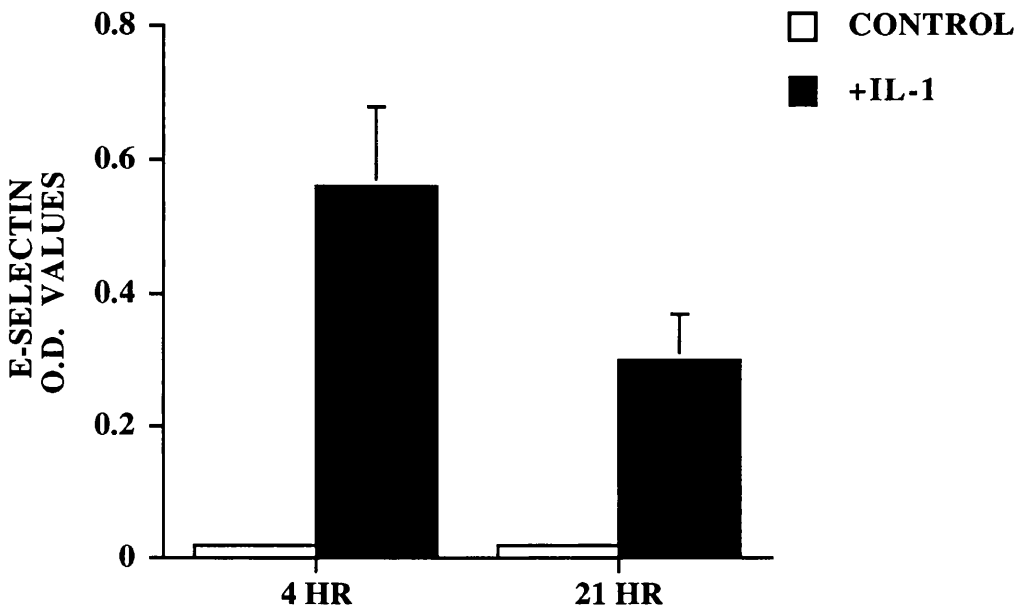

Figure 3.1a IL-1 induces E-selectin protein expression on endothelial cells. Endothelial cells were cultured as described in the Methods, and were grown to confluence in 96 well plates over 48 hours in complete growth media. The endothelial cells were then washed and incubated with IL-1 (10U/ml). E-selectin protein expression was measured using ELISA at 4 and 21 hours of stimulation. Data shown are mean ± s.e.m. of 3 experiments.

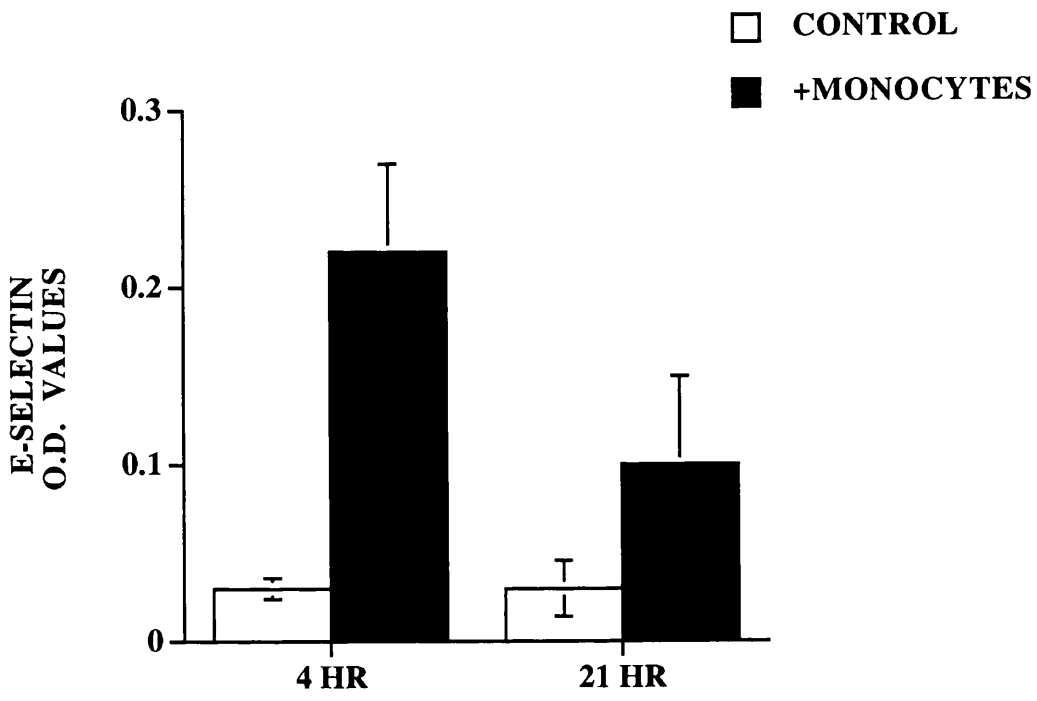

Figure 3.1b Monocyte coculture induces E-selectin protein expression on endothelial cells. Endothelial cells were cultured as detailed in Figure 3.1a and monocytes were isolated as described in the Methods. Endothelial cells were washed prior to the addition of monocytes and E-selectin protein expression determined by ELISA after 4 and 21 hours of coculture. Data are mean ± s.e.m. of 3 experiments.

Coculture of monocytes with endothelial cells leads to a significant induction of E-selectin protein expression on endothelial cells (Figure 3.1b). At 4 hours the O.D. on endothelial cells cocultured with monocytes is 0.22±0.05 compared to 0.03±0.01 on control endothelial cell (n=3, $p<0.05$, Figure 3.1b), and at 21 hours E-selectin protein is still detectable on endothelial cells cocultured with monocytes (O.D. 0.1±0.05 with monocyte coculture compared to 0.03±0.02 on control cells, n=3, Figure 3.1b).

The induction of E-selectin was directly proportional to the number of monocytes seeded, with maximal induction seen at an endothelial cell/monocyte ratio of 1:1 (Figure 3.1c). In addition, there was no specific binding of anti-E-selectin monoclonal antibody to monocytes under the conditions used (data not shown).

Neutrophils and Lymphocytes do not induce E-selectin protein expression on endothelial cells

In order to investigate whether other leukocytes, when cocultured with endothelial cells, also induce endothelial E-selectin protein expression, neutrophils and lymphocytes were cultured with endothelial cells for 21 hours before an ELISA was performed to detect E-selectin protein expression. Whilst monocytes significantly upregulated E-selectin expression on endothelial cells (O.D. 0.58±0.07 on endothelial cells cocultured with monocytes compared to 0.1±0.04 on control endothelial cells, n=3, $p<0.005$, Figure 3.2) neither lymphocytes nor neutrophils induced E-selectin expression on endothelial cells at this time point (Figure 3.2). In addition, in one experiment, the monocytic cell line, THP-1, induced E-selectin upregulation on endothelial cells (O.D. of 0.45 at 4 hours and sustained at 21 hours with O.D. of 0.33, compared to 0.05 on

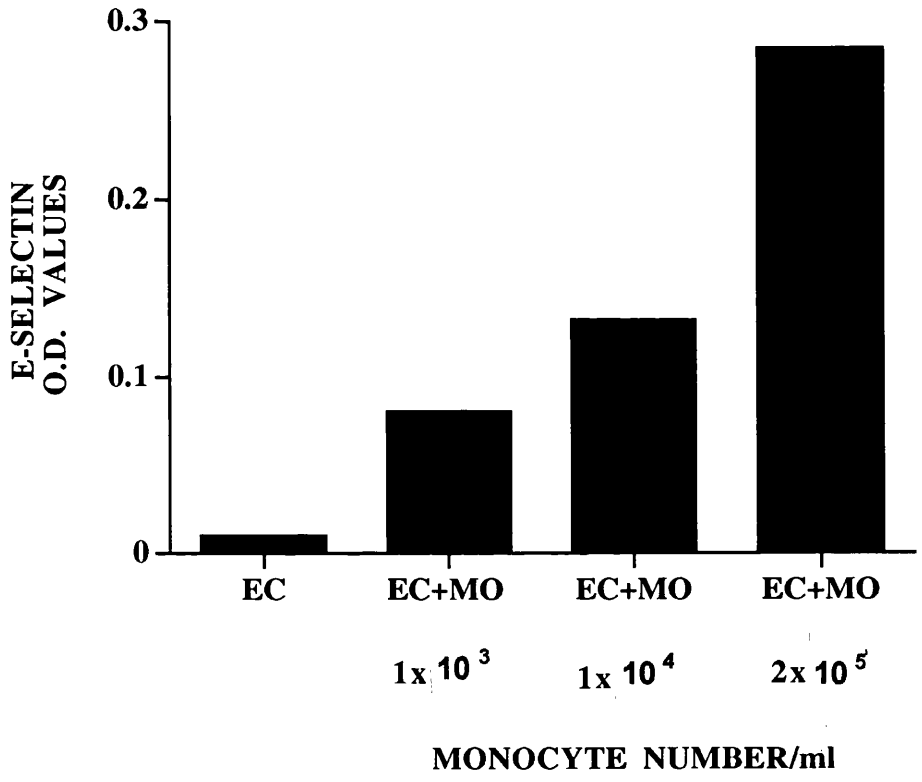

Figure 3.1c Monocyte induction of E-selectin protein is dependent on monocyte number. Endothelial cells and monocytes were cultured as detailed in Figure 3.1b. Three concentrations of monocytes were used and E-selectin protein expression determined after 4 hours. Data are from one representative experiment.

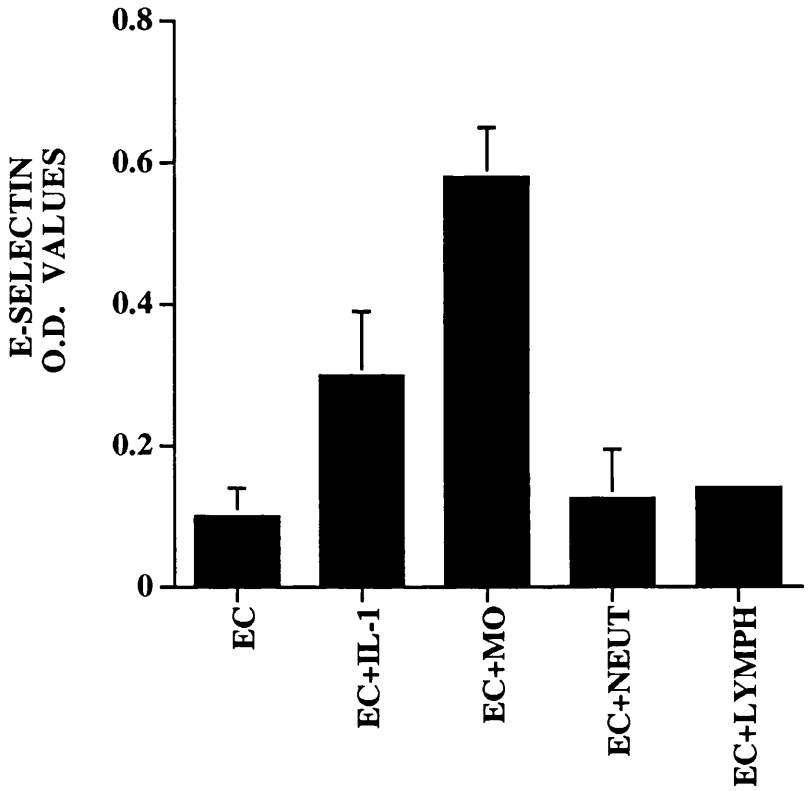

Figure 3.2 Neutrophils and lymphocytes do not induce E-selectin protein expression on endothelial cells. Endothelial cell cultures were set up as described for Figure 3.1b, except that neutrophils or lymphocytes were also added, at the same concentration as the monocytes ($2x10^5$/ml) for 21 hours. Data are mean ± s.e.m. of 3 experiments for IL-1, monocytes and neutrophils, and one representative experiment for lymphocytes. IL-1 (10U/ml).

unstimulated endothelial cells, data not shown). There is some platelet contamination of the monocyte preparation (< 1%) but the ability of THP-1 cells to induce E-selectin on endothelial cells suggests that platelets contribute little to monocyte induction of E-selectin in this system.

Monocytes induce E-selectin mRNA in endothelial cells

Next, I investigated whether increased E-selectin protein in endothelial cells cocultured with monocytes was due to changes in gene transcription. Control endothelial cells do not express any E-selectin mRNA but following 3 hours of coculture with monocytes E-selectin mRNA appears (E-selectin/actin ratio 0.75 in endothelial cells cocultured with monocytes for 3 hours, Figure 3.3) and remains present up to 30 hours after the start of the coculture (E-selectin/actin ratio 0.48 in endothelial cells cocultured with monocytes for 30 hours, Figure 3.3). In contrast, when endothelial cells were stimulated with IL-1, E-selectin mRNA was strongly induced by 6 hours (E-selectin/actin ratio 0.7 in endothelial cells treated with IL-1 for 6 hours) but was markedly reduced at 24 hours (E-selectin/actin ratio 0.2 in endothelial cells treated with IL-1 for 24 hours compared to 0.6 in endothelial cells cocultured with monocytes for 24 hours, Figure 3.3). Monocytes do not express E-selectin mRNA (Figure 3.3).

Monocytes enhance and sustain the response to IL-1

As illustrated above, IL-1 acts to upregulate E-selectin expression in endothelial cells, and this occurs in a dose dependent manner, with near maximal effects seen at 10U/ml. Coculture with monocytes enhances the response of endothelial cells to suboptimal concentrations of IL-1 at 4 hours (Figure 3.4a), however, the most striking effect of monocytes was seen at 21 hours when IL-1 induced E-selectin expression began to decline (Figure 3.4b).

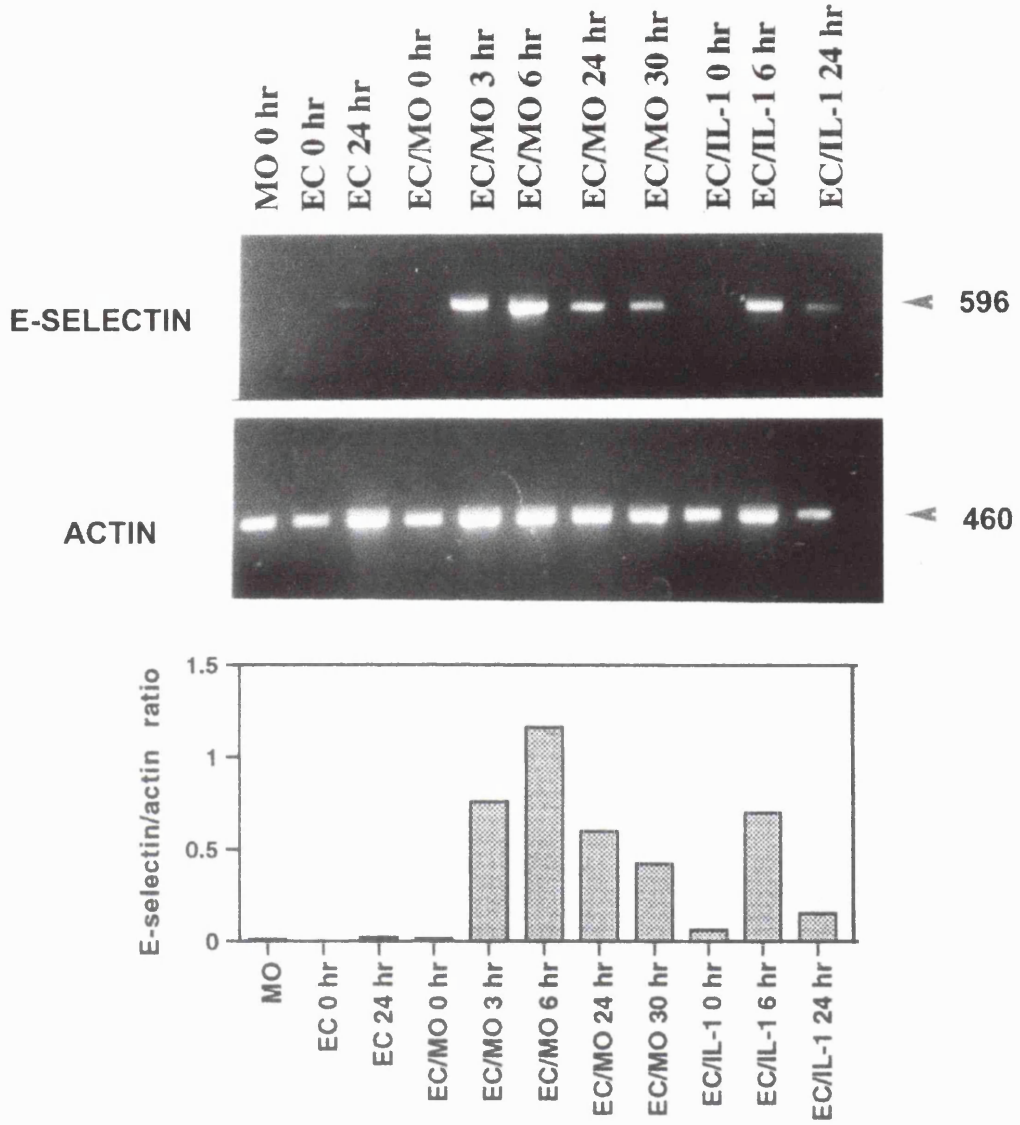

Figure 3.3 Monocytes induce E-selectin mRNA expression in endothelial cells. Confluent endothelial cells were washed before the addition of monocytes or IL-1(10U/ml) for 3, 6, 24 and 30 hours. RNA isolation and RT-PCR were performed as described in the Methods. MO=monocytes; EC/MO=endothelial cells cocultured with monocytes; EC/IL-1= endothelial cells stimulated with IL-1. Bands from one representative experiment. The histogram displays E-selectin/actin ratios. Molecular weights of the products are indicated.

In the presence of monocytes, E-selectin expression remains high, even at substimulatory concentrations of IL-1 (Figure 3.4b).

Cell:cell contact is required for optimal monocyte induction of E-selectin

Having shown that monocytes cocultured with endothelial cells induce upregulation of E-selectin in endothelial cells, I proceeded to investigate whether this induction was dependent on contact between the two cell types. Monocytes were cultured above endothelial cells on 0.45 micron filters in a Transwell system. Monocytes in contact with endothelial cells increased E-selectin expression from an O.D. of 0.002±0.001 to 0.34±0.12 at 21 hours (n=3, $p<0.05$, Figure 3.5a). When monocytes were separated from the endothelial cells, E-selectin expression was reduced by 87±5% to 0.04±0.03 (n=3, $p<0.05$, Figure 3.5a).

These results suggest that contact between monocytes and endothelial cells is needed for maximal induction of E-selectin. One possible mechanism is that adhesion molecule ligation generates a signal in the endothelial cells leading to the induction of E-selectin gene expression. Ligation could also stimulate cytokine release or, since the cells are in close contact, membrane bound cytokines such as mTNF on monocytes could bind to receptors on endothelial cells.

In order to investigate whether monocyte derived soluble factors were inducing E-selectin expression on endothelial cells, supernatants from monocytes cultured for 21 hours were added to endothelial cells for a further 4 and 21 hours. In two experiments, the addition of monocyte derived supernatants to endothelial cells increased E-selectin expression at 4 hours (Figure 3.5b). At 21 hours, endothelial cells treated with monocyte derived supernatants still expressed E-selectin protein (Figure 3.5c). The addition of monocyte derived

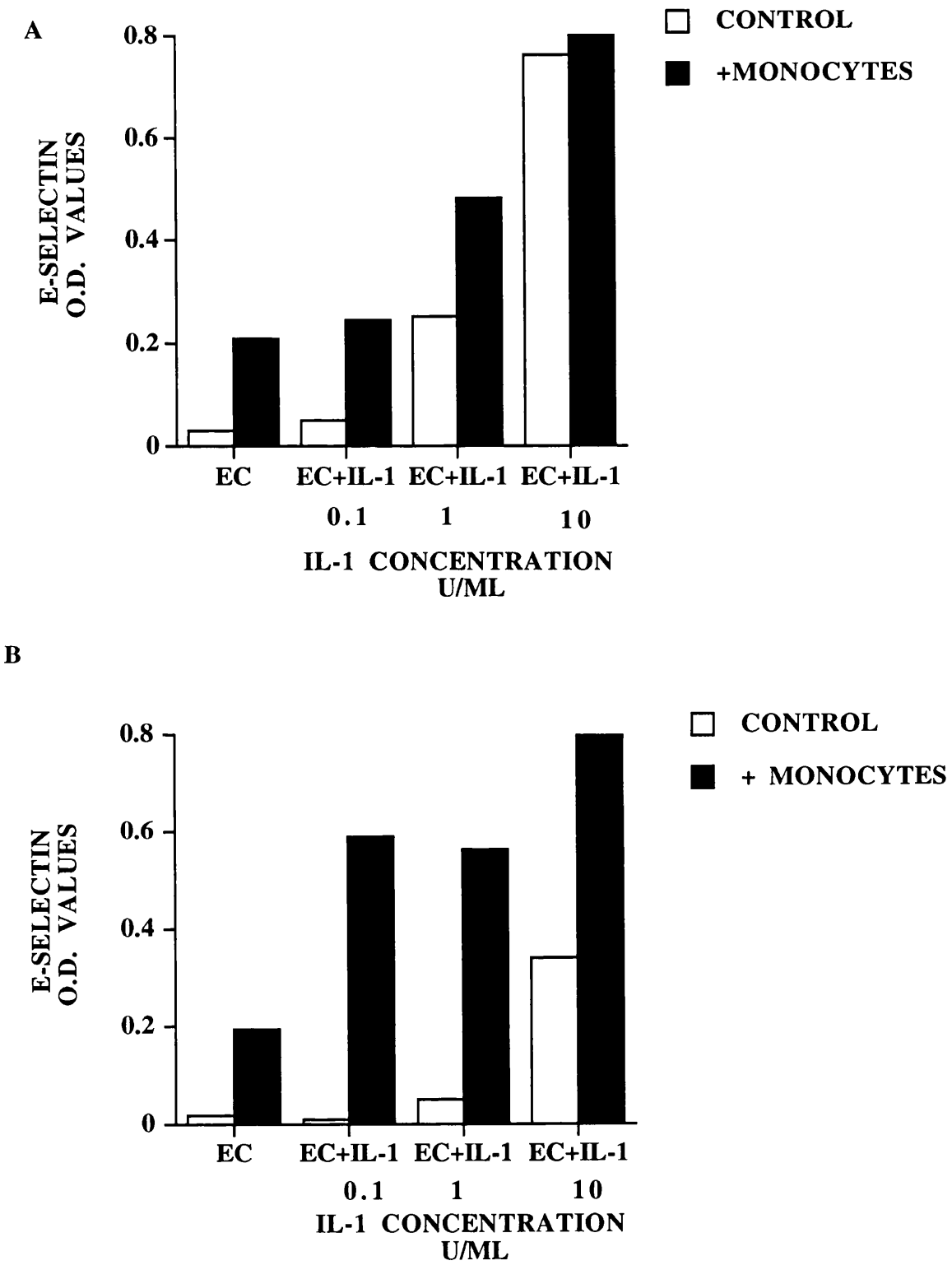

Figure 3.4 Monocytes sustain and enhance the IL-1 induction of E-selectin protein expression on endothelial cells. Endothelial cells and monocytes were prepared as detailed in Figure 3.1b. IL-1 at varying concentrations was added to the endothelial cells in the presence or absence of monocytes. Monocytes were added at a cell concentration of $2x10^5$/ml. An ELISA was used to determine E-selectin protein expression on endothelial cells after A) 4 hours and B) 21 hours. Data shown from one representative experiment.

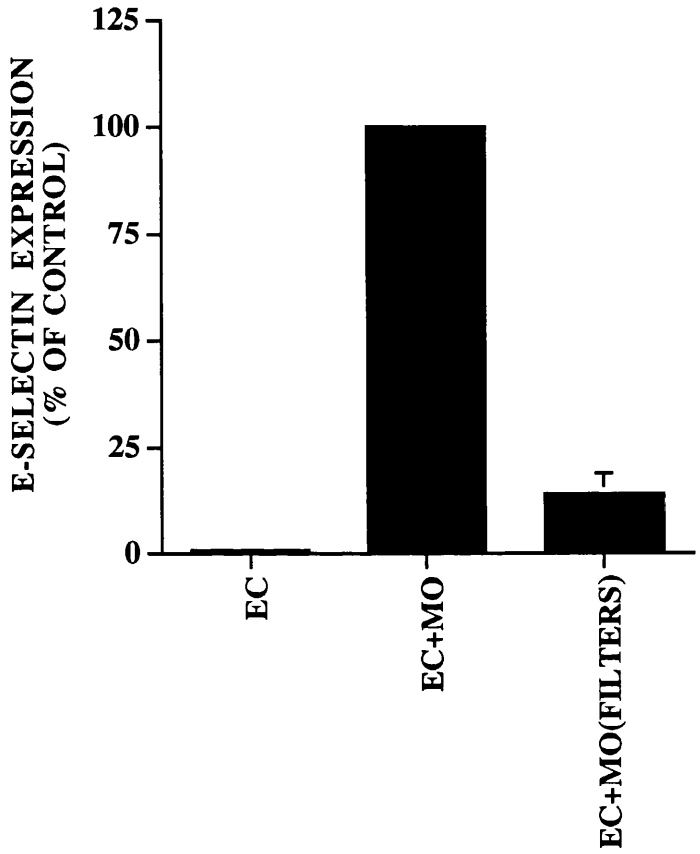

Figure 3.5a Cell:cell contact is required for maximal monocyte induction of E-selectin protein on endothelial cells. Endothelial cell cultures were set up as described, except that contact between endothelial cells and monocytes was prevented by placing the monocytes on a 0.45-μm pore filter above the endothelial cells, EC+MO(FILTER). Control cocultures with endothelial cells and monocytes in contact were conducted in parallel, EC+MO. E-selectin protein expression was determined at 21 hours by ELISA. Data have been normalised so that the E-selectin expression on endothelial cells in contact with monocytes is designated 100%. Data are mean ± s.e.m. of 3 experiments.

supernatants to IL-1 stimulated endothelial cells enhances the IL-1 response at suboptimal concentrations, at both 4 hours (Figure 3.5b) and at 21 hours (Figure 3.5c).

In one experiment, supernatants from 21 hour monocyte/endothelial cell cocultures were added to endothelial cells for 4 and 21 hours. There was no induction of E-selectin on endothelial cells treated with monocyte/endothelial cell coculture supernatant at 4 hours (O.D. 0.03 on endothelial cells treated with the supernatant compared to 0.02 on endothelial cells alone, data not shown), nor at 21 hours (O.D. 0.02 on endothelial cells treated with the supernatant compared to 0.01 on endothelial cells alone, data not shown).

Role of TNF in monocyte induction of E-selectin

TNF induces E-selectin expression in endothelial cells maximally at 4 hours (O.D. 0.57 on endothelial cells treated with 100U/ml TNF at 4 hours compared to 0.05 on control cells, one representative experiment, Figure 3.6a), after which the expression falls by 62% over 24 hours (Figure 3.6a).

TNF is synthesised by stimulated monocytes and the binding of monocytes to endothelial cells enhances TNF production by monocytes (Fan et al, 1993), thus it is possible that TNF could be involved in the monocyte induction of E-selectin on endothelial cells cocultured with monocytes. In order to investigate the role that TNF plays in this induction, an anti-TNF monoclonal antibody (mAb), was included in 21 hour cocultures and E-selectin protein expression on endothelial cells determined by ELISA. Preincubation of monocytes with anti-TNF mAb (20μg/ml), which was also present throughout the experiment, produced a 66±5% reduction in E-selectin upregulation in unstimulated endothelial cell/monocyte cocultures (O.D. 0.34±0.07 on endothelial cells cocultured with monocytes compared to 0.15±0.05 on endothelial cell/monocyte

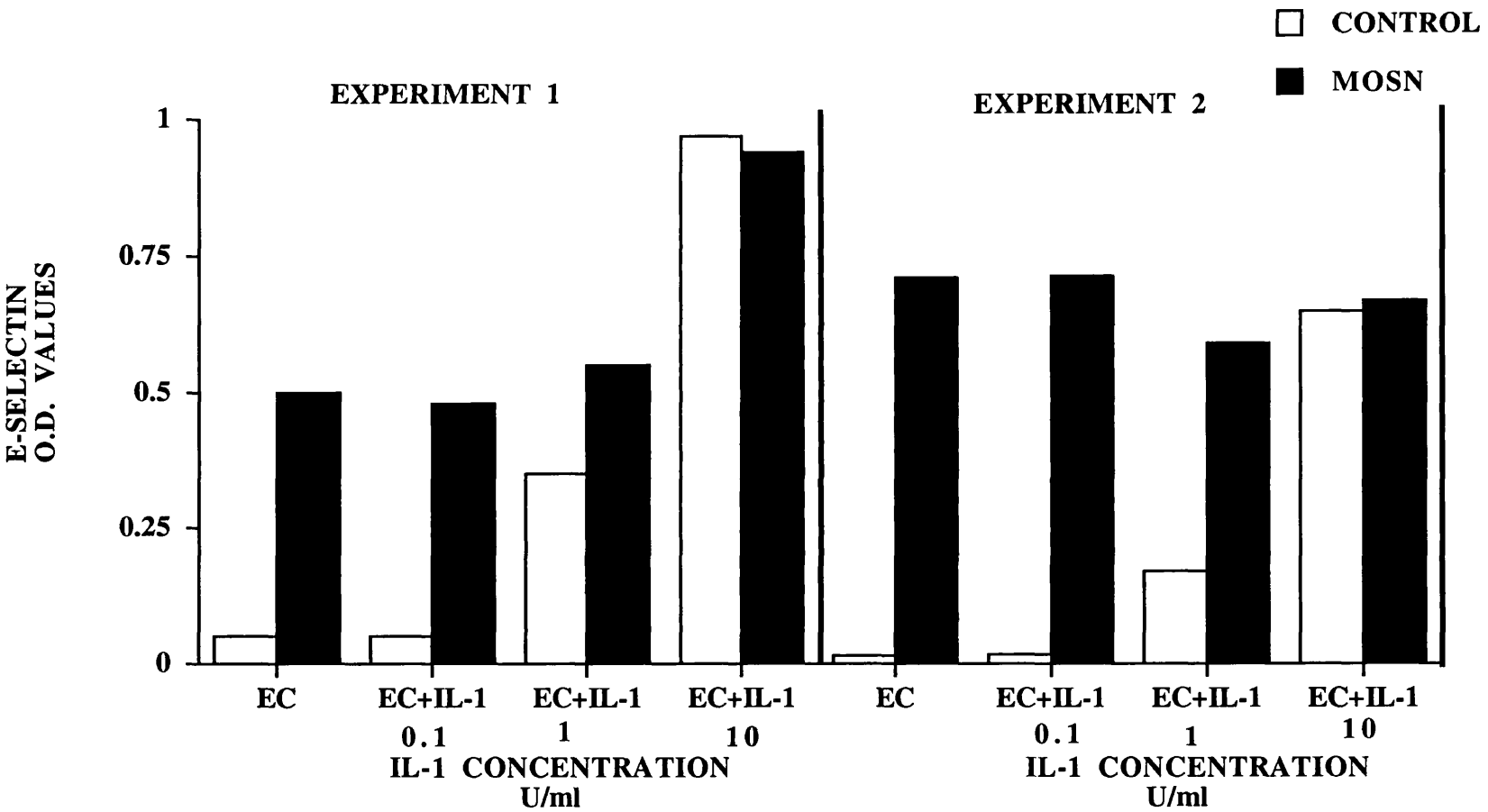

Figure 3.5b Effect of monocyte supernatants on E-selectin protein expression on endothelial cells. Monocyte supernatants were collected from monocytes cultured alone in growth medium for 21 hours. Endothelial cells were cultured as described, and were then treated with monocyte supernatants (MOSN) for 4 hours in the absence or presence of varying concentrations of IL-1 (EC+IL-1). CONTROL=endothelial cells not treated with MOSN. E-selectin protein expression was determined by ELISA. Two separate experiments are shown.

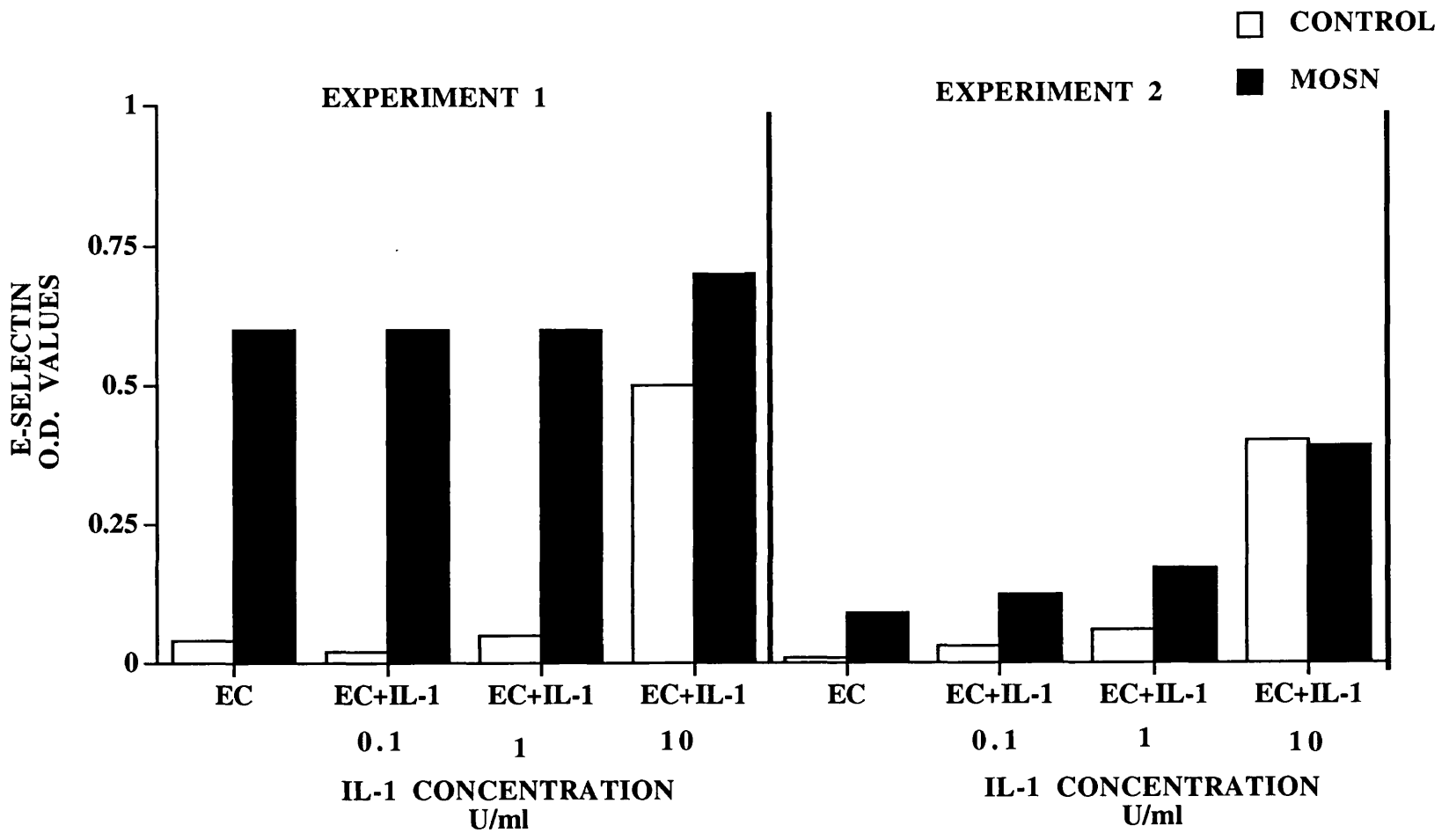

Figure 3.5c Effect of monocyte supernatants on E-selectin protein expression on endothelial cells. The experiment was set up as described for Figure 3.5b, except that the monocyte supernatants were incubated with the endothelial cells for 21 hours. Two separate experiments are shown.

cocultures with anti-TNF mAb, n=3, $p<0.05$, Figure 3.6b). Anti-TNF mAb also reduced the effect of monocytes in IL-1 stimulated cocultures with an inhibition of 43±21% with 1U/ml of IL-1 (n=3, Figure 3.6b), while on endothelial cells treated with 10U/ml of IL-1, anti-TNF inhibited the monocyte response by 57±14% (n=3, Figure 3.6b). Anti-TNF had no effect on the IL-1 induction of E-selectin (Figure 3.6b)

Anti-TNF mAb did not however completely block the monocyte induction of E-selectin in control cocultures. In addition, when used in cocultures also treated with IL-1 the anti-TNF antibody did not bring E-selectin expression down to the levels of expression on endothelial cells treated only with IL-1(n=3, Figure 3.6b). This suggests that there is a significant component of the response that is independent of TNF, and that the ability of monocytes to regulate E-selectin levels on endothelial cells cannot solely be attributed to monocyte-derived TNF.

Effect of antibodies to CD11b/CD18 on monocyte induction of E-selectin

Ligation of CD11b/CD18 by agonistic monoclonal antibodies has been shown to enhance the TNF response in monocytes (Fan et al, 1994). Thus, this receptor-ligand pathway might also be important in monocyte induced E-selectin expression, partly by augmenting TNF production in monocytes, but also by directly activating monocytes and/or endothelial cells. In addition these antibodies can block monocyte adhesion and reduced monocyte adhesion may also alter E-selectin induction. Inclusion of 7E3, an anti-CD11b antibody that recognises an activation induced epitope on the CD11b molecule, inhibited monocyte induction of E-selectin in cocultures by 29±8% at 21 hours (O.D. 0.24±0.03 on endothelial cells cocultured with monocytes and 7E3 compared to 0.36±0.03 on control endothelial cells, n=4, $p<0.05$, Figure 3.7). M170, another anti-CD11b antibody, that has been shown to produce significant inhibition of

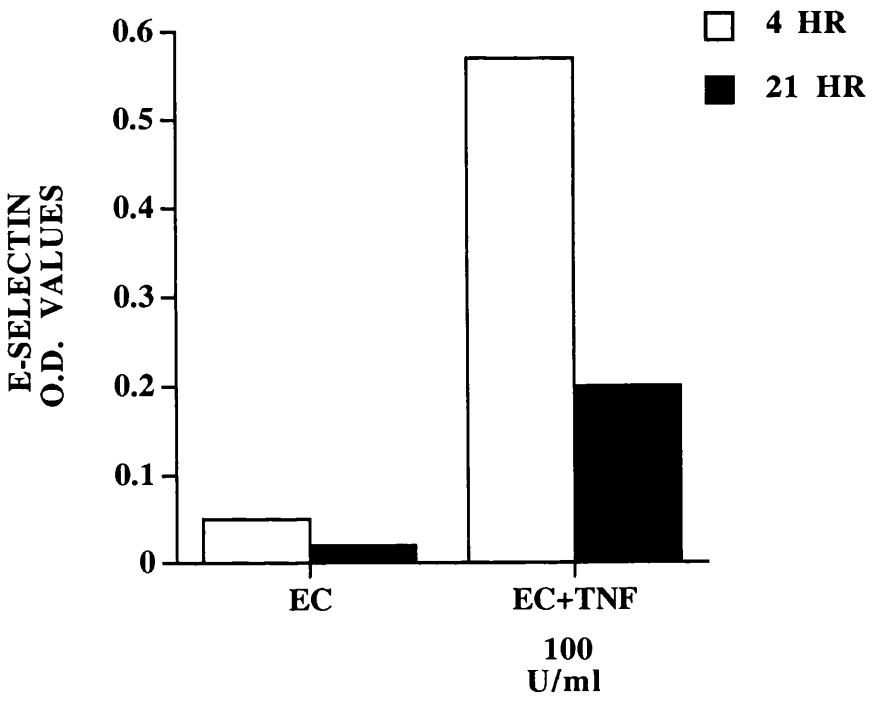

Figure 3.6a TNF induces E-selectin protein expression on endothelial cells. Endothelial cells were set up as described and TNF (100U/ml) added to washed confluent endothelial cells. E-selectin protein expression was determined by ELISA after 4 and 21 hours. Data from one representative experiment.

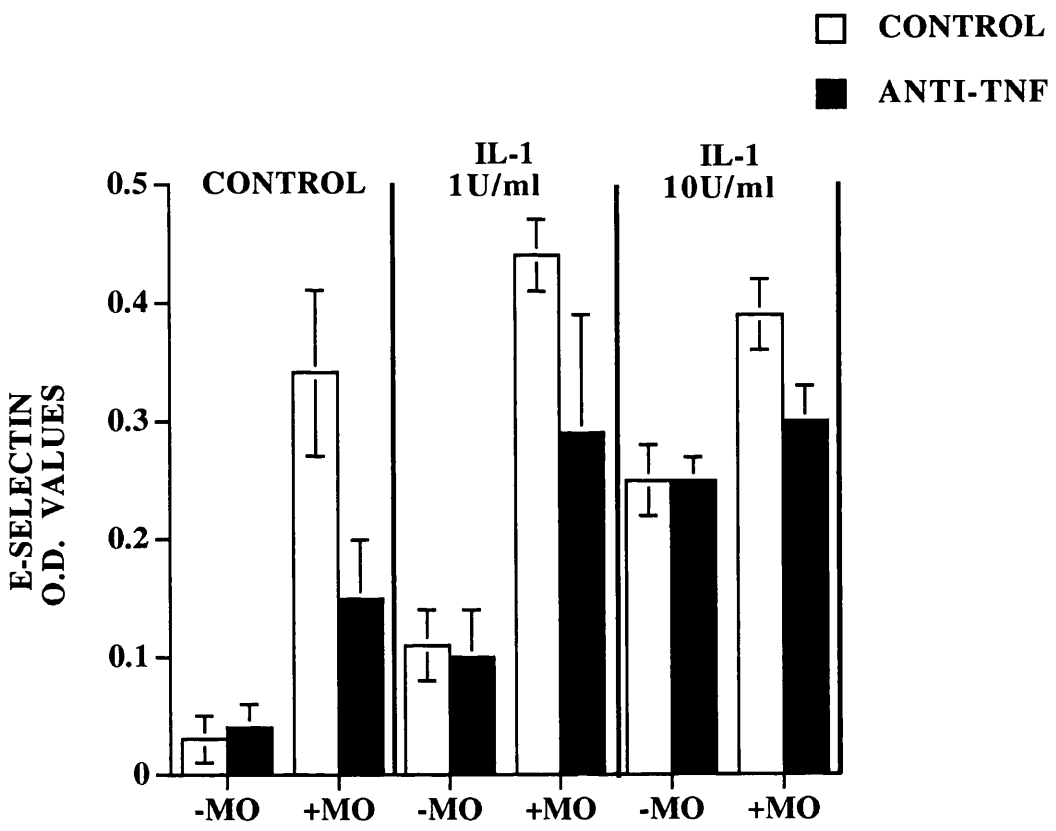

Figure 3.6b Role of TNF in monocyte induction of E-selectin protein on endothelial cells. Endothelial cells were cultured as described. Monocytes were preincubated with anti-TNF mAb (20μg/ml) for 15 minutes prior to addition to endothelial cells, and remained present throughout the experiment, 18 hours. An isotype IgG control mAb was included in control cocultures. IL-1 was added at two concentrations concurrently with the monocytes. An ELISA was used to determine E-selectin protein expression on endothelial cells. Data are mean ± s.e.m. of 3 experiments.

TNF release by monocytes binding to endothelial cells (Fan et al, 1993), also reduced the E-selectin expression on endothelial cells cocultured with monocytes at 21 hours, from O.D. of 0.3±0.04 to 0.16±0.6 (50±16% inhibition, n=6, Figure 3.7). The anti-CD18 antibody, 60.3, also reduced the monocyte induction of E-selectin expression at 21 hours (36±12% inhibition, n=5, Figure 3.7). In addition, anti-PECAM-1 reduced E-selectin mRNA expression in endothelial cells cocultured with monocytes, at both 4 and 21 hours. Anti-PECAM-1 inhibited the monocyte induction of E-selectin mRNA by 35% at 4 hours and by 86% at 21 hours (Figure 3.8). Anti-PECAM-1 had no effect on the ability of TNF and IL-1 to induce E-selectin mRNA expression at either 4 or 21 hours (Figure 3.8).

Antibody against VLA-4 had no inhibitory effect on the monocyte induction of E-selectin at 21 hours. In two experiments, E-selectin expression on endothelial cells cocultured with monocytes in the presence of anti-VLA-4 was 114% and 175% of that seen on cocultures in the presence of control IgG (data not shown). In addition, DREG-56, an antibody against L-selectin had no effect on the monocyte induction of E-selectin at 21 hours when included with cocultures in two experiments (E-selectin expression in the presence of anti-L-selectin was 133% and 84% of that seen on control endothelial cell/monocyte cocultures, data not shown).

Role of ECM proteins in the monocyte induction of E-selectin

The adherence of monocytes to extracellular matrix proteins has been reported to induce expression of inflammatory genes (Lin et al, 1994). Thus in my experiments, monocytes adhering to ECM proteins on endothelial cells may be triggered to upregulate TNF production. The endothelial cells are cultured on fibronectin-coated plastic and hence it is also possible that monocytes adhering

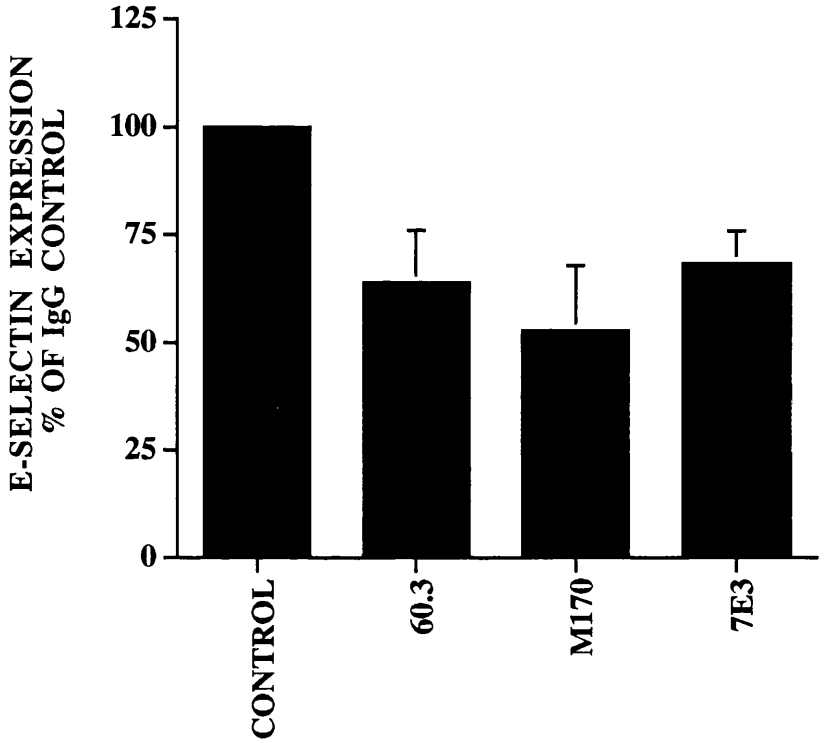

Figure 3.7 Role of adhesion molecules in monocyte induction of E-selectin on endothelial cells. Endothelial cells were cultured as described. mAb's were added to the monocytes for 15 minutes before the start of the coculture period and were present throughout the duration of the experiment, 18 hours. Results are the mean ± s.e.m. of 4 experiments for 7E3, 5 experiments for 60.3 and 6 experiments for M170. The data have been normalised so that the E-selectin expression on endothelial cell cocultures in the presence of an isotype control mAb (Control) is designated 100%.

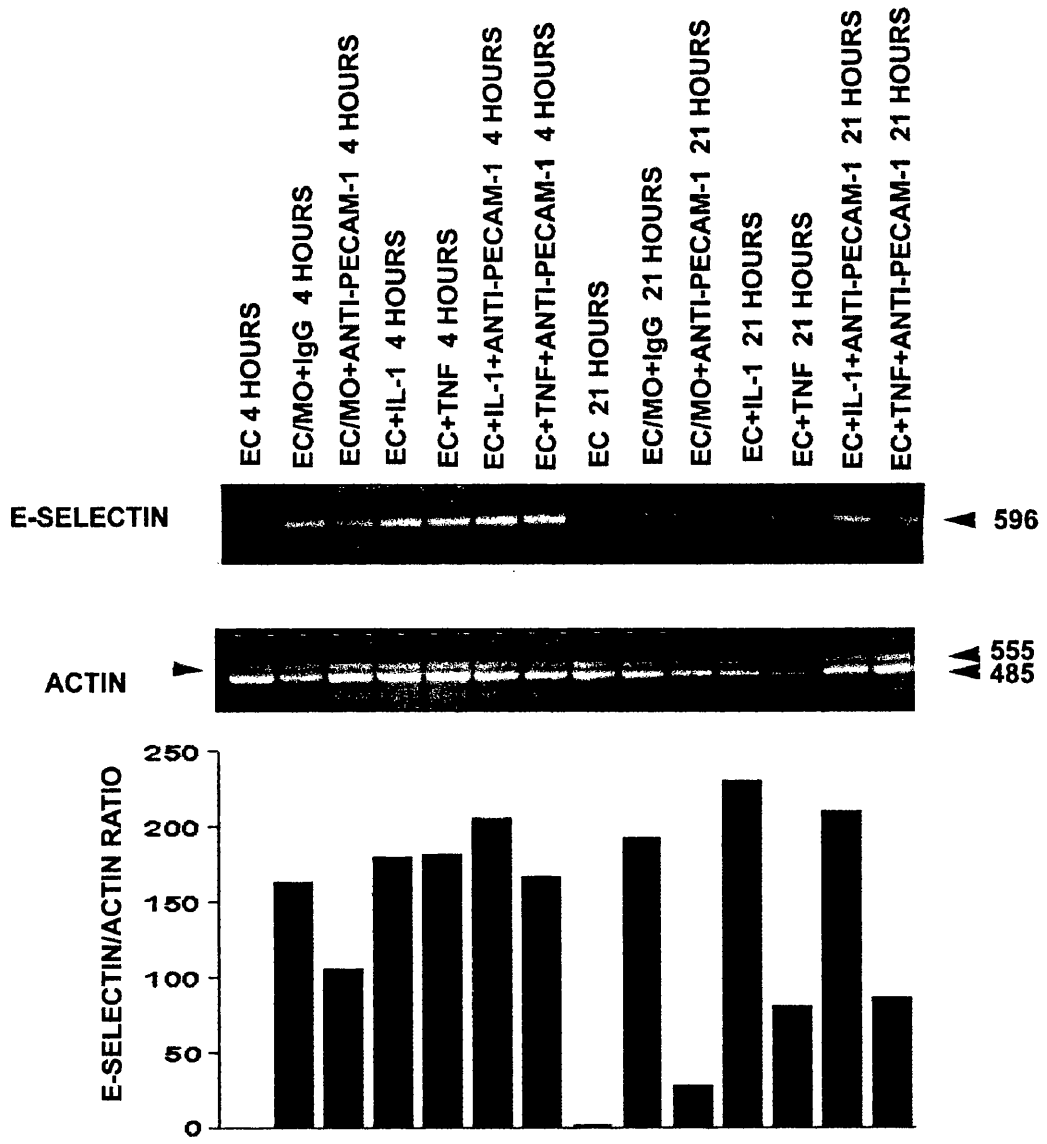

Figure 3.8 Role of PECAM-1 in monocyte induction of E-selectin on endothelial cells. Confluent endothelial cells were washed before the addition of monocytes or IL-1 (20U/ml) or TNF (100U/ml). Monocytes were preincubated with anti-PECAM-1 mAb (20μg/ml) for 15 minutes prior to the start of the experiment and the mAb was present throughout the duration of the coculture; endothelial cells stimulated with IL-1 or TNF had anti-PECAM-1 mAb added concurrently with the cytokines. RNA isolation and RT-PCR were performed as described in the Methods. Notations are as detailed in Figure 3.3; EC/MO+IgG=endothelial cells cocultured with monocytes in the presence of isotype IgG control; EC/TNF=endothelial cells stimulated with TNF. Bands are from one representative experiment. Actin PCR incorporates a mimic template, highlighted by arrow. The histogram displays E-selectin/actin ratios. Molecular weights are indicated.

to exposed fibronectin could become activated. Coculture experiments were therefore conducted using endothelial cells grown on gelatin-coated plastic and as seen in Figure 3.9a, these cultures responded to monocyte coculture in a similar manner to endothelial cells cultured on fibronectin. Coculture with monocytes increased E-selectin expression and enhanced the IL-1 response in endothelial cells grown on gelatin (Figure 3.9a).

To investigate whether interactions with ECM proteins might be responsible for monocyte induction of E-selectin on endothelial cells, soluble forms of such proteins were tested for the ability to inhibit E-selectin induction in the coculture system. The inclusion of collagen I reduced E-selectin expression in endothelial cell/monocyte cocultures from 0.18±0.09 on control cocultures to 0.13±0.08 (33±16% inhibition of the monocyte induction, n=4, Figure 3.9b) while collagen IV was also inhibitory (32±0.9% inhibition of the monocyte induction, n=3, Figure 3.9b). Fibronectin inhibited the monocyte induction of E-selectin by a smaller degree than collagen I and IV, reducing expression from O.D. 0.38±0.02 on control cocultures to 0.32±0.02 on cocultures treated with fibronectin, n=5, Figure 3.9b). The addition of laminin to monocyte cocultures had no effect on E-selectin induction. In two experiments, E-selectin expression on endothelial cells following monocyte coculture in the presence of laminin was 108% and 93% of that seen in endothelial cells cocultured with monocytes (data not shown).

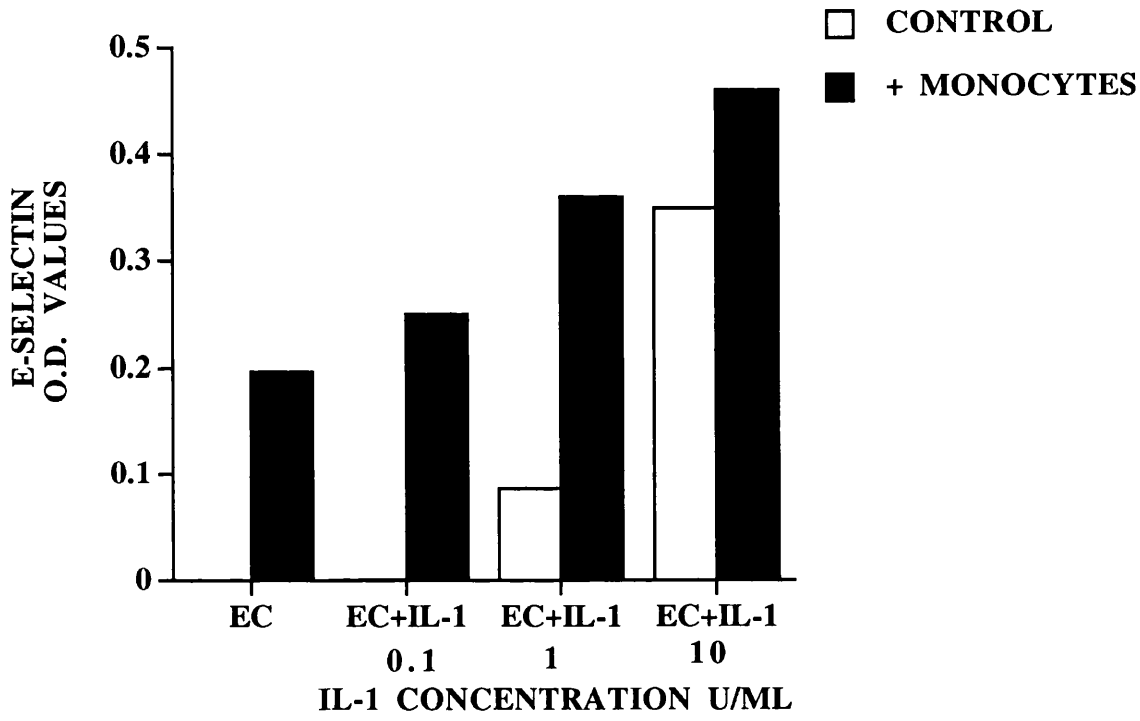

Figure 3.9a Effect of IL-1 and monocytes on endothelial cells cultured on gelatin. Endothelial cells were grown to confluence on gelatin coated 96 well plates, washed and monocytes ± IL-1 were added. E-selectin expression was determined by ELISA after 18 hours incubation. Data from one representative experiment.

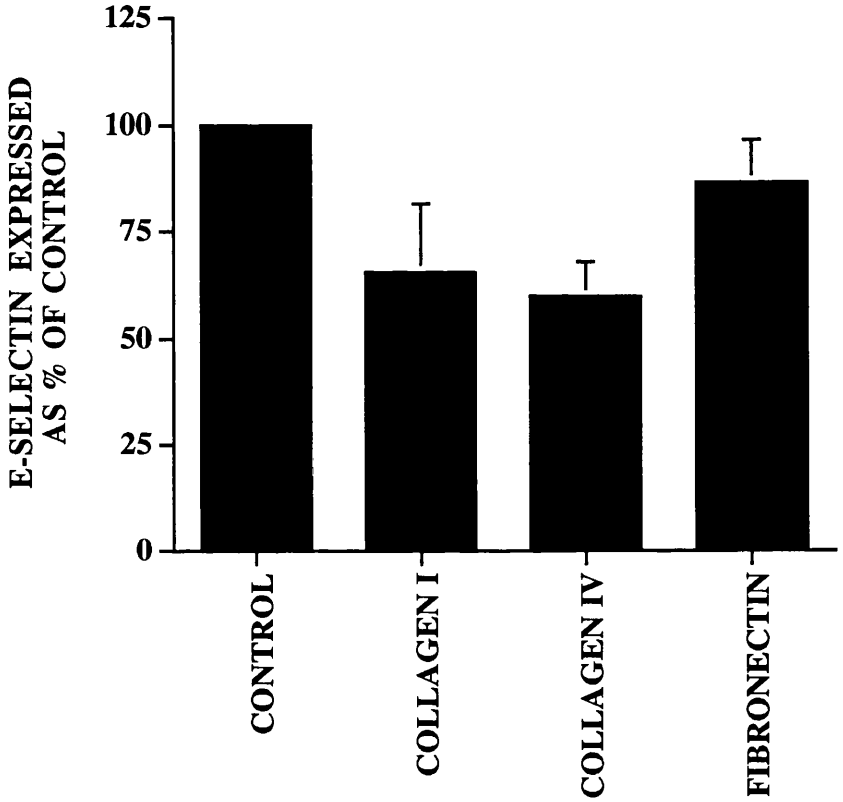

Figure 3.9b Role of extracellular matrix proteins in monocyte induction of E-selectin protein on endothelial cells. Endothelial cells were cultured as described. Monocytes were preincubated with 50μg/ml of fibronectin, and 60μg/ml of collagen I and IV for 30 minutes at room temperature prior to addition to endothelial cells, and ECM proteins remained present throughout the experiment. Results are the mean ± s.e.m. of 5 experiments for fibronectin, 4 experiments for collagen I and 3 experiments for collagen IV. The data have been normalised so that the E-selectin expression on endothelial cells cocultured with monocytes alone (Control) is designated 100%.

Role of NF-κB in the monocyte induction of E-selectin

TPCK, a protease inhibitor, blocks monocyte induction of E-selectin on endothelial cells

Induction of E-selectin by TNF and other inflammatory cytokines is regulated at the level of gene transcription and is mediated by the transcription factor nuclear factor-κB (Pierce et al, 1996; Min et al, 1997). In order to investigate whether monocyte induction of E-selectin was also dependent on NF-κB activation, n-tosyl-Phe-chloromethylketone (TPCK), a protease inhibitor that blocks activation of NF-κB was added to the cocultures and endothelial E-selectin expression was then measured using ELISA. Monocytes cocultured with endothelial cells for 4 hours increased endothelial E-selectin expression ($p<0.01$, n=3, Figure 3.10a). Preincubation of monocytes with 25μM or 50μM of TPCK for 30 minutes prior to addition to endothelial cells resulted in significant inhibition of the monocyte induction of E-selectin (Figure 3.10a). Using 25μM of TPCK monocyte induction of E-selectin was inhibited by 78±8% ($p<0.05$, n=3, Figure 3.10a). A similar degree of inhibition was seen using 50μM of TPCK (Figure 3.10a). In these experiments, monocytes were washed to remove excess TPCK before addition to endothelial cells. To ensure that the TPCK had been effectively removed by washing and was not exerting a direct effect on endothelial cells, endothelial cells cocultured with TPCK treated monocytes were stimulated with IL-1(1U/ml) for 4 hours. These endothelial cells responded to IL-1 stimulation with upregulation of E-selectin comparable to the levels on endothelial cells treated only with IL-1(data not shown). E-selectin upregulation was also inhibited by pretreating endothelial cells with TPCK prior to the addition of monocytes. Treatment with 25μM of TPCK inhibited E-selectin upregulation by 75±13% ($p<0.05$, n=3, Figure 3.10a), while at the higher

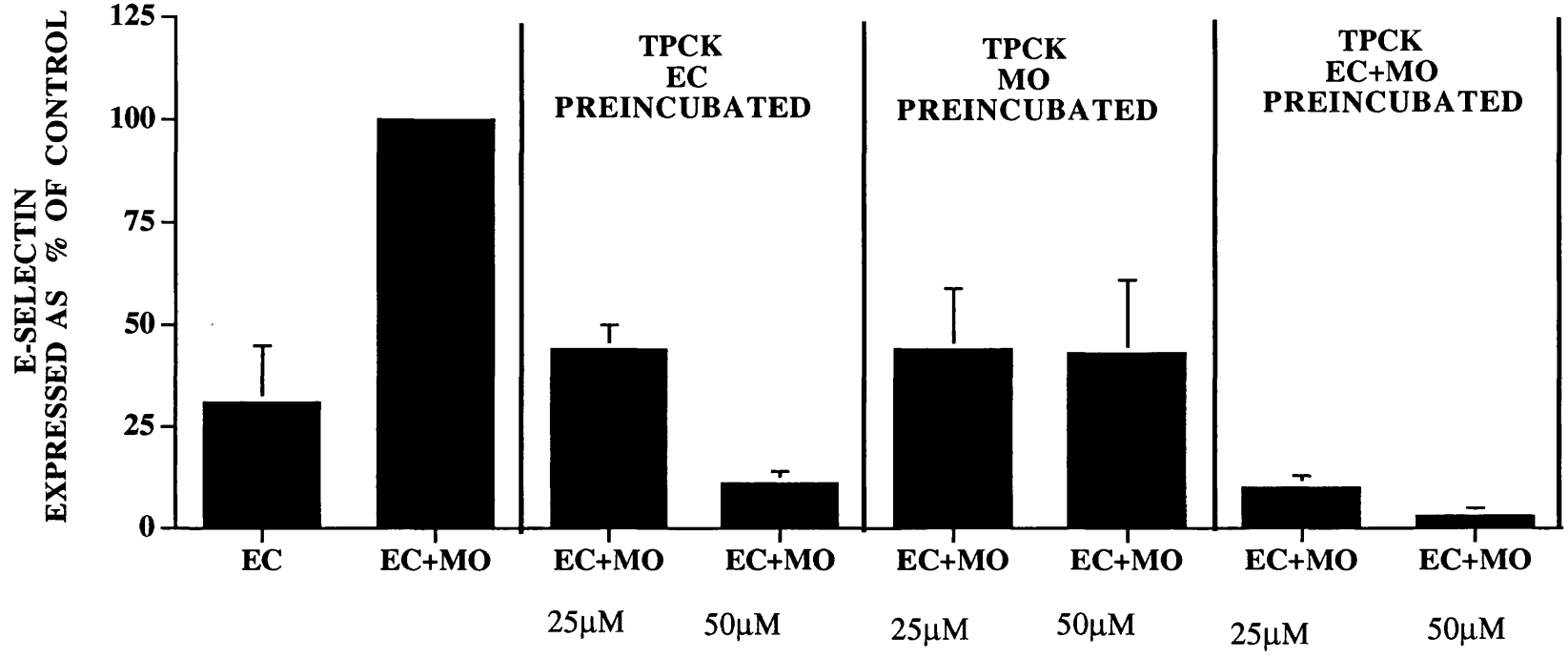

Figure 3.10a Effect of TPCK on monocyte induction of E-selectin on endothelial cells. Endothelial cells and monocytes were cultured as described. Monocytes and endothelial cells were preincubated with two concentrations of TPCK for 30 minutes at room temperature, and then washed twice before monocytes were added to the endothelial cells. Following 4 hours incubation an ELISA was used to determine E-selectin expression on endothelial cells. The data have been normalised so that the E-selectin expression on control cocultures in the presence of DMSO, TPCK diluent, is designated 100%. Results are the mean ± s.e.m. of 3 experiments.

concentration of 50μM TPCK E-selectin expression was reduced to below the levels on control cultures ($p<0.001$, $n=3$, Figure 3.10a). IL-1 induction of E-selectin was also inhibited on endothelial cells treated with TPCK despite monocyte coculture (data not shown).

When both endothelial cells and monocytes were pretreated with TPCK E-selectin expression was reduced to levels below that of control endothelial cells cultured alone.

To confirm that TPCK blocked NF-κB activation I used Western blotting to examine levels of IκB-α in cytoplasmic lysates of endothelial cells stimulated with LPS (10μg/ml). IκB-α is rapidly degraded in response to numerous stimuli such as TNF and LPS and proteasome inhibitors, for example, TPCK, prevent IκB-α proteolysis and ultimately NF-κB binding activity (Steffan et al, 1995). In endothelial cells stimulated with LPS, IκB-α disappeared from the cytoplasm at 15 minutes post stimulation, with maximal effect at 30 minutes (Figure 3.10b). After 60 minutes stimulation with LPS, IκB-α was still absent from the cytoplasm (Figure 3.10b). However, pretreatment with TPCK (25μM) for 30 minutes, prevented the degradation of IκB-α at all time points (Figure 3.10b).

The effect of monocyte coculture on endothelial cell IκB-α and IκB-β degradation

TNF rapidly activates NF-κB with degradation of the inhibitory proteins, IκB-α and IκB-β. These two proteins differ however, in the kinetics of degradation by TNF in that IκB-α protein disappears rapidly but reappears by 4 hours. IκB-β protein whilst also being degraded rapidly does not recover even after 22 hours

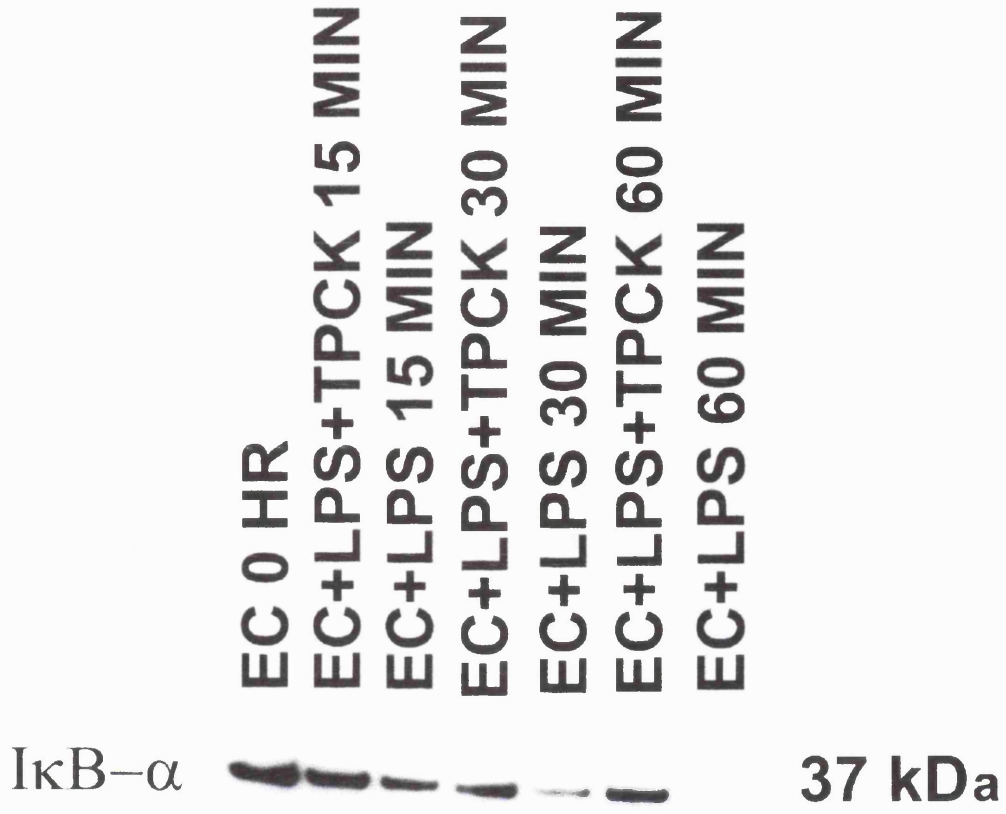

Figure 3.10b Effect of TPCK on LPS induced IκB-α degradation in endothelial cells. Endothelial cells were grown to confluence in culture flasks, pretreated with 25μM of TPCK for 15 minutes and LPS (10μg/ml) added. At 15, 30 and 60 minutes post LPS addition cytoplasmic lysates were made as described in the Methods and the lysates probed for IκB-α by Western blotting. Control endothelial cells were incubated with ethanol, TPCK diluent.

(Johnson et al, 1996). It is thought that TNF causes persistent activation of NF-κB in endothelial cells through this sustained reduction in IκB-β levels. The sustained expression of E-selectin on endothelial cells cocultured with monocytes may be due to different kinetics in IκB-α and IκB-β degradation and protein recovery.

Monocytes were cocultured with endothelial cells over 30 hours and at varying time points, cytoplasmic extracts of endothelial cells were made and probed for IκB-α and IκB-β by Western blotting. Endothelial cells were also stimulated with IL-1 and assayed in the same manner. Unstimulated endothelial cells express IκB-α in the cytoplasm (EC 0 HR, Figure 3.10c). Stimulation of endothelial cells with IL-1 causes complete disappearance of IκB-α in cytoplasmic extracts by 30 minutes (EC+IL-1 30 MIN, Figure 3.10c). By 4 hours after IL-1 stimulation, IκB-α protein levels have recovered (EC+IL-1 4 HR) and remain detectable up to 18 hours after IL-1 treatment (EC+IL-1 18 HR). Coculture with monocytes also causes IκB-α disappearance at 30 minutes (EC+MO 30 MIN, Figure 3.10c) and the protein returns to the cytoplasm by 4 hours (EC+MO 4 HR) and is still detectable at 18 hours (EC+MO 18 HR, Figure 3.10c).

IκB-β is also expressed in the cytoplasm of unstimulated endothelial cells (EC 0 HR, Figure 3.10d). Stimulation of endothelial cells with IL-1 causes disappearance of IκB-β by 30 minutes (EC+IL-1 30 MIN, Figure 3.10d), and IκB-β protein levels remain low at 4 hours (EC+IL-1 4 HR) and at 18 hours (EC+IL-1 18 HR, Figure 3.10d). By 30 hours, IκB-β protein levels have recovered (EC+IL-1 30 HR, Figure 3.10d).

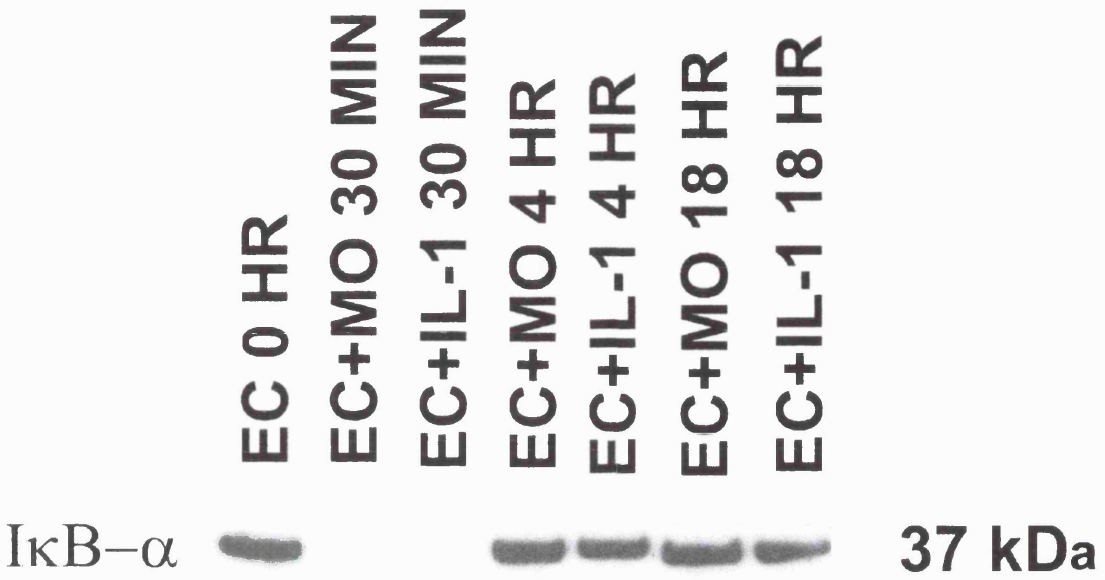

Figure 3.10c Effect of monocytes and IL-1 on IκB-α degradation in endothelial cells. Endothelial cells were grown to confluence in culture flasks and monocytes or IL-1 (10U/ml) were added. After 30 minutes, 4 and 18 hours incubation cytoplasmic lysates were made as described in the Methods and the lysates probed for IκB-α by Western blotting.

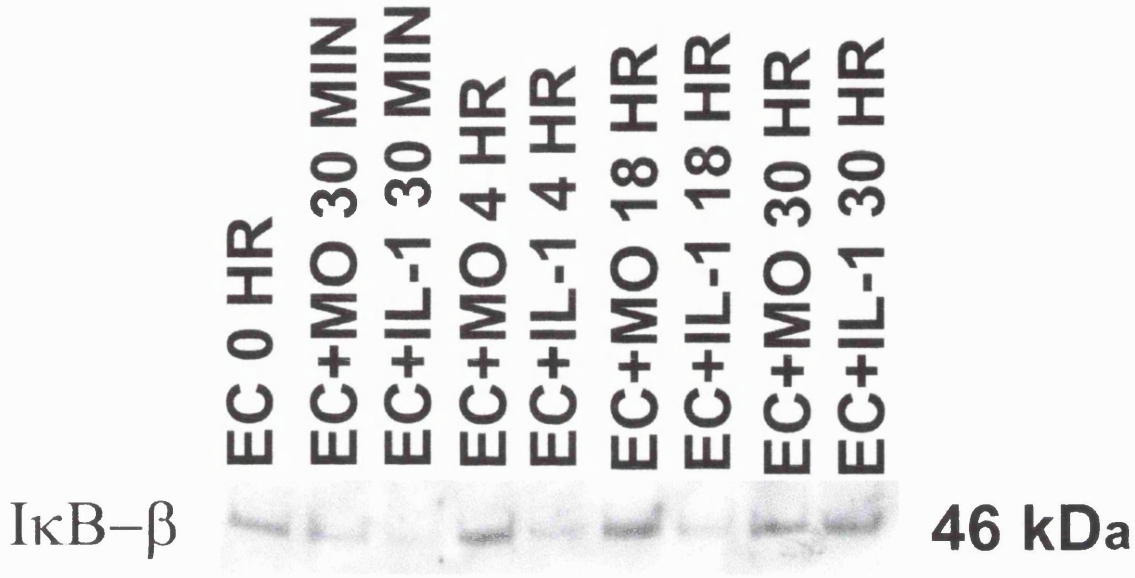

Figure 3.10d Effect of monocytes and IL-1 on IκB-β degradation in endothelial cells. Endothelial cells were grown to confluence in culture flasks and monocytes or IL-1 (10U/ml) were added. After 30 minutes, 4, 18 and 30 hours incubation cytoplasmic lysates were made as described in the Methods and the lysates probed for IκB-β by Western blotting.

In endothelial cells cocultured with monocytes, IκB-β protein partially disappears from the cytoplasm at 30 minutes (EC+MO 30 MIN, Figure 3.10d). By 4 hours (EC+MO 4 HR) IκB-β protein has returned to control levels and remained at these levels for up to 30 hours (EC+MO 30 HR, Figure 3.10d).

NAC, an antioxidant, blocks monocyte induction of E-selectin on endothelial cells

Reactive oxygen species can regulate NF-κB activation, and N-acetylcysteine, a potent antioxidant inhibits NF-κB activation induced by TNF (Staal et al, 1990). In order to investigate whether monocyte induction of E-selectin could be influenced by antioxidants in a similar way, NAC was included in monocyte/endothelial cell cocultures and E-selectin expression on endothelial cells measured using ELISA. Preliminary experiments were carried out to determine the optimal incubation conditions with NAC, and endothelial cells were preincubated with NAC (80mM) for 1 hour, after which the cells were washed, and monocytes or IL-1 added for 4 and 21 hours. At 4 hours, NAC (80mM) inhibited IL-1 induction of E-selectin by 81±17% ($p<0.05$, n=4, Figure 3.11) and the monocyte induction of E-selectin was reduced at 4 hours to below the levels on endothelial cells treated with NAC ($p<0.005$, n=4, Figure 3.11). At 21 hours both the IL-1 and monocyte inductions of E-selectin were inhibited to levels comparable to those on endothelial cells treated with NAC ($p<0.05$, n=3, Figure 3.11)

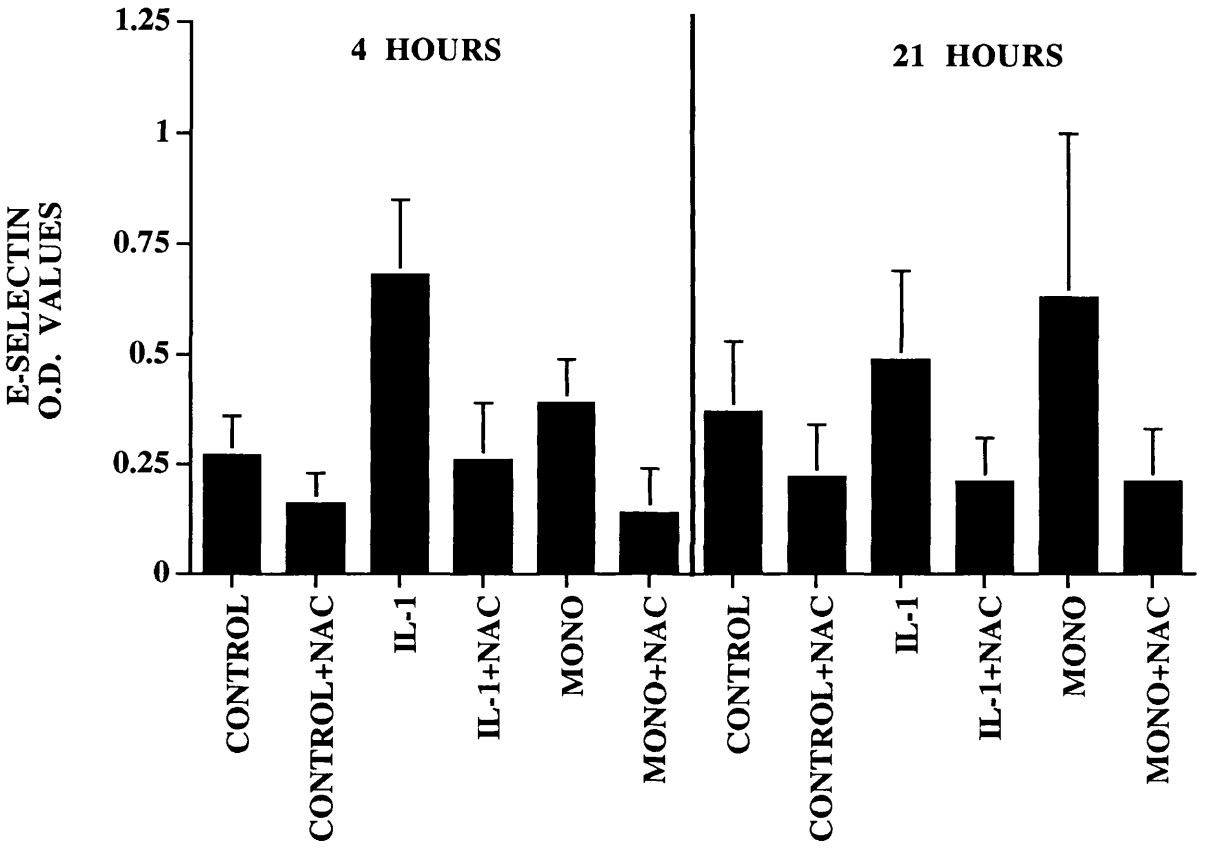

Figure 3.11 Effect of NAC on monocyte induction of E-selectin on endothelial cells. Endothelial cells were cultured as described, preincubated with 80mM NAC for 1 hour and washed before the addition of monocytes/IL-1 (10U/ml) for 4 and 21 hours. E-selectin protein expression was determined by ELISA. Data are mean ± s.e.m. of 4 experiments at 4 hours and 3 experiments at 21 hours.

Monocyte coculture with endothelial cells does not cause upregulation of the endothelial cell adhesion molecule VCAM-1

VCAM-1 is an adhesion molecule that is not constitutively expressed on endothelial cells but is upregulated following stimulation with TNF, IL-1 and IL-4 with peak expression by 6 hours which persists for at least 72 hours after induction by TNF (Carlos et al, 1994). It is postulated to play an important role in the pathogenesis of atherosclerosis because of its role in monocyte recruitment. I investigated whether interaction of monocytes with endothelial cells could also induce VCAM-1 expression similar to the upregulation of E-selectin. The addition of TNF (100U/ml) to endothelial cells for 8 hours induced VCAM-1 expression on endothelial cells (n=3, Figure 3.12a) which persisted for up to 21 hours (n=3, Figure 3.12b). Stimulation of endothelial cells with IL-1(100U/ml) also induced VCAM-1 expression on endothelial cells at 8 hours but levels were returning to baseline after 21 hours in IL-1 stimulated endothelial cells (n=3, Figure 3.12b).

The addition of monocytes to control endothelial cells had no effect on VCAM-1 expression at 8 or at 21 hours (Figure 3.12a and b). In addition monocytes did not enhance the cytokine responses at either 8 or 21 hours (Figures 3.12a and b). In a parallel experiment, the same monocytes induced E-selectin levels on endothelial cells (increasing O.D. from 0.06 to 0.42 at 8 hours, data not shown) confirming that the endothelial cells were responsive to monocyte stimulation.

Discussion

Monocytes cocultured with endothelial cells induce E-selectin mRNA and protein expression on endothelial cells with different kinetics to that induced by the cytokines TNF and IL-1. With these cytokines E-selectin expression peaks

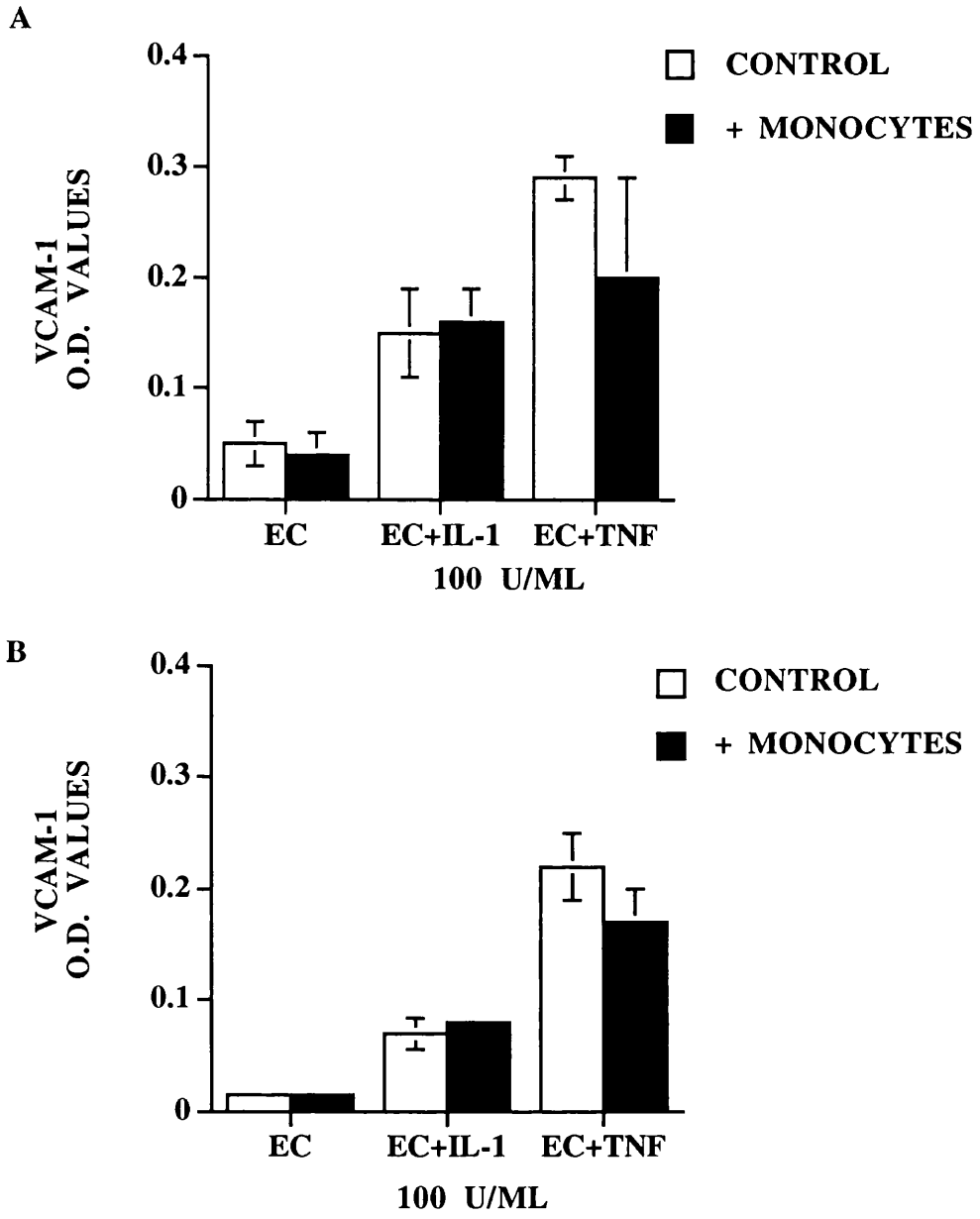

Figure 3.12 VCAM-1 expression on endothelial cells cocultured with monocytes. Endothelial cells were cultured to confluence as described, washed and monocytes ± IL-1 (100U/ml) or TNF (100U/ml) added. VCAM-1 protein expression was determined by ELISA after A) 8 hours and B) 21 hours. Data are mean ± s.e.m. of 3 experiments.

at 4 hours post stimulation and declines to baseline over 24 hours. Such transient kinetics are not consistent with *in vivo* observations of E-selectin expression in chronic inflammation where E-selectin may be persistently expressed (Norris et al, 1991; Groves et al, 1991; Koch et al, 1991), thus suggesting that other factors are involved in the regulation of E-selectin expression *in vivo*. Endothelial cells cocultured with monocytes display elevated E-selectin levels over 24 hours. The ability of monocytes to induce E-selectin on endothelial cells has been confirmed by two other studies, with one also demonstrating that E-selectin expression can be sustained by monocyte coculture over 24 hours (Rainger et al, 1996). However the other study showed that whilst monocytes do induce E-selectin expression on endothelial cells, they do so following the same kinetics to TNF, with levels almost down to background by 24 hours (Lidington et al, 1998). Suggestions as to other factors responsible for the extended E-selectin expression on endothelial cells cocultured with monocytes include human serum (Sepp et al, 1994) or sensitised T lymphocytes (Lidington et al, 1998). Human serum is absent from my system, whilst the small number of T cells present will have no effect over 21 hours, the time period studied. In addition, foetal calf serum was not found to have an influence since E-selectin expression was still sustained at 21 hours on endothelial cells cultured in 0.1% FCS (data not shown). I have also shown that the induction of E-selectin is monocyte specific, and this was confirmed by other studies showing that whilst highly purified T cells (Lidington et al, 1998) and neutrophils (Takahashi et al, 1996) do bind to E-selectin they do not induce E-selectin expression on endothelial cells. A possible reason for this is that neutrophils do not produce TNF and T cells that do release TNF do so after 3-5 days (Von Fliedner et al, 1992).

The induction of E-selectin on endothelial cells was dependent on monocyte cell number, with greatest induction at a ratio of 1:1 or $2x10^5$ monocytes/ml, a finding in keeping with the effect of monocyte coculture on ICAM-1 and VCAM-1 expression on endothelial cells (Takahashi et al, 1996). Monocytes enhance the induction of E-selectin by IL-1 induction particularly at 21 hours and with substimulatory concentrations of IL-1.

Monocyte induction of E-selectin is dependent on cell contact between the monocytes and endothelial cells. One possible explanation is that actual cell:cell contact between the two cell types generates intracellular signals within the endothelial cells causing initiation of transcription of the E-selectin gene, as has been demonstrated for the induction of tissue factor (Edgington et al, 1991) and plasmingen activator inhibitor type (PAI)-1 (Millan et al, 1997) on endothelial cells cocultured with monocytes. Alternatively, cell:cell contact could induce cytokine production from activated monocytes and endothelial cells. Monocyte interaction with unstimulated endothelium results in induction of IL-8 from both cell types and MCP-1 from the endothelial cells only and this increase was abrogated when monocytes were separated from endothelial cells on filters (Lukacs et al, 1995). Monocytes are induced to synthesise MIP-1α (Lukacs et al, 1994), TNF-α (Takahashi et al, 1996; Lidington et al, 1998; Fan et al, 1993) and IL-1β (Lidington et al, 1998; Takahashi et al, 1996) when cocultured with endothelial cells. Another possible mechanism for the induction of E-selectin would be the activation of endothelial cells engaging with mTNF or mIL-1β on the surface of monocytes. The role of membrane bound cytokines in monocyte induction of E-selectin is suggested by the finding that fixed monocytes positive for both mTNF and mIL-1β were able to induce detectable levels of E-selectin on endothelial cells (Lidington et al, 1998). In addition, human monocytes

cocultured with porcine endothelial cells were proposed to induce E-selectin protein expression on endothelial cells via mTNF-α (Millan et al, 1997).

Monocyte derived soluble factors appear to play a role in the upregulation of E-selectin since supernatants harvested from 21 hour monocyte cultures induced E-selectin protein expression at 4 hours on endothelial cells with some expression at 21 hours. Monocyte cell adherence to plastic tissue culture dishes results in a rapid induction of multiple inflammatory genes such as TNF-α and IL-1β (Haskill et al, 1991) and this mimics the activation of monocytes cocultured with endothelial cells and thus it is possible that these cytokines play a role in monocyte induction of E-selectin. The ability of conditioned media from monocytes to induce endothelial cell activation has been shown but with much less effect than direct cell-cell interaction in endothelial PAI-1 (Hakkert et al, 1990) and PGI_2 production (Hakkart et al, 1992), as well as in another study on E-selectin upregulation (Lidington et al, 1998). The soluble component of the monocyte induction of E-selectin was not identified in my studies however it could be TNF since the addition of anti-TNF mAb to monocyte/endothelial cells cocultures inhibited E-selectin induction at 21 hours by 66%. These results suggest that TNF plays a partial role in the ability of monocytes to regulate E-selectin expression on endothelial cells in my system. On the other hand, blocking antibodies against TNF-α and to IL-1β had little or no effect on the induction of E-selectin by monocytes in another study (Lidington et al, 1998), however these antibodies did inhibit induction by coculture supernatants. The separate use of these antibodies also had no significant inhibitory effect on monocyte induced ICAM-1 and VCAM-1 expression, but when used in combination the expression of these adhesion molecules were significantly inhibited by about 40% (Takahashi et al, 1996). Furthermore, in another study,

the addition of neutralizing antibodies to TNF-α and to IL-1β to monocyte-endothelial cell cocultures had no effect on monocyte induced IL-8 or MCP-1 production (Lukacs et al, 1995). Thus the relative contribution of TNF to the induction of endothelial cell responses by monocytes may depend upon the gene in question, and in addition whether preincubation of monocytes with anti-cytokine antibodies occurred.

Supernatants harvested from monocyte/endothelial cell cocultures had no effect on E-selectin induction in my system. In contrast, coculture supernatants were able to induce endothelial adhesion molecules in the study by Lidington et al, however, maximal effect was achieved by supernatants collected at 3 hours, while in my experiments, I used supernatants collected following 21 hours of coculture. The lack of effect of coculture supernatants in my studies may be attributable to endothelial cell receptors binding monocyte derived cytokines, which would thus not be detectable by ELISA. Endothelial cells express both TNF receptors, TNFR60 and TNFR80 (Schmid et al, 1995).

There is evidence from other studies that cocultures of monocytes and endothelial cells leads to production of IL-1β and TNF-α (Takahashi et al, 1996; Millen et al, 1997). In conclusion, it is likely that monocyte derived TNF does contribute to E-selectin induction in monocyte/endothelial cell cocultures, but mechanisms independent of TNF may also participate in monocyte/endothelial interactions resulting in endothelial activation.

Monocytes adhere to endothelial cells through various receptor-ligand pathways and my studies suggest that CD11b may play a role in monocyte induction of E-selectin. Inhibiting CD18 also reduced monocyte induction of E-selectin, however an antibody against VLA-4 had no inhibitory effect on the monocyte induction of E-selectin. Integrin ligation of adherent monocytes can lead to

intracellular signals that change the phenotype, movement, gene expression or activation state of the monocyte (Yurochko et al, 1992). β2 integrins are important in monocyte adhesion to unstimulated endothelium (Beekheizen et al, 1991) whilst the cytokine mediated increase in the binding of monocytes to endothelial cells is partially mediated by E-selectin, but not by either ICAM-1 on endothelial cells or CD11/CD18 molecules on monocytes. Evidence for a role for β2 integrins in activation of monocytes includes one study where CD11b/CD18 dependent adhesion of monocytes to endothelial cells enhanced TNF-α mRNA and protein secretion (Fan et al, 1993). The reduction in monocyte adhesion caused by antibody to CD11/CD18 may be partly responsible for the inhibition of monocyte induction of E-selectin. There was not, however, complete inhibition of monocyte induction of E-selectin suggesting that multiple receptor ligand systems may be involved in monocyte activation to endothelial cells. Only partial inhibition of adhesion can be obtained with single use of mAbs directed against adhesion molecules, as such, adhesion molecules are considered to act synergistically and strengthen in concert the binding between the cells (Leeuwenberg et al, 1992). Anti-VLA-4 mAb had little or no effect on binding when used alone, but when combined with anti-CD18 there was enhanced inhibition of monocyte adhesion to endothelial cells (Jonjic et al, 1992). Evidence for a role of β1 integrins in monocyte activation comes from a study in which ligation of β1 integrins induced expression of immediate early genes such as IL-1β and TNF-α in monocytes (Lin et al, 1994; Juliano et al, 1993).

Blocking monocyte adherence to the extracellular matrix proteins namely collagen I and collagen IV also inhibited monocyte induction of E-selectin on endothelial cells. Previous studies have identified matrix protein interactions as

inducing monocyte cytokine release; TNF is rapidly secreted following binding of monocytes to ECM (Darville et al, 1992), monocytes coincubated with immobilised fibronectin secrete TNF-a (Hershkoviz et al, 1993) and when monocytes were preincubated with soluble fibronectin or collagen type 1 to prevent monocyte engagement with ECM, there was significant inhibition in IL-8 production during cell-cell interactions (Lukacs et al, 1995). MCP-1 production was inhibited only by the addition of soluble collagen. In addition, engagement of the common β chain of the β1 subfamily of integrins that recognise ECM proteins results in expression of IL-1β in monocytes (Yurochko et al, 1992). The fact that laminin did not inhibit monocyte induction of E-selectin may mean there is some specificity for matrix proteins used, as laminin did not inhibit either IL-8 or MCP-1 production from monocytes cocultured with endothelial cells which were blocked by the addition of collagen (Lukacs et al, 1995).

Sustained E-selectin protein expression on endothelial cells cocultured with monocytes could be due to increased transcription of the E-selectin gene, increased mRNA stability or due to altered turnover of E-selectin molecules at the endothelial cell surface. E-selectin expression on endothelial cells stimulated with cytokines is transient with a peak at 4 to 6 hours, and by 24 hours the molecule is no longer detectable on the cell surface. The fate of E-selectin subsequent to its placement in the plasma membrane is not fully known. Some studies suggest that TNF-activated endothelial cells constitutively internalise E-selectin into lysosomal compartments (von Asmuth et al, 1992; Kuijpers et al, 1994; Smeets et al, 1993) whilst other reports have identified soluble forms of E-selectin in supernatants of cytokine activated endothelial cells which are smaller than the membrane bound form, consistent with the molecule having been cleaved from the membrane (Pigott et al, 1992; Newman

et al, 1993; Leeuwenberg et al, 1992). Maximal release of E-selectin into the supernatant was observed 6-12 hours after activation of endothelial cells and decreased to almost zero after 24 hours (Leeuwenberg et al, 1992). Since the presence of sE-selectin is not related to a rapid decrease in membrane expression of E-selectin, it is not likely that shedding contributes significantly to the downregulation of the expression of this adhesion molecule, suggesting internalisation as an additional mechanism. The nature of the mechanism of release of E-selectin has not been revealed as yet and thus a putative role for monocytes in preventing internalisation and cleavage, possibly through multiple receptor-ligand engagements providing a preventative signal cannot be ruled out. Alternatively, elevated surface E-selectin protein at 21 hours on endothelial cells cocultured with monocytes could be the result of prolonged transcription or increased stability of mRNA. This would be consistent with the demonstration of elevated E-selectin mRNA expression in endothelial cells cocultured with monocytes at 30 hours, compared to mRNA in IL-1 stimulated endothelial cells which had disappeared by 24 hours.

Induction of E-selectin by TNF and other inflammatory cytokines is regulated at the level of gene transcription and is mediated by the transcription factor NF-κB. Monocyte induction of E-selectin also appears to be regulated by NF-κB since TPCK, a protease inhibitor that blocks activation of NF-κB (Kim et al, 1995) inhibited monocyte induction of E-selectin on endothelial cells. Maximal inhibition of monocyte NF-κB blocks release of TNF-α and IL-1 (Kim et al, 1995) but does not completely inhibit E-selectin induction in monocyte/endothelial cell cocultures. When TPCK is applied directly to endothelial cells, however, E-selectin induction is completely inhibited. This would suggest that there is a component of the E-selectin response in

monocyte/endothelial cell cocultures which is independent of cytokine stimulation.

TPCK inhibits NF-κB activation by interfering with the degradation of IκB, an inhibitory protein. The dissociation of the cytoplasmic NF-κB complexes is thought to be triggered by the phosphorylation and subsequent degradation of the IκB protein. There are two major biochemically characterised forms of IκB proteins in mammalian cells, IκB-α and IκB-β (Thompson et al, 1995). The primary difference between the two is in their response to different inducers of NF-κB activity. One class of inducers causes rapid but transient activation of NF-κB by primarily affecting IκB-α complexes, whereas another class of inducers causes persistent activation of NF-κB by affecting both IκB-α and IκB-β (Johnson et al, 1996). Therefore the overall activation of NF-κB consists of two overlapping phases, a transient phase mediated through IκB-α and a persistent phase mediated through IκB-β. In endothelial cells TNF activates NF-κB rapidly and persistently and IL-1 activates NF-κB rapidly but with less persistence (Johnson et al, 1996), the difference between these cytokines in the kinetics of NF-κB activation being ascribed to the prolonged disappearance of IκB-β produced by TNF. Experiments were therefore conducted to investigate the kinetics of IκB-α and IκB-β isoforms in monocyte treated endothelial cells. Following incubation with IL-1 or monocytes, cytoplasmic extracts of endothelial cells revealed that IκB-α was quickly degraded and reappeared by 4 hours, a finding in keeping with previous published studies (Johnson et al, 1996; Read et al, 1994). There was, however, a difference in IκB-β kinetics. In endothelial cells stimulated with IL-1, IκB-β disappeared from the cytoplasm by 30 minutes and remained absent up to 30 hours post

stimulation. These results slightly differ to those already published where IL-1 more slowly induced the reduction of IκB-β to approximately 40% of control IκB-β levels by 4 hours, returning to control levels by 20 hours of IL-1 treatment (Johnson et al, 1996). TNF rapidly induced the reduction to 25% of control IκB-β levels at 30 minutes, and levels of IκB-β remained low throughout the period of treatment (Johnson et al, 1996). With monocytes IκB-β partially disappeared from the cytoplasm at 30 minutes but returned and remained at baseline levels for up to 30 hours. Thus, there is no evidence that prolonged absence of IκB is responsible for sustained monocyte activation of endothelial cells. Whether monocytes affect p50-p65 DNA binding is as yet unclear.

Recent findings suggest that reactive oxygen species can regulate NF-κB activation and N-acetylcysteine (NAC), a potent antioxidant, inhibits NF-κB activation induced by TNF (Staal et al, 1990). In my studies, monocyte induction of E-selectin was inhibited at 4 and 21 hours when incubated with NAC. Whilst information is lacking on the effects of antioxidants on monocyte induction of adhesion molecules there have been reports showing that E-selectin and VCAM-1 protein expression and mRNA in IL-1 treated endothelial cells were inhibited with similar concentrations of NAC as used in my studies (Faruqi et al, 1997). NAC did not, however, inhibit IL-1 induced E-selectin expression by reduced binding of κB dimers to the DNA binding sites. NAC supposedly affects one of the other regulatory elements rather than NF-κB in attenuating E-selectin gene expression (Faruqi et al, 1997).

Finally, in my system, monocytes cocultured with endothelial cells did not induce expression of VCAM-1 protein on endothelial cells. These results are in contrast to studies showing VCAM-1 induction on endothelial cells when cultured with monocytes (Lidington et al, 1998; Takahashi et al, 1996) and are

surprising in view of the ability of TNF to upregulate VCAM-1 expression. TNF is produced in monocyte/endothelial cells cocultures and has been shown to play a role in the monocyte induction of E-selectin on endothelial cells. These results could suggest that soluble TNF plays a limited role in adhesion molecule induction on endothelial cells cocultured with monocytes and that possibly mTNF is more important for monocyte induction of E-selectin, but does not induce VCAM-1 protein expression. In addition, TNF only played a partial role in the monocyte induction of E-selectin with other mechanisms such as receptor-ligand interactions also contributing. The lack of induction of VCAM-1 by monocytes may suggest that the molecular mechanisms involved in monocyte induction of E-selectin and VCAM-1 differ. Both these adhesion molecules are NF-κB dependent with p50-p65 being the NF-κB species binding to the DNA elements, but the the promoter regions of the genes differ in their construction. The E-selectin promoter contains 4 positive regulatory domains (PDs), of which PDI is the concensus κB element and PDII an ATF-like site, which is mainly occupied by ATF2 dimers which play a major role in cytokine inducibility of the gene (Mantovani et al, 1997). Occupancy of the ATF-like element by ATF2/c-jun confers significantly stronger response to cytokines than that by NF-κB alone (Min et al, 1997). TNF and IL-1 activate ATF2 and c-jun by kinases (JNK).These phosphorylate ATF2 and c-jun and these proteins are more potent enhancers of transcription than unphosphorylated forms, therefore TNF-mediated activation of JNK in endothelial cells results in functional activation of ATF2/c-jun complex, markedly enhancing NF-κB dependent transciption of E-selectin. Phosphorylations of ATF2 by JNK is less important than that of c-jun in regulation of TNF-induced E-selectin transciption. Thus ATF2 phosphorylation may be critical for responses to signals other than TNF (Min et al, 1997). PDI

and PDIII are simultaneously occupied while PDIV is not since it appears that NF-κB binding to PDIII sterically interferes with binding at PDIV. The high mobility group (HMG) protein I (Y) is required for cytokine-induced E-selectin expression, binds to both PDIII and PDIV, and enhances the binding of NF-κB to these elements (Collins et al, 1995).

The promoter for VCAM-1 is a tandem of NF-κB binding elements necessary for cytokine-mediated expression, and which are targets for binding of p50-p65 heterodimers or p65 homodimers. p65 acts as a powerful activator of VCAM-1, and higher levels of p50 can result in transcription repression. There is also an IRF-1 and Sp1 binding site which binds the transcriptional activator interferon regulatory factor-1 (IRF-1), which is important in cytokine-induced VCAM-1 gene expression. Thus while the expression of E-selectin and VCAM-1 requires NF-κB, each promoter is regulated by a distinct combination of transcription factors that contribute to cytokine-induced gene expression. In fact, evidence that these two adhesion molecules are regulated differently comes from experiments using antioxidants. NAC inhibited both VCAM-1 and E-selectin mRNA and protein expression in IL-1 stimulated endothelium. However, whilst NAC reduced binding of the VCAM-1 κB dimers to DNA elements, E-selectin binding was not affected, even under differing redox conditions suggesting different redox sensitivities of NF-κB binding to its consensus binding sequences in the promoter region of these different genes (Faruqi et al, 1997). This suggests that NF-κB appears to be necessary but not sufficient for E-selectin gene induction, however, when NF-κB binding is inhibited, VCAM-1 expression is abrogated. E-selectin gene expression is also insensitive to inhibition by pyrrolidine dithiocarbamate (PDTC), another antioxidant, that reduces VCAM-1 expression on IL-1 stimulated endothelium (Marui et al,

1993). TNF induced E-selectin was partially inhibited by PDTC. Thus these results illustrate that different stimuli activate gene expression differently and that adhesion molecule expression is not solely reliant on NF-κB. How exactly monocytes cause sustained E-selectin expression on endothelial cells remains to be elucidated.

In conclusion the inflammatory consequences of monocyte/endothelium interactions demonstrate a potentially important mechanism for enhanced monocyte recruitment, and endothelial cell activation under such circumstances may have wider applications in situations such as atherosclerosis.

Chapter Four

The Effect of Monocytes on Expression of A1 and Survival in Serum Starved Endothelial Cells

Introduction

Homeostasis of the vascular endothelium is maintained by the balance between cell survival, cell proliferation and cell death. While proliferation and migration of endothelial cells are crucial to embryogenesis and tissue repair, apoptosis of endothelial cells may be important in tissue remodelling and inflammation, as well as contributing to the vascular pathology of atherosclerosis and immune rejection. In the steady state, endothelial cells do not extensively proliferate, with an overall doubling time, *in vivo*, in the order of months to years depending on the vascular bed (Hobson and Denekamp,1984). This suggests that there is tight control of the endothelial cell population size and that endothelial cell death must be a rare event. Vascular endothelial cell apoptosis does arise, however, in normal situations such as in angiogenesis and vascular restructuring that occur throughout growth and development in conjunction with proliferation. In addition, apoptosis may be one cause of endothelial cell damage contributing to various inflammatory disorders. After bone marrow transplantation, transplant related complications such as endothelial leakage syndrome are due in part to apoptosis of damaged endothelium possibly as a result of total body irradiation. Ionizing radiation has been found to induce endothelial cell apoptosis *in vitro* and *in vivo* (Fuks et al, 1994). Endothelial cell apoptosis also contributes to disease states, such as vascular disease associated with diabetes where high levels of glucose have been reported to promote endothelial cell apoptosis (Baumgartner-Parzer et al, 1995). In addition, whilst unperturbed endothelial cells provide anticoagulant properties, endothelial cells which have become apoptotic as a result of exposure to inflammatory stimuli become procoagulant (Bombeli et al; 1997). It is important that endothelial cells remain non apoptotic, and since endothelial

cell death is such a rare event it suggests that these cells receive signals confering survival.

The endothelium is strategically placed to receive signals from humoral factors, inflammatory mediators and physical forces from both the circulation and tissue. Inflammatory processes centred on the endothelium could result in damage to, and apoptosis of endothelial cells mediated perhaps by the influx of leukocytes and inflammatory mediators. Lipopolysaccharide (LPS), TNFα, and other cytokines have been shown in several studies to participate in cell death of endothelial cells (Tsukada et al, 1995). In addition, transforming growth factor β1 (TGF-β1) inhibits HUVEC growth by the induction of apoptosis (Tsukada et al, 1995). On the other hand, additional growth factors such as fibroblast growth factor (FGF) (Langley et al, 1997) and vascular endothelial growth factor (VEGF) have been found to delay apoptosis (Karsan et al, 1997, Gerber et al, 1998). Maintenance of cell survival in normal tissues is dependent on extracellular factors that transmit signals to the interior of the cell resulting in expression of molecules that promote cell viability. Such extracellular factors include growth factors, serum and extracellular matrix molecules. Many cell types undergo cell death when deprived of adhesion to the appropriate extracellular matrix; for example endothelial cells die by apoptosis when attachment is prevented by growing them in suspension (Bozzo et al, 1997).

Direct cell:cell contact can also influence cellular responses and adherent leukocytes recruited to an area of inflammation may alter endothelial cell function and survival as has been shown for the induction of E-selectin expression by monocytes in Chapter 3.

Interactions between monocytes and endothelial cells have been shown to enhance production of cytokines such as TNF-α and IL-1. Human endothelial

cells are not directly killed by TNF but the cells can be rendered sensitive to it if the cells are given RNA or protein synthesis inhibitors (Karsan et al, 1996). These findings suggest the existence of TNF inducible genes which confer a protective effect on cells. A1, a Bcl-2 homologue (Lin et al, 1993) is associated with increased survival of endothelial cells (Karsan et al, 1996) and has recently been shown to be upregulated by TNF and IL-1. Proteins belonging to the Bcl-2 family appear to play a central and prominent role in maintaining cell viability. Bcl-2 is an intracellular protein that blocks apoptosis and prolongs survival but does not independently induce proliferation. Previous studies have shown that there is very little if any Bcl-2 present in endothelial cells but several of the Bcl-2 homologues such as A1 (Lin et al, 1996) and Bcl-x_L which promote cell survival, as well as Bax which causes accelerated apoptosis, are present in endothelial cells.

In this study using a model of serum starved endothelial cells I have set out to investigate if monocytes interacting with endothelial cells could influence expression of A1 in endothelial cells; whether coculture with monocytes can decrease the levels of EC apoptosis seen with serum deprivation, and to determine what cellular and extracellular adhesive pathways are involved in this interaction.

Results

Monocytes stimulate A1 mRNA expression in endothelial cells

The experimental design and kinetics of serum starvation, addition of monocytes or IL-1 and harvesting of the endothelial cells are illustrated in Figure 4.1a.

Endothelial cells grown in complete growth media express A1 mRNA (EC -5HR, A1/actin ratio 0.14±0.06, n=3, Figure 4.1b and c). Following serum starvation

the levels of A1 mRNA in endothelial cells decrease over time, falling to 0.08±0.07 at 3 hours (EC -2HR) and 0.02±0.01 at 5 hours (EC 0HR). When monocytes were added to serum starved endothelial cells at 0HR and incubation carried on for a further 6 hours, there was an increase in A1 mRNA compared to serum starved endothelial cells which were cultured alone (A1/actin ratio 0.33±0.01 in endothelial cells cocultured with monocytes, EC+MO +6HR, compared to 0.00 in endothelial cells cultured alone, EC +6HR, $p<0.01$, n=3, Figures 4.1b and c). Peripheral blood monocytes (MO) do not express A1 mRNA under these conditions and thus could not have contaminated the RT-PCR of endothelial cells. In addition, endothelial cells harvested for RT-PCR contained less than 1% contaminating monocytes, as determined by staining with anti-CD14 using the APAAP technique (Data not shown).

Addition of IL-1 (100U/ml) to serum starved endothelial cells at 0HR also increased levels of A1 mRNA after 6 hours incubation (A1/actin ratio 0.29±0.12 in endothelial cells treated with IL-1, EC+IL-1 +6HR, compared to 0.00 for endothelial cells cultured alone, EC +6HR, n=3, Figures 4.1b and c). Expression of A1 in response to IL-1 was shortlived, in that it declined by greater than 50% at 21 hours (A1/actin ratio 0.03 ±0.01 at 21 hours , EC+IL-1 +21 HR, compared to 0.29±0.12 at 6 hours, EC+IL-1 +6HR, n=3). In contrast, the increase in A1 mRNA expression in endothelial cells induced by monocytes was sustained (A1/actin ratio 0.35±0.08 at 21 hours, EC+MO +21 HR, $p<0.05$, n=3, Figure 4.1c), and levels were not significantly different to those at 6 hours.

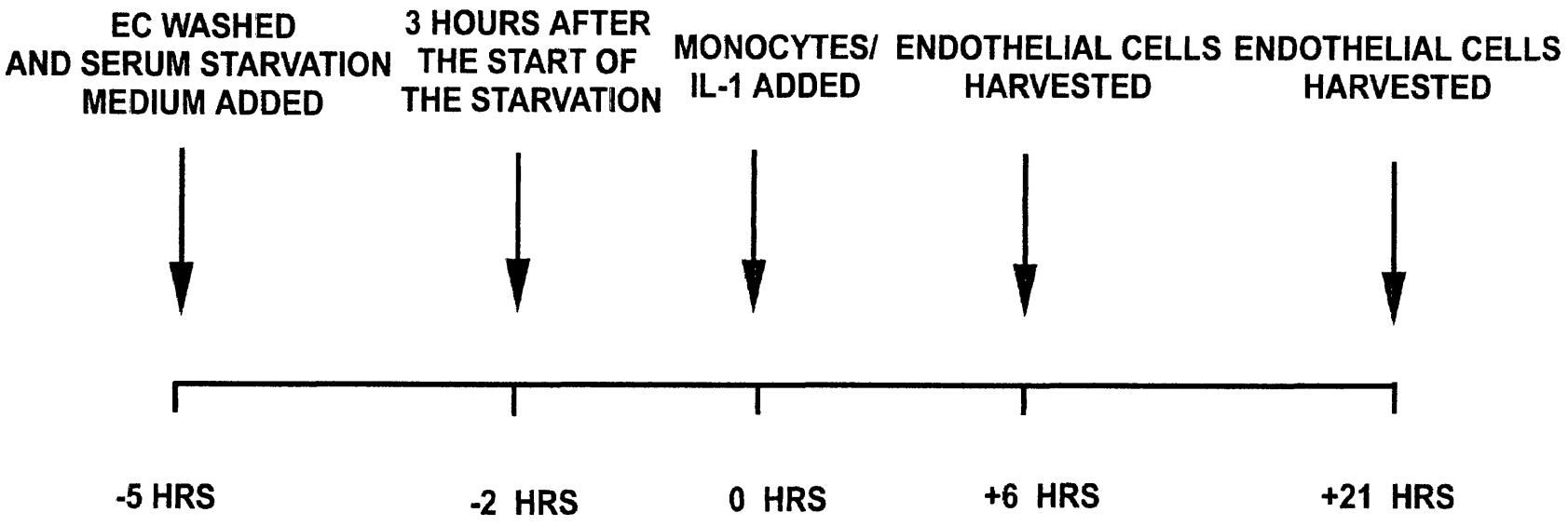

Figure 4.1a Experimental design for studies on serum starved endothelial cells

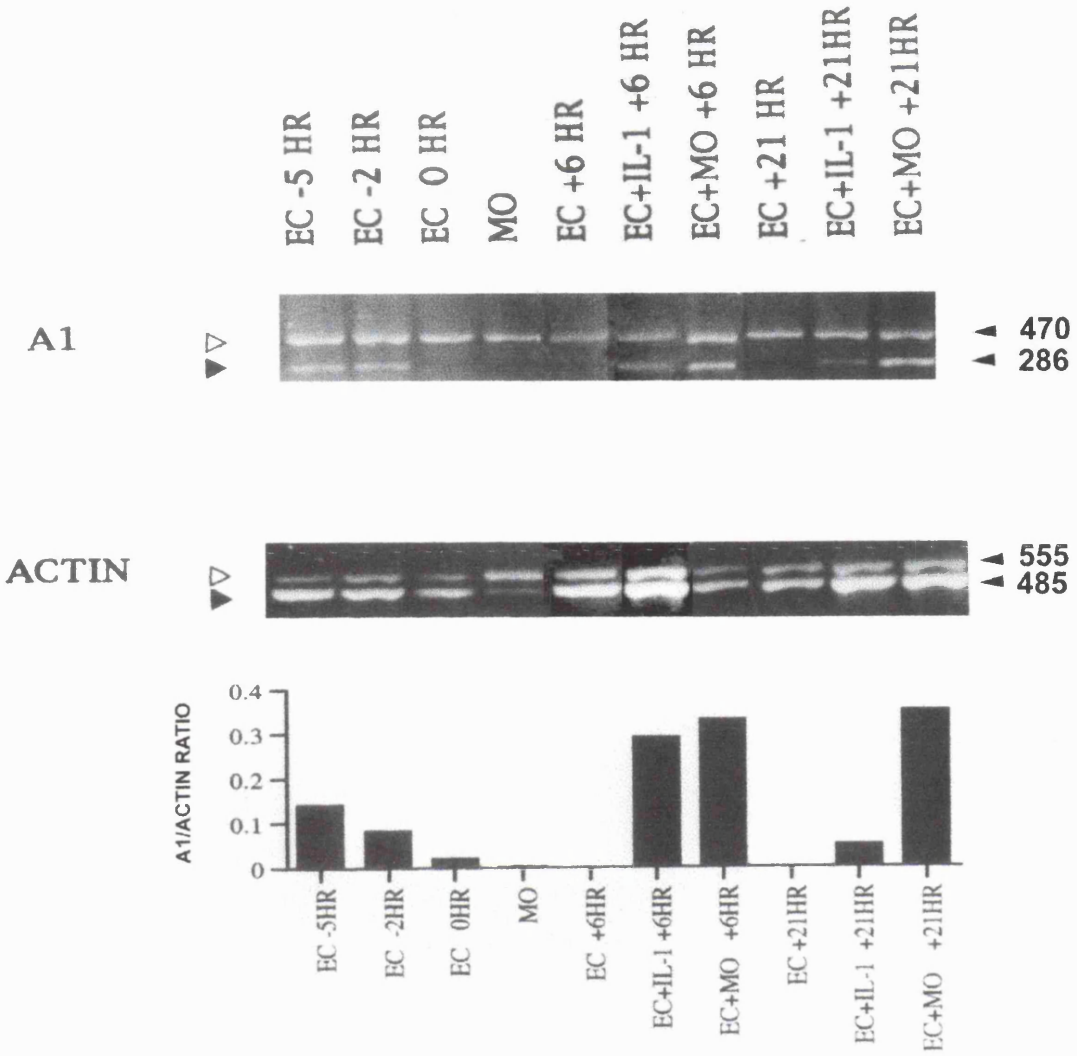

Figure 4.1b Monocyte coculture stimulates A1 mRNA expression in endothelial cells. Endothelial cells were serum starved for 5 hours prior to the addition of monocytes (1:1) or IL-1(100U/ml) as detailed in Figure 4.1a. Monocytes and IL-1 were incubated with the endothelial cells for up to 21 hours, after which the monocytes were removed with 2mM EDTA. Total RNA was then isolated from the endothelial cells and RT-PCR reactions performed as described in the Methods. Times are given relative to the addition of monocytes/IL-1 to serum starved endothelial cells. MO=monocytes; EC -5HR=endothelial cells grown in complete medium; EC -2HR=endothelial cells after 3 hours serum starvation; EC+MO=endothelial cells cocultured with monocytes; EC+IL-1=endothelial cells stimulated with IL-1.The bands shown are from one representative experiment and the histogram shows the mean optical density ratios from 3 experiments. The open triangles represent the mimic bands and the closed triangles the specific PCR product. The m.wt. of the products are indicated.

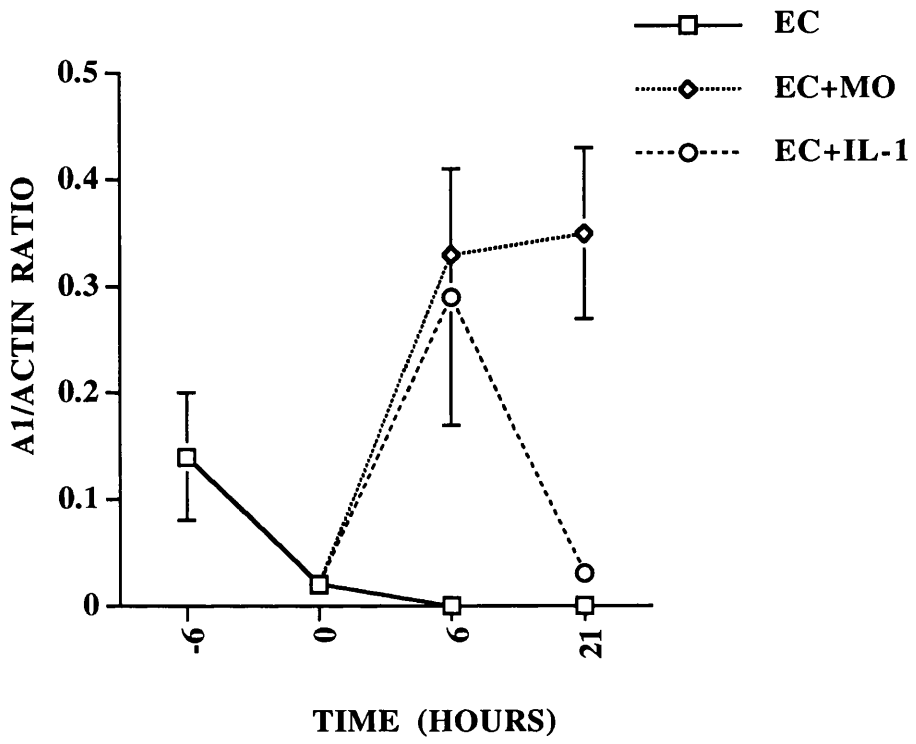

Figure 4.1c Monocytes stimulate endothelial cell expression of A1 mRNA with different kinetics compared to IL-1. Endothelial cell cultures, RNA isolation and RT-PCR reactions were set up as described for Figures 4.1a and b. Data from 3 experiments are given as the mean ± s.e.m. of the A1/actin ratios.

The ability of monocytes to augment A1 expression was tested more rigorously by varying the input of selected cDNA samples over 100-fold range while maintaining constant levels of mimic templates in PCR reactions (Figure 4.2). When cDNA from serum starved endothelial cells cocultured with monocytes (EC+MO +6 HR) was tested, the relative intensity of the A1 amplification product was at least equivalent to or exceeded that of the actin product when 0.1 or 1 μl of cDNA was used. When cDNA from control endothelial cells (EC +6HR) was tested the relative intensities of A1 PCR products were substantially lower than that of actin products at all input levels (Figure 4.2). The A1 band intensity became equivalent to the A1 mimic band intensity at cDNA inputs between 0.1 and 1μl in the EC+MO +6 HR sample. In contrast, A1 bands did not achieve equivalence with the mimic bands when the EC+6HR sample was tested, even at cDNA input levels of 1μl. A similar titration using cDNA from EC after five hours of serum starvation (EC 0HR), additionally confirmed that A1 mRNA expression was very low in these cells (Data not shown).

Neutrophils and Lymphocytes do not stimulate A1 expression in endothelial cells

To investigate whether the monocyte induced stimulation of A1 mRNA was specific to monocytes, neutrophils and lymphocytes were cocultured with endothelial cells under the same conditions. As seen in figure 4.3, stimulation with IL-1 (100U/ml) increased A1 mRNA levels at 11 hours (EC+IL-1 +6HR), however the addition of neutrophils and lymphocytes had no significant effect on the expression of A1 mRNA in the endothelial cells at this time point. In addition, no induction of A1 mRNA in endothelial cells by neutrophils and lymphocytes was detected at 21 hours (Figure 4.3). Despite the inability of

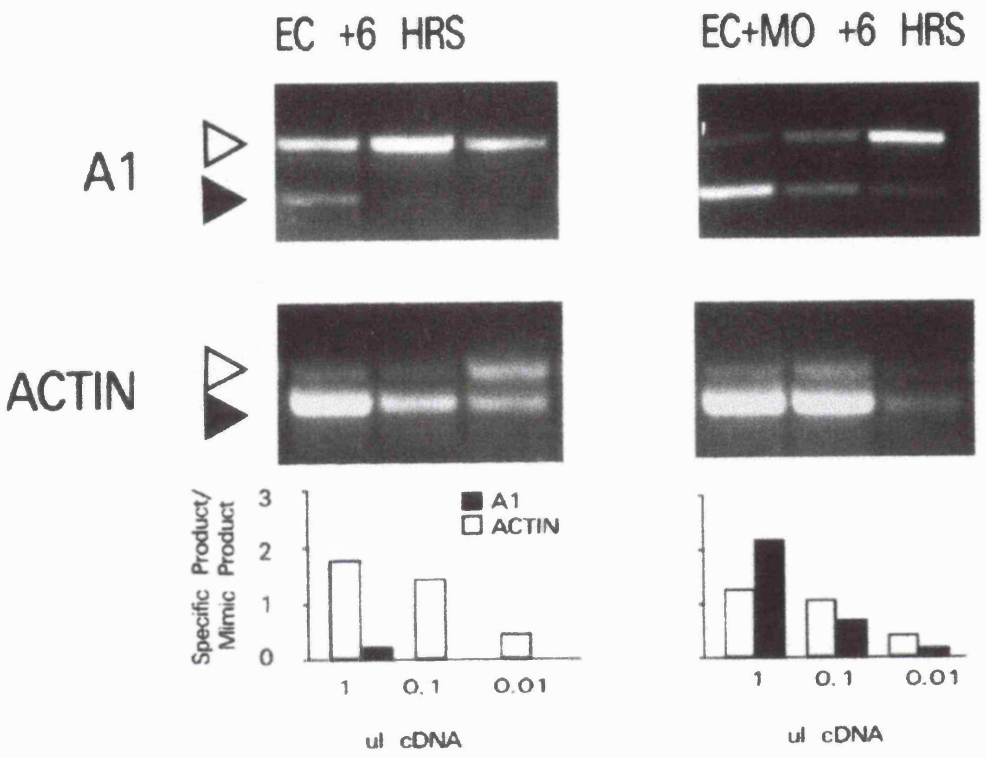

Figure 4.2 Comparison of A1 mRNA and actin mRNA levels at varying dilutions of input cDNA. cDNA (0.01-1μl) from endothelial cells after 11 hours of serum starvation (EC +6HRS), or after 5 hours of serum starvation, followed by 6 hours incubation with monocytes (EC+MO +6HRS), were used in competitive PCR assays for actin and A1 sequences. A1 mimic templates were used at a 100-fold lower concentration than in Figure 4.1b.

lymphocytes themselves to stimulate A1 mRNA expression in endothelial cells it is not possible to discount the possible contribution of a small number of lymphocytes to the A1 response seen in our endothelial cell/monocyte cocultures. Monocyte suspensions contain a variable (5-15%) number of contaminating lymphocytes which, despite several attempts to deplete the CD3 positive cells, were impossible to eradicate.

Monocytes reduce damage caused by serum starvation

The preceding observations suggest that monocytes stimulate A1 mRNA expression in serum starved endothelial cells but it is not clear whether monocytes confer protection against cell death at the same time. To study cell viability endothelial cells were morphologically assessed using phase contrast microscopy. Endothelial cells grown to confluence in complete growth media exhibit a characteristic morphology of tightly adherent cobblestone monolayers (Figure 4.4a). Serum starvation of endothelial cells over 11 hours (Figure 4.4b) causes a breakdown of the cobblestone appearance and damage to the confluent layer with large areas devoid of cells (Figure 4.4b). Cells that have detached are floating in the media. The addition of IL-1 (Figure 4.4c) to the serum starved cells has little effect, whilst the addition of monocytes (Figure 4.4d) markedly decreases the damage and endothelial cells are displaying a confluent monolayer with few gaps developed between the cells.

Monocytes increase survival of serum starved endothelial cells

To quantify cell survival the degree of apoptosis in endothelial cell cultures was assessed by morphological analysis of cytospin preparations. Endothelial cells cultured in 20% FCS show no features of apoptosis (Figure 4.5a), but endothelial cells serum starved for 21 hours exhibit alterations of nuclear

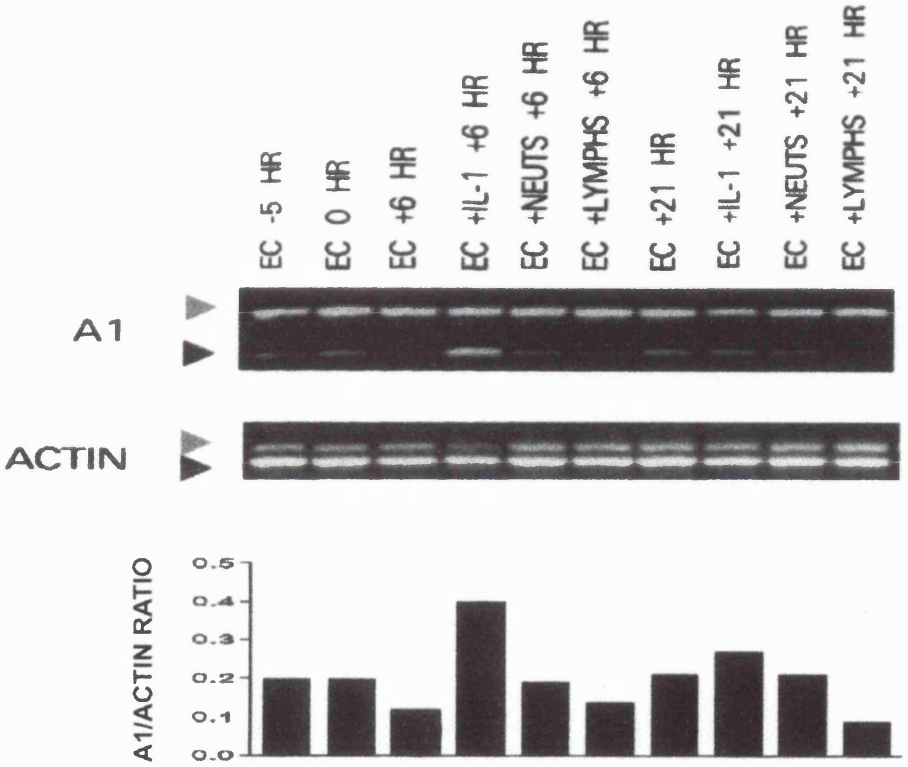

Figure 4.3 Neutrophils and lymphocytes do not alter A1 mRNA levels in endothelial cells. Endothelial cell cultures were set up essentially as described in Figure 4.1a, except that neutrophils or lymphocytes were added in place of monocytes. IL-1 (100U/ml) was used as a positive control. The bands shown are from one representative experiment and the histogram shows the optical density ratios from that experiment. The grey triangles represent the mimic product and the black triangles the specific PCR product.

morphology with chromatin condensation and nuclear and cell fragmentation (Figure 4.5b) all of which are characteristic of apoptosis. The addition of IL-1 does not appear to reduce the severity of apoptosis (Figure 4.5c), however with the addition of monocytes very few endothelial cells are apoptotic (Figure 4.5d). The small cells highlighted by the arrow are monocytes and not apoptotic endothelial cells. These observations are confirmed by counting morphologically live and apoptotic cells on cytospin preparations (Figure 4.6). Serum starvation over 21 hours decreased the percentage of live endothelial cells from 93.65±2.13% to 76.15±3.9% ($p<0.005$, $n=5$, Figure 4.6). When serum starved endothelial cells were cultured in the presence of monocytes, the percent of live endothelial cells increased to 83.5±3.2% ($n=5$). The addition of IL-1 to serum starved endothelial cells had no effect on the percent of live endothelial cells over 21 hours (75.4±2.9% with IL-1 compared to 76.5±3.9% for endothelial cells alone, Figure 4.6).

The evaluation of cell survival can be difficult since apoptosis can be underestimated when counting morphological apoptotic cells, as this method ignores cells engaged in early steps of the apoptotic process and cells completely disrupted into small fragments (apoptotic bodies), characteristic of late steps of apoptosis. It is also difficult to distinguish cells that are necrotic. To try and overcome this problem, the percent of live endothelial cells or those undergoing apoptosis or necrosis were measured by FACS analysis using annexin V-FITC/PI staining. Apoptotic cells stain positive for annexin V, while necrotic cells take up both PI and annexin V. Thus live cells are found in the dual negative (lower left) quadrant.

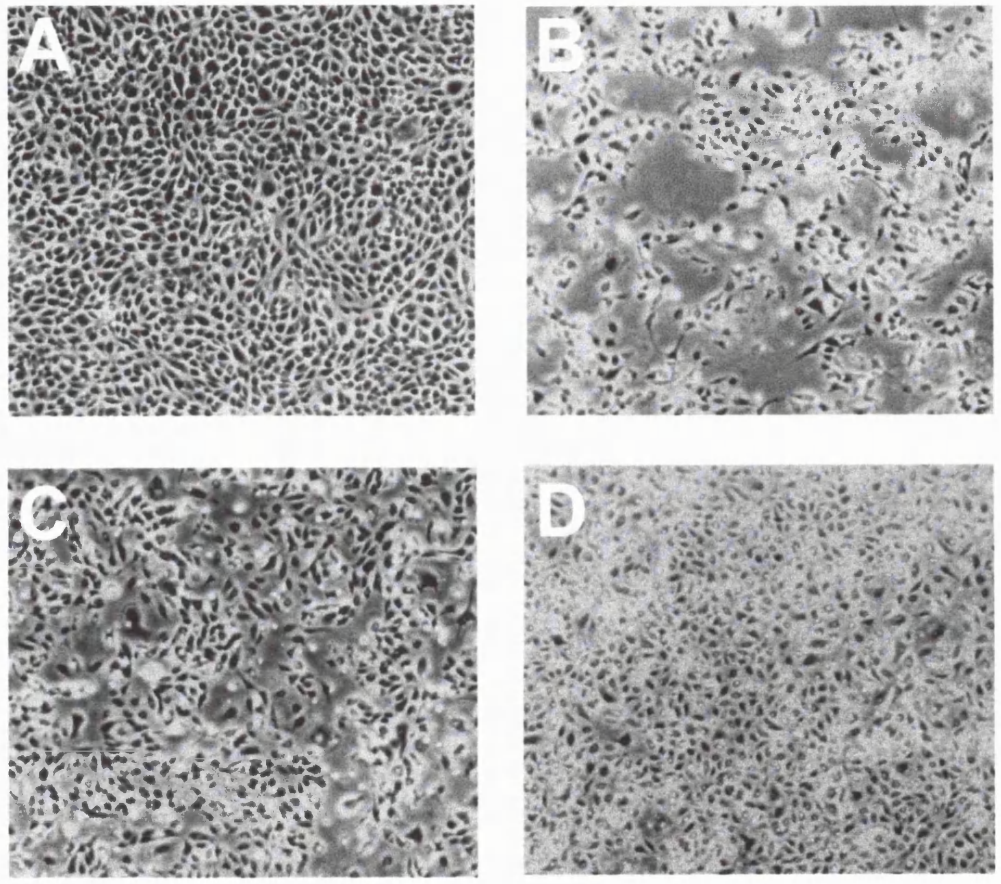

Figure 4.4 Monocytes increase the survival of endothelial cells. Phase contrast micrographs of endothelial cells that were serum starved for 5 hours before the addition of monocytes or IL-1 for a further 6 hours. A) endothelial cells grown in complete growth medium (20% FCS) B) serum starved endothelial cells C) serum starved endothelial cells stimulated with IL-1 (100U/ml) and D) serum starved endothelial cells cocultured with monocytes. Magnification=x100

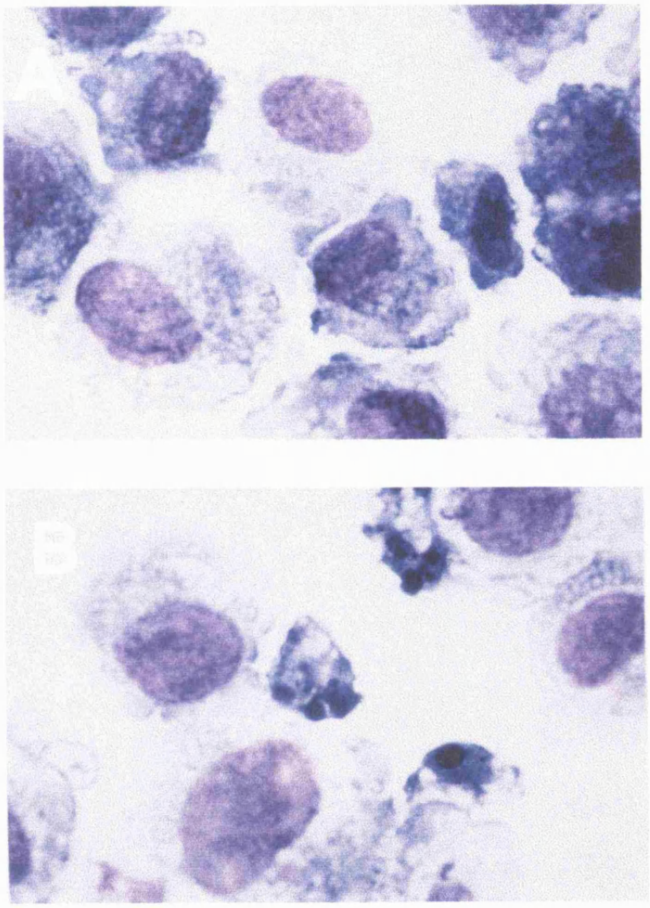

Figure 4.5 Monocytes decrease apoptosis in serum starved endothelial cells. Light micrographs of cytospin preparations stained with MGG stain. Endothelial cells were cultured as described in Figure 4.1a and monocytes and IL-1 were incubated with the endothelial cells for 21 hours. After 21 hours the endothelial cell cultures complete with monocytes were removed from the tissue culture flasks with trypsin/EDTA and cytospin preparations made as described in the Methods. A) endothelial cells grown in complete growth medium (20% FCS) B) serum starved endothelial cells

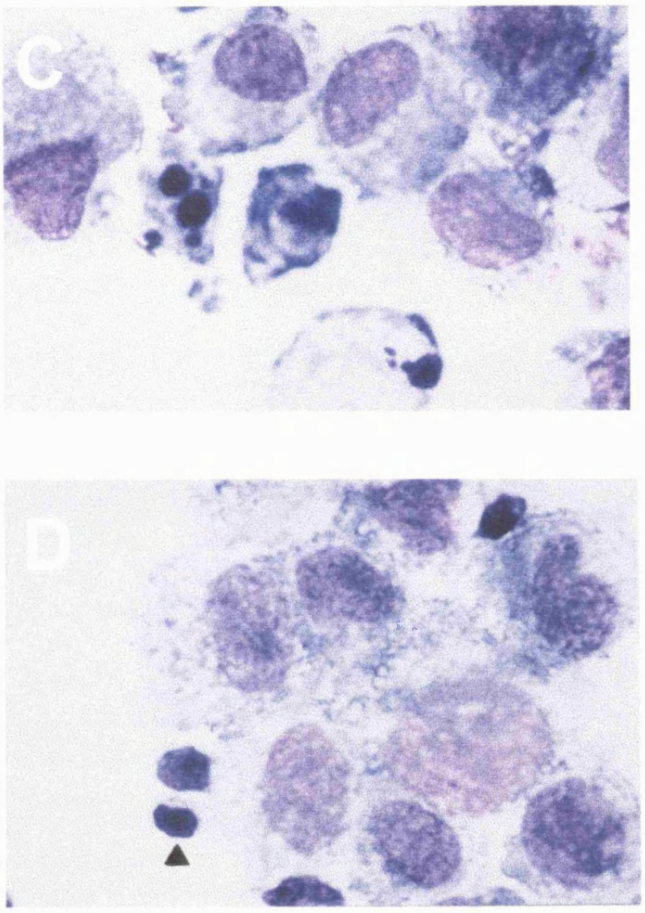

Figure 4.5 Monocytes decrease apoptosis in serum starved endothelial cells. C) serum starved endothelial cells stimulated with IL-1 (100U/ml) and D) serum starved endothelial cells cocultured with monocytes. The black arrow is highlighting a monocyte. Magnification=x600.

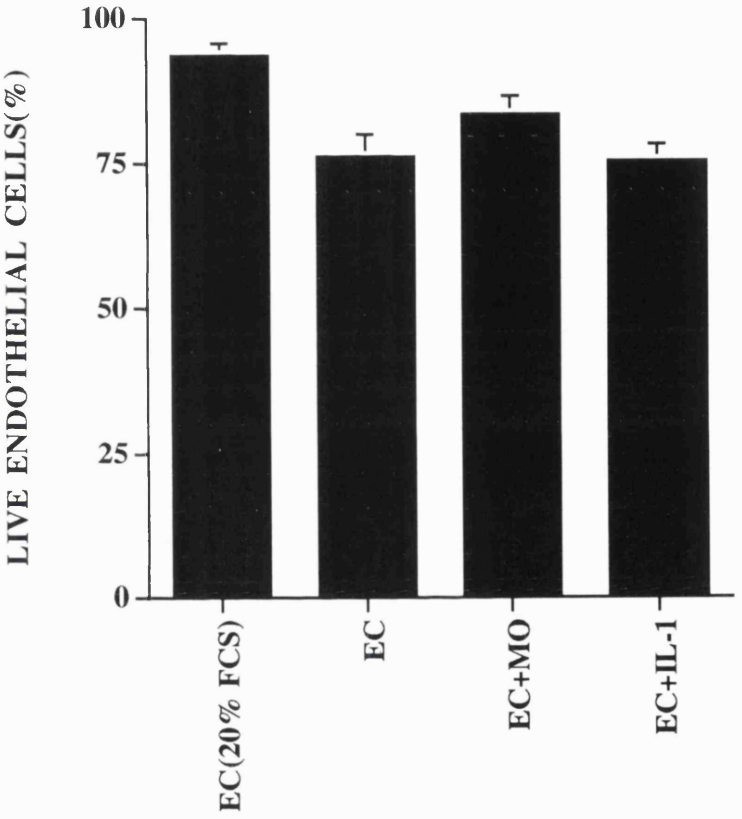

Figure 4.6 Monocyte coculture increases the percentage of live endothelial cells following serum starvation. Endothelial cells were cultured as detailed in Figure 4.1a and cells were harvested at 21 hours for survival analysis. The percentage of live endothelial cells was determined by morphological assessment of cytospin preparations stained with MGG. Data are mean ± s.e.m. of live endothelial cells from 5 experiments.

Figure 4.7 shows FACS histograms from one representative experiment where the percent of live endothelial cells falls from 90.5% when cultured in complete growth media (20% FCS) to 61.2% when serum starved. The addition of monocytes increases the percent of live cells to 83.1%. The percent of apoptotic endothelial cells increases from 1.9%

to 11.8% when cells are serum starved and decreases to 3.6% with monocytes. In four experiments, the percentage of live endothelial cells in 21 hour serum starved cultures (EC) was 63±4.9%, compared with 87.7±2.6% live cells in cultures grown in complete media (20% FCS, $p<0.01$, $n=4$, Figure 4.8). Coculture with monocytes (EC+MO) increased the proportion of live endothelial cells in serum starved cultures from 63±4.9% to 75.8±5.5% (n=4). Serum starvation increased the percentage of apoptosis from 3.1±0.5% to 10.27±1.3% ($p<0.01$, $n=4$) whilst the addition of monocytes to serum starved cells reduces apoptosis (6.28±2.5%). Serum starvation increases endothelial cell necrosis (from 9.25±2.2% to 26.8 ±4%) while the addition of monocytes reduces necrosis of serum starved cells (to 17.8±3.1%). IL-1 showed no protection at 21 hours (Figure 4.8).

The percentage of apoptotic endothelial cells determined by the FACS method is considerably lower than that obtained by morphologic analysis. Apoptotic cells eventually take up PI because of loss of integrity of the cell membrane, and thus appear in the annexin positive, PI positive upper right quadrant. In addition, when counting cells on cytospin preparations, necrotic cells can be mistaken for late apoptotic cells thus artificially increasing the percentages of apoptotic cells.

Thus monocyte coculture induced A1 mRNA in serum starved endothelial cells and increased the survival of these cells, probably by reducing apoptosis. Next I

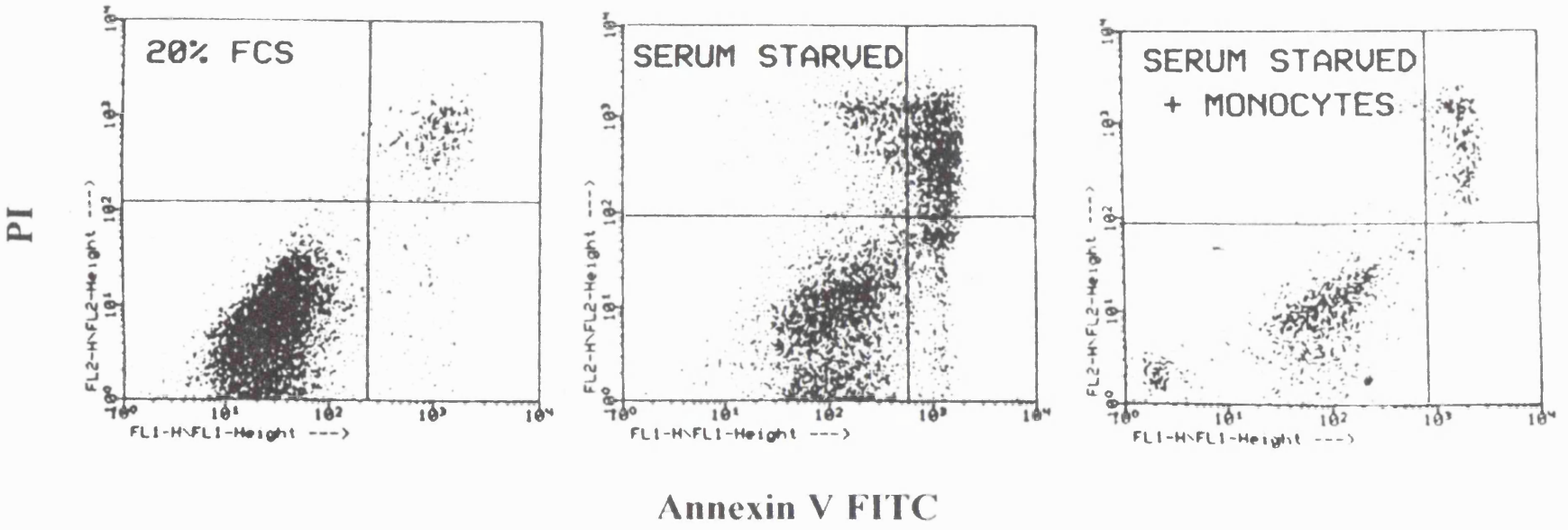

Figure 4.7 Reduction in endothelial cell apoptosis and necrosis in serum starved cells cocultured with monocytes. Endothelial cells cultured as described in Figure 4.1a. Following 21 hours of monocyte or IL-1 incubation, endothelial cells were dual stained with annexin V-FITC/PI and the percentages of live, apoptotic and necrotic endothelial cells determined by FACS analysis. FACS histograms from one representative experiment are shown. Live endothelial cells are found in the dual negative quadrant, apoptotic cells in the bottom right quadrant and necrotic cells in the dual positive quadrant.

wanted to investigate the mechanisms involved in generating a signal in endothelial cells that leads to the induction of A1.

Monocyte stimulation of A1 expression requires cell:cell contact

In order to determine whether contact between monocytes and endothelial cells was needed to stimulate A1 expression, experiments were conducted where monocytes were separated from the endothelium by culturing on a 0.45 micron filter above the endothelial cells. When contact between monocytes and endothelial cells was prevented in this way (EC+MO (FILT), Figure 4.9), endothelial cells displayed lower A1 mRNA levels when compared with endothelial cells that had been cocultured in contact with monocytes (EC+MO, Figure 4.9). In two experiments analysed at 6 hours, the level of A1 mRNA in endothelial cells cultured beneath filters was reduced by 100% and 57% (Figure 4.10a) when compared with parallel cultures in which endothelial cells were cultured in contact with monocytes (Figure 4.10a) .

When A1 expression was determined at 21 hours there was a 51±7% reduction in monocyte induction of A1 mRNA levels in endothelial cells cultured below filters, compared with control cocultures (Figure 4.10b, n=3). This suggests that contact between monocytes and endothelial cells is required to achieve optimal induction of A1 expression in endothelial cells.

One possible explanation for these findings is that adherent monocytes are necessary to generate a signal in endothelial cells that leads to increased A1 expression. Alternatively, monocyte adherence may result in the release of a soluble factor that stimulates A1 expression. I attempted to distinguish between these two possibilities by plating endothelial cells on a 0.45 micron filter above an endothelial cell/monocyte coculture (Figure 4.11).

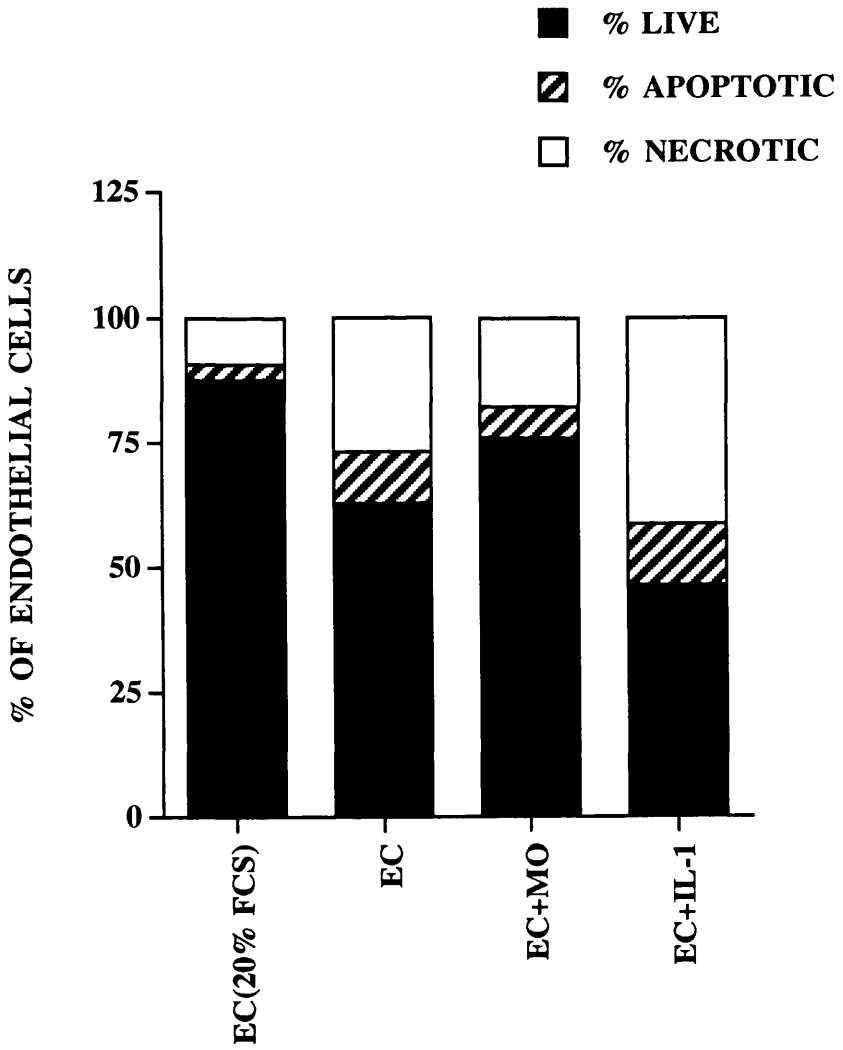

Figure 4.8 Monocytes reduce apoptosis and necrosis in serum starved endothelial cells. Endothelial cells were cultured as in Figure 4.7. Data expressed as percentages of live, apoptotic and necrotic endothelial cells from four separate experiments assessed by FACS analysis using annexin V-FITC/PI staining. The s.e.m.'s have been omitted for clarity but did not exceed 5% of the mean values shown.

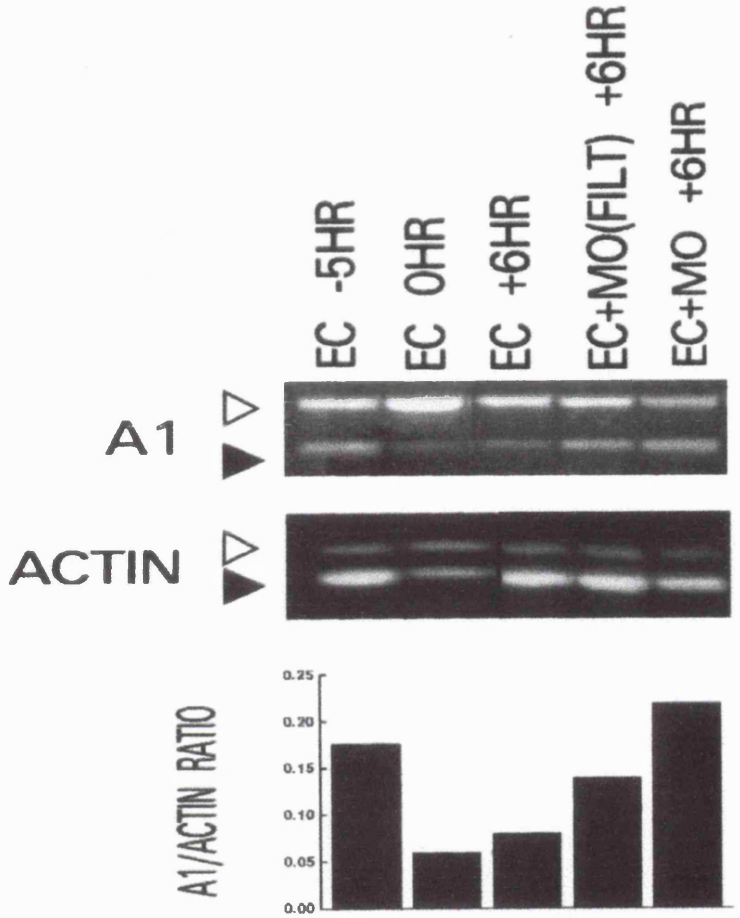

Figure 4.9 Monocyte stimulation of A1 mRNA requires cell:cell contact
Endothelial cell cultures were set up essentially as described in Figure 4.1a, except that contact between endothelial cells and monocytes was prevented by placing the monocytes on a 0.45μm pore filter above the endothelial cells for 6 hours. Endothelial cells were harvested for RNA isolation and RT-PCR performed as described in Methods. Notations are as defined in Figure 4.1b, EC+MO(FILT)=endothelial cells separated from monocytes by filter. Bands from one representative experiment are shown, and the corresponding optical density ratios are displayed as a column histogram.

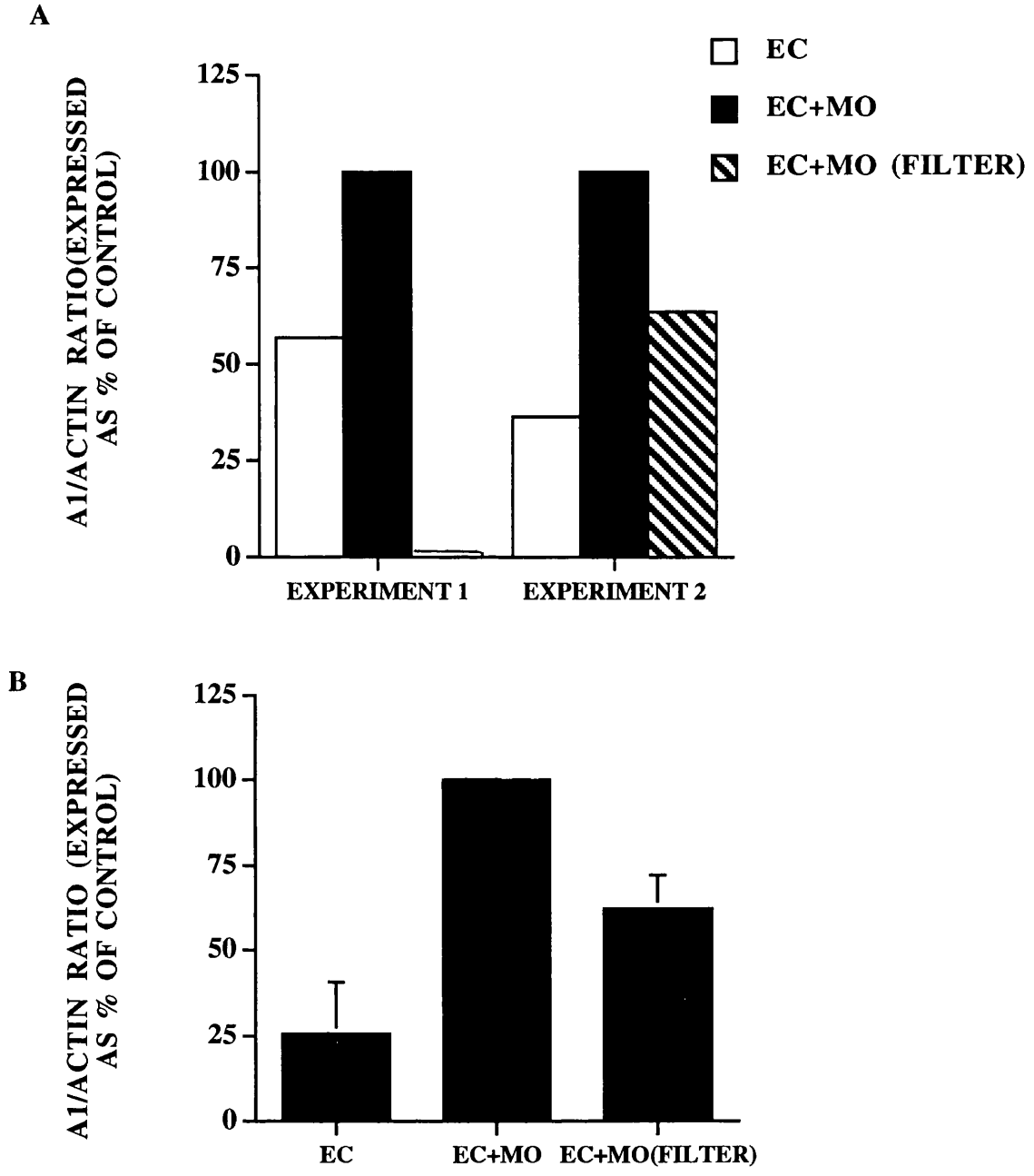

Figure 4.10 Cell:cell contact is required for maximal upregulation of A1 mRNA by monocytes. Endothelial cell cultures were set up as described in Figure 4.9. A) 6 hours and B) 21 hours. Data shown as 2 separate experiments in A and the mean±s.e.m. of 3 experiments in B. A1/actin ratios have been normalised so that the expression in endothelial cells cocultured with monocytes is designated 100%. EC=serum starved endothelial cells; EC+MO= serum starved endothelial cells cocultured with monocytes; EC+MO(FILTER)=serum starved endothelial cells separated from monocytes by filter.

A1 levels in endothelial cells cocultured with monocytes (EC+MO +21HR, A1/actin ratio 0.44) showed an increase over levels in endothelial cells cultured alone (A1/actin ratio 0.02). Endothelial cells that had been cultured on the filter above the coculture (EC above EC+MO +21HR) showed a small increase in A1 levels (A1/actin ratio 0.08) over endothelial cells cultured alone, but this represented only 14% of the response seen in endothelial cells cocultured with monocytes (Figure 4.11). In addition endothelial cells cultured above monocytes alone (EC above MONO +21HR) showed no increase in A1 levels (A1/actin ratio 0.03) compared to endothelial cells cultured alone (A1/actin ratio 0.02). Endothelial cells cultured on filters showed increased A1 expression when stimulated with IL-1 (A1/actin ratio 0.16) which was similar to the IL-1 reponse in endothelial cells cultured in wells (A1/actin ratio 0.22, data not shown) confirming that culture on filters per se does not alter the ability of the cells to respond to agonists.

Role of adhesion molecules in monocyte stimulation of A1 mRNA expression in endothelial cells

Having shown that monocytes induce A1 expression in endothelial cells at least in part by contact dependent mechanisms I went on to investigate whether the β2 integrin/ICAM-1 adhesion pathway was important for this induction. Monocyte adherence to endothelium is mediated partly by β2 integrins. FACS analysis of serum starved endothelial cells confirmed that these cells expressed low/intermediate levels of ICAM-1, but undetectable levels of VCAM-1 and E-selectin (data not shown), a pattern similar to that in non-cytokine stimulated endothelial cells grown in complete growth media. Experiments of monocyte adhesion to serum starved endothelial cells (Figure 4.12a) confirmed that mAbs directed at either CD11b (M170) or CD18 (60.3) were effective in blocking

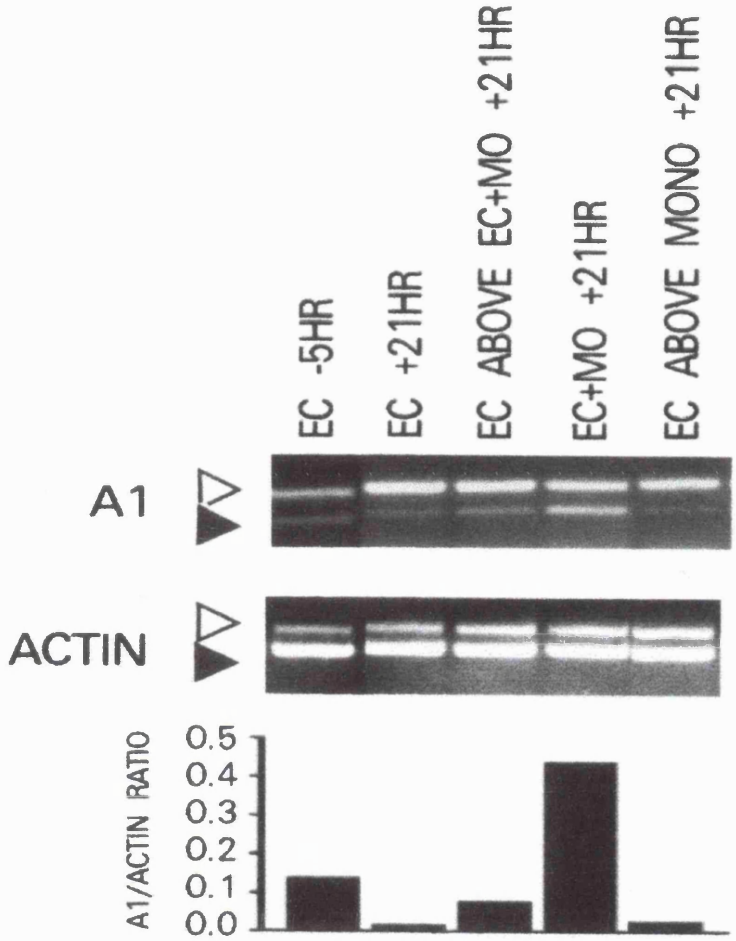

Figure 4.11 Direct contact between monocytes and endothelial cells is needed for A1 mRNA stimulation. Endothelial cells were grown to confluence on a 0.45μm filter, serum starved as detailed in Figure 4.1a, and then placed above a control endothelial cell/monocyte coculture, or above monocytes alone, and analysed for A1 expression after 21 hours. Notations the same as in Figure 4.1b, EC above EC+MO +21 HRS=endothelial cells placed above a normal endothelial cell/monocyte coculture; EC above MONO +21 HRS= endothelial cells placed above monocytes only. The bands shown are from one representative experiment and the corresponding optical density ratios are displayed as a histogram.

monocyte adhesion to serum starved endothelial cells (4.5% adhesion with M170 compared to 7.7% with control isotype IgG_2 and 2.9% adhesion with 60.3 compared to 4.6% with control isotype IgG_1 in one representative experiment). When these same monoclonal antibodies were included in endothelial cell/monocyte cocultures, however, there was no alteration in A1 mRNA levels compared with control cocultures incubated with isotype-matched IgG (Figure 4.12b).

In contrast, the inclusion of anti-PECAM-1 (CD31) to endothelial cell/monocyte cocultures reduced A1 expression both at 6 hours, from A1/actin ratio 0.37±0.13 to 0.25±0.04 (n=5, Figure 4.13a) and at 21 hours from 0.47±0.16 to 0.28±0.09 (n=5, Figure 4.13b). Anti-CD31 had no effect on basal levels of A1 mRNA in endothelial cell cultures (data not shown). In addition since blocking PECAM-1 has an inhibitory effect on A1 expression in endothelial cells cocultured with monocytes I investigated whether there was a concurrent decrease in endothelial cell survival when anti-PECAM-1 mAb was included in the cocultures. In one experiment, the inclusion of this antibody in monocyte/endothelial cell cocultures reduced cell survival from 93% to 85.5% (data not shown). These results suggest that by blocking PECAM-1 there is not only a reduction in A1 expression but also a decrease in cell survival in serum starved endothelial cells cocultured with monocytes.

Role of TNF in induction of A1

TNF is produced by monocytes and has been reported to cause up regulation of A1 gene expression in endothelial cells (Karsan et al, 1996). To determine the contribution of TNF to the induction of A1 in endothelial cells cocultured with monocytes, an anti-TNF mAb was added at the start of the coculture.

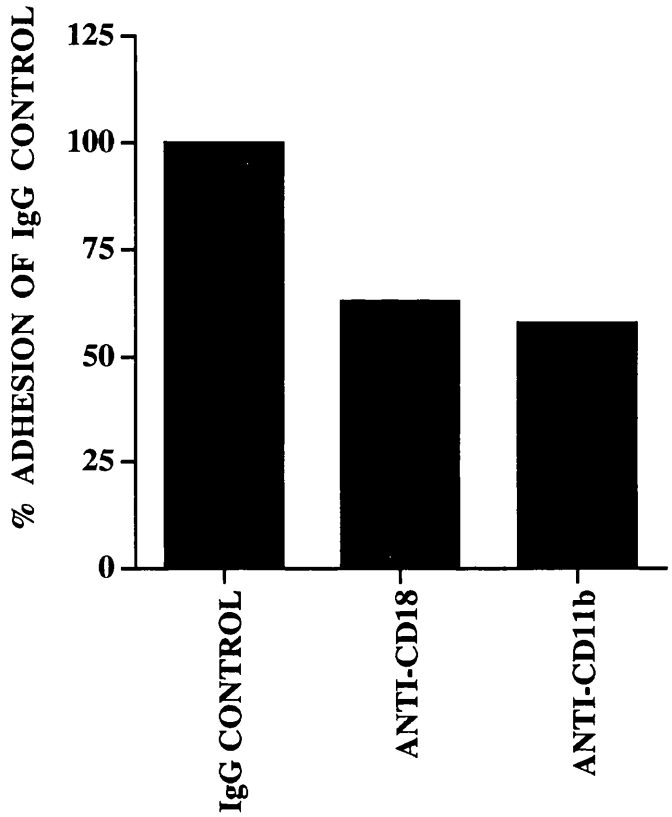

Figure 4.12a Role of adhesion molecules in monocyte adhesion to serum starved endothelial cells. Endothelial cells were set up as described in Figure 4.1a. Monocytes were preincubated with mAbs (60.3, anti-CD18; M170, anti-CD11b or IgG control) for 15 minutes prior to the adhesion assay, which was performed as detailed in the Methods. Antibodies were present throughout the assay (2 hours). The data have been normalised so that the adhesion of monocytes to endothelial cells in the presence of an isotype control mAb (control IgG) is designated 100%. Results are from one representative experiment.

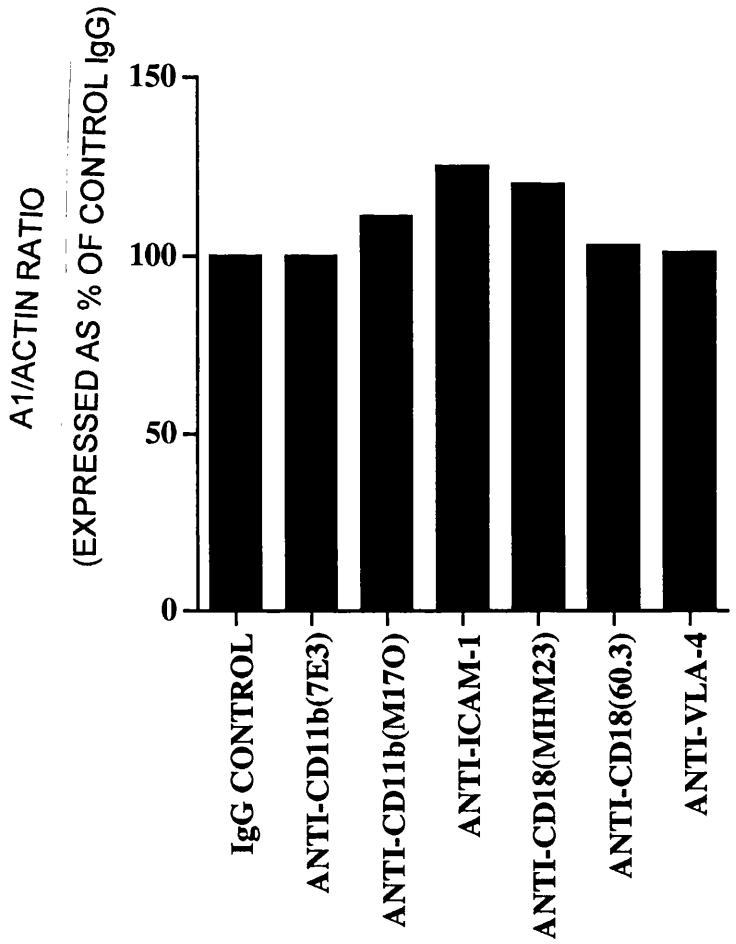

Figure 4.12b Role of adhesion molecules in monocyte stimulation of A1 mRNA expression in endothelial cells. Endothelial cells were set up as described in Figure 4.1a. mAbs were preincubated with monocytes (7E3, MI70, 60.3, MHM23) or endothelial cells (anti-ICAM-1) for 15 minutes and were present throughout the duration of the coculture (21 hours). Results are the mean of two experiments each with ICAM-1, MHM23, VLA-4, 60.3 and MI70, and one experiment with 7E3. The data have been normalised so that the expression of A1 mRNA in monocyte:endothelial coculture in the presence of an isotype control mAb (control IgG) is designated 100%.

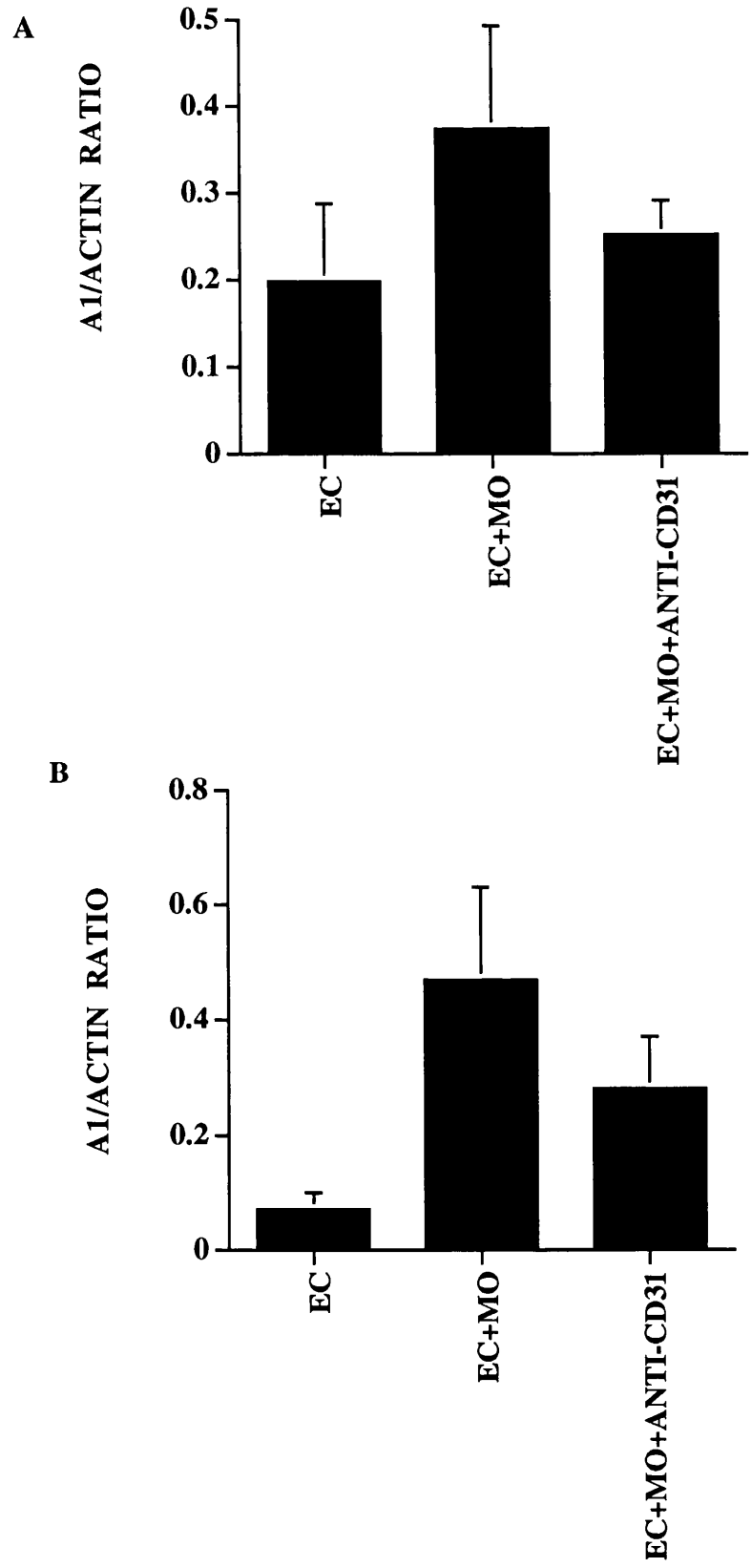

Figure 4.13 Blocking PECAM-1 in endothelial cell/monocyte cocultures inhibits monocyte stimulation of A1 mRNA. Endothelial cells were set up as described in Figure 4.1a. Monocytes were preincubated with anti-PECAM-1 mAb or IgG control for 15 minutes and mAbs remained present throughout the duration of the experiment. A) 6 hours B) 21 hours. Data are the mean ±s.e.m. of 5 experiments. Isotype control IgG was included in the monocyte coculture

Anti-TNF mAb abolished the monocyte induction of A1 at 6 hours (A1/actin ratio 0.24±0.09 in endothelial cell/monocyte cocultures with anti-TNF compared to 0.47± 0.29 in control endothelial cell/monocyte cultures (n=3, $p<0.05$); A1/actin ratio in endothelial cells cultured alone was 0.27±0.13, Figure 4.14a) After 21 hours, the inhibitory effect of anti-TNF antibody on the monocyte induction of A1 was less than at 6 hours (35±15% inhibition, n=7, Figure 4.14b).

Interestingly, the inclusion of anti-TNF mAb at 21 hours completely abolished any increase in A1 expression when monocytes were separated from endothelial cells by filters (data not shown), suggesting that monocyte-derived TNF may be responsible for the soluble component of the A1 response in endothelial cell/monocyte cocultures. In contrast, anti-IL-1 mAb did not affect A1 mRNA expression either in endothelial cell/monocyte cocultures or when monocytes were cultured on filters (data not shown).

The addition of exogenous TNF to serum starved endothelial cells led to an increase in A1 expression at 6 hours, which was 163±33% of that induced by monocytes in parallel cultures (A1/actin ratio 0.14±0.01 in endothelial cells cultured with monocytes compared to 0.19±0.02 in endothelial cells stimulated with TNF, n=4, Figure 4.15a). At 21 hours however, TNF induced A1 expression was less than 50% of the levels in monocyte/endothelial cell cocultures (n=4, Figure 4.15b). These results suggest that TNF contributes more to the early increase at 6 hours in A1 levels in endothelial cell/monocyte cocultures, but is less important for the sustained increase in A1 at 21 hours, consistent with the lesser inhibitory action of anti-TNF mAb at 21 hours, as described above.

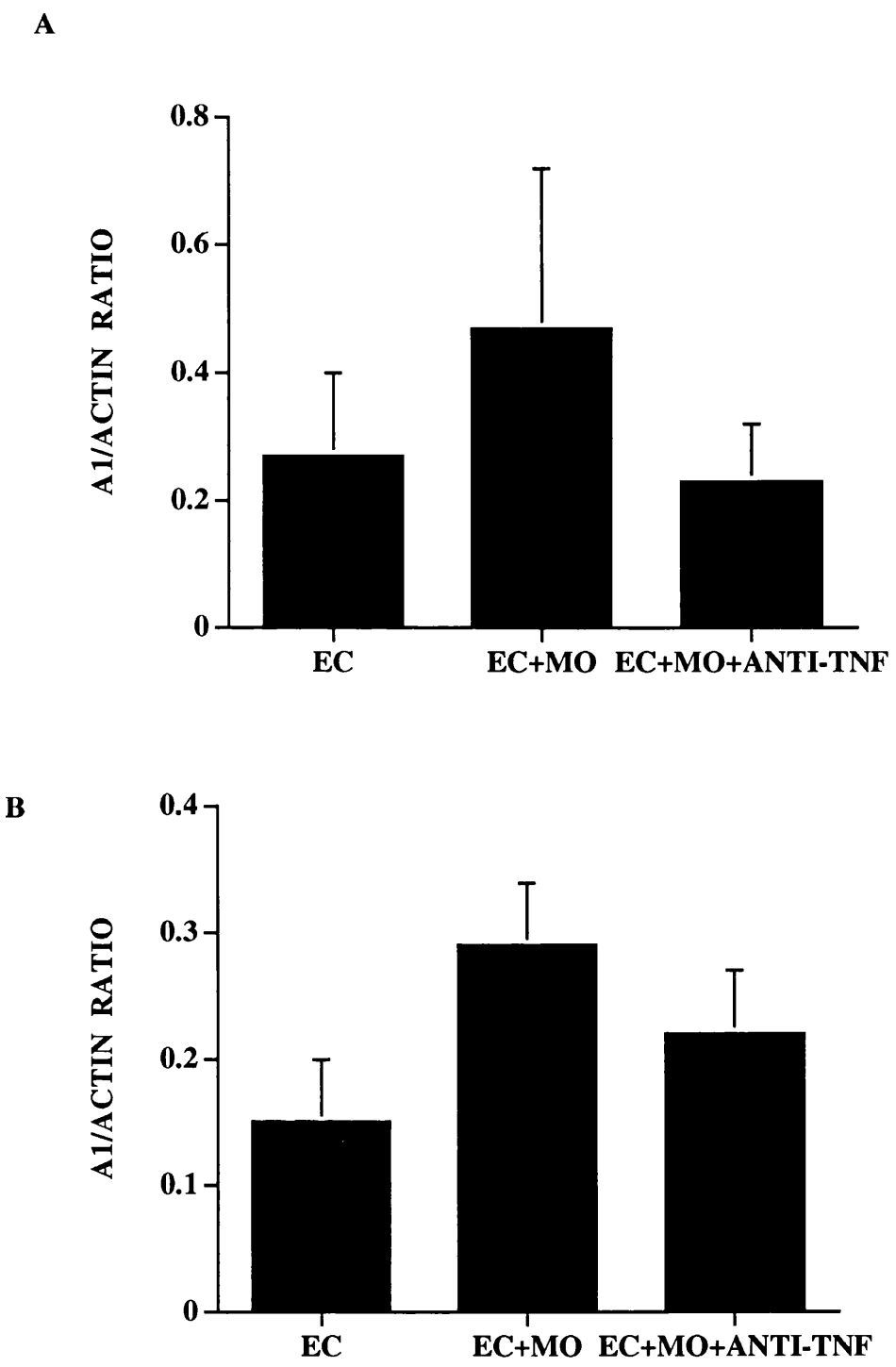

Figure 4.14 Role of TNF in monocyte stimulation of A1 mRNA in endothelial cells. Endothelial cells were set up as described in Figure 4.1a. Monocytes were preincubated with anti-TNF mAb (20μg/ml) (EC+MO+ANTI-TNF) or IgG control at the same concentration (EC+MO) for 15 minutes and mAbs remained present throughout the duration of the experiment. A1 expression was determined at A) 6 hours and B) 21 hours. Data are the mean ±s.e.m. of 3 experiments at 6 hours and 7 experiments at 21 hours.

Effect of IL-10 on monocyte induced A1 expression in endothelial cells

Stimulation of TNF synthesis in monocytes by TNF or LPS is followed by the induction of IL-10, which acts in an autocrine fashion to inhibit further TNF production, thus limiting the inflammatory response. In two experiments, the addition of IL-10 to endothelial cell/monocyte cocultures reduced A1 expression at 6 hours by 37% and 70% (Figure 4.16a). At 21 hours monocyte induction of A1 was inhibited by 32±15% (n=6, Figure 4.16b). These results provide further support for a role for TNF in the initial induction of A1 expression by monocytes, while suggesting that non TNF dependent pathways mediate the sustained levels of A1 at later time points.

This inhibitory effect of IL-10 on A1 expression in endothelial cells cocultured with monocytes could have a negative effect on the increased survival of endothelial cells cocultured with monocytes. In one experiment, the addition of IL-10 to 6 hour cocultures reduced the number of live cells from 95±0.7% to 91.5±2% (data not shown). The addition of IL-10 to 21 hour cocultures also reduced endothelial cell survival and the effects of IL-10 inclusion on endothelial cell survival are illustrated in Table 4.1. Thus IL-10 inhibited A1 induction and also decreased survival of endothelial cells cocultured with monocytes.

The role of NF-κB activation in A1 induction

TNF induction of A1 in endothelial cells is dependent on NF-κB activation (Karsan et al, 1997). Results presented in Chapter 3 support the notion that coculture of monocytes with endothelial cells activates NF-κB dependent genes, and in addition I have shown that TNF plays a role in the monocyte

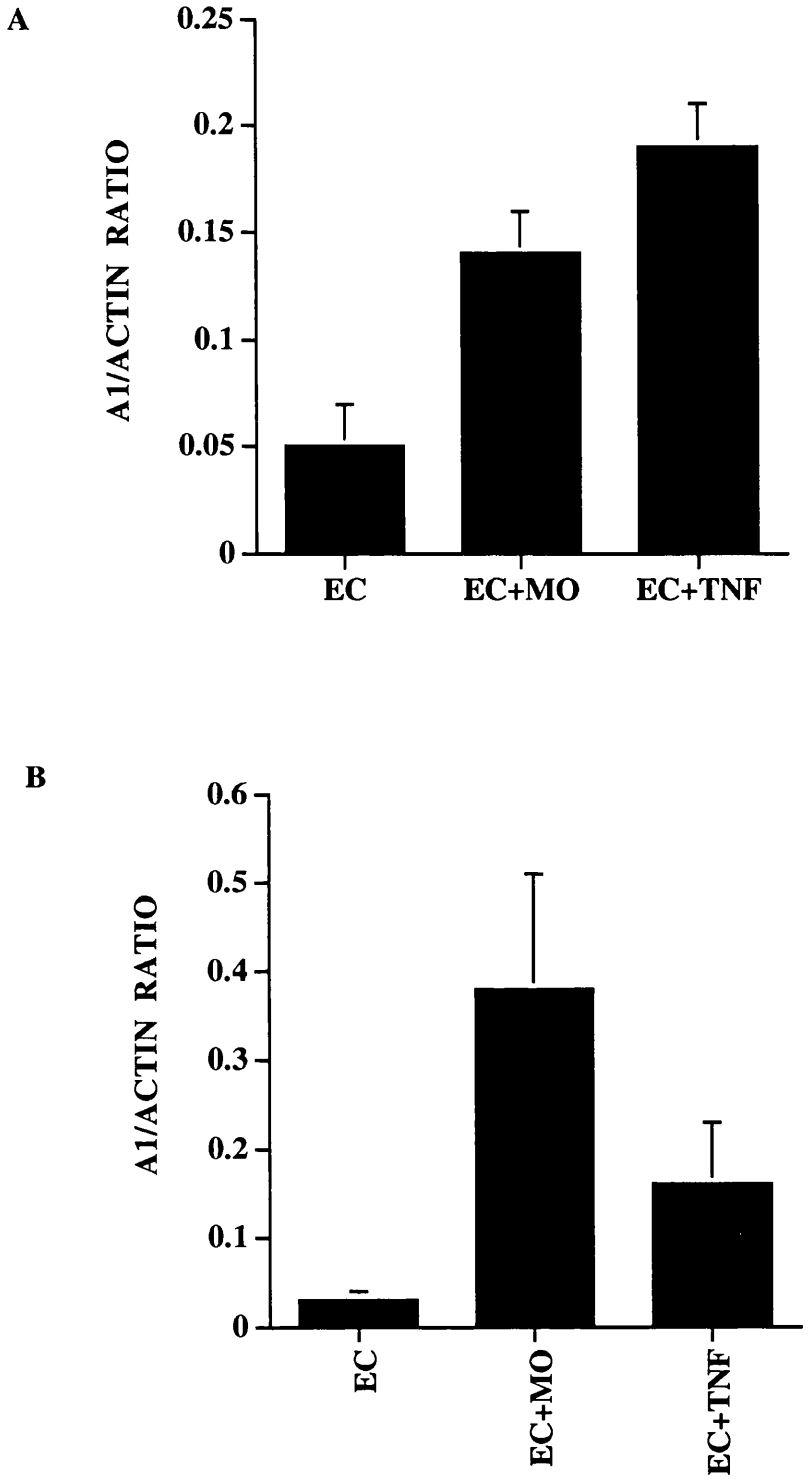

Figure 4.15 TNF induction of A1 mRNA in endothelial cells. Endothelial cells were serum starved as described in Figure 4.1a. Monocytes or exogenous TNF (100U/ml) were added to serum starved endothelial cells for A) 6 hours and B) 21 hours. Data are the mean ± s.e.m. of 4 experiments at both time points.

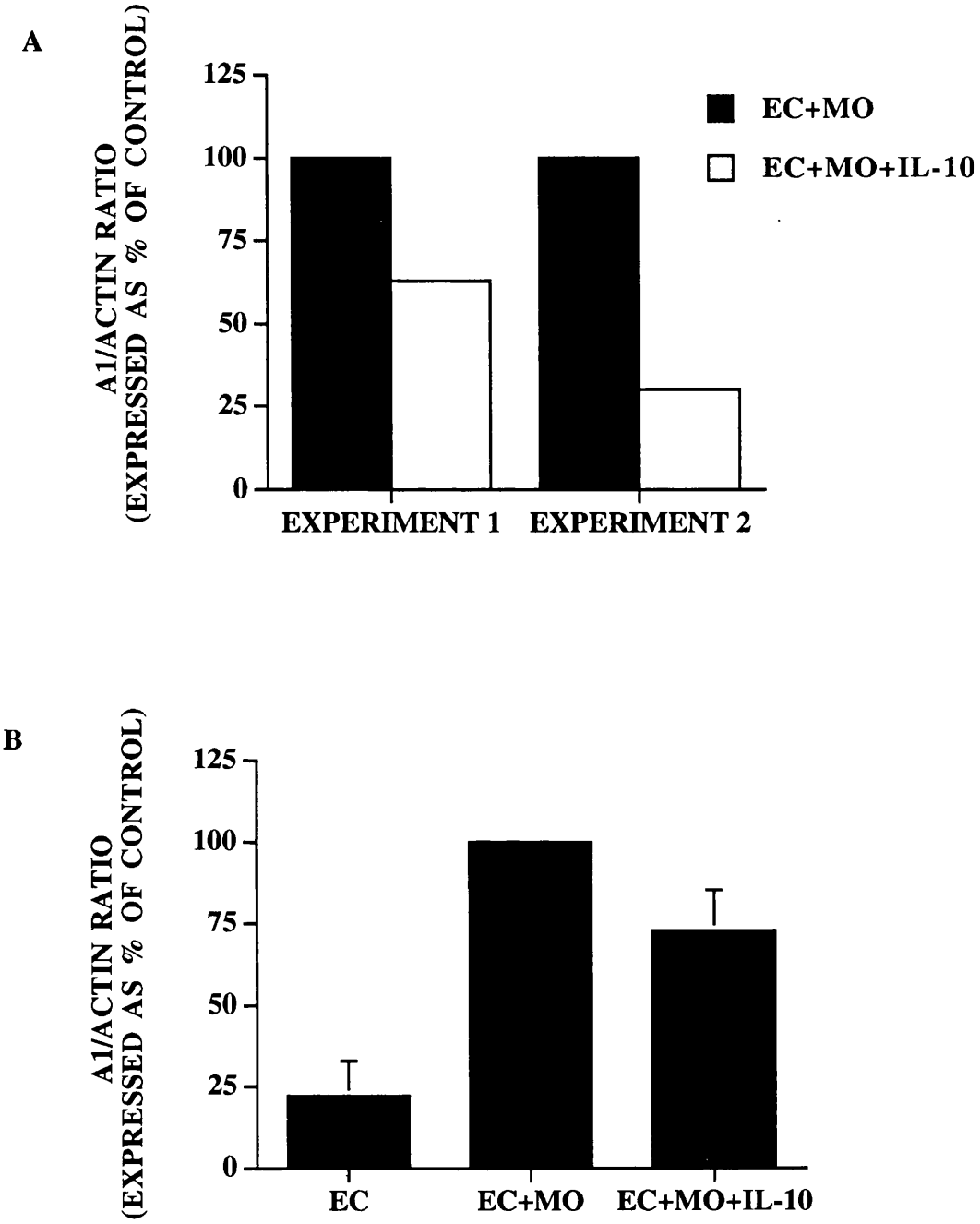

Figure 4.16 IL-10 inhibits monocyte induced A1 mRNA expression in endothelial cells. Endothelial cells were serum starved with monocytes as described in Figure 4.1a. IL-10 (100ng/ml) was added concurrently with the monocytes and was present throughout the assay. A1 expression was determined at A) 6 hours B) 21 hours. Data normalised so that A1 mRNA expression in monocyte cocultures is designated 100%. Results in A) are from two separate experiments and in B) the mean ± s.e.m. of 6 experiments.

CULTURE CONDITIONS	% SURVIVAL EXPERIMENT 1	% SURVIVAL EXPERIMENT 2
EC	83.8±0.6	86.5±1.8
EC+MO	95.8±1.3	94.5±0.86
EC+MO+IL-10	88.3±1.4	91.3±1.25

Table 4.1 Effect of IL-10 on monocyte induced survival of endothelial cells cocultured with monocytes. Endothelial cells were serum starved with monocytes as detailed in Figure 4.1a. IL-10 (100ng/ml) was added concurrently with the monocytes and was present throughout the assay. After 21 hours, the endothelial cell cultures were removed from the tissue culture plates with trypsin/EDTA and cytospin preparations made as described in the Methods. The percentage of live endothelial cells was determined by morphological assessment of the cytospin preparations stained with MGG. Four fields of view with 100 cells in each were counted for each cytospin slide. Data are the mean ± sd of 4 fields of view for each experiment. EC = serum starved endothelial cells; EC+MO=serum starved endothelialcell/monocyte cocultures; EC+MO+IL-10=serum starved endothelial cell/monocyte cocultures in the presence of IL-10.

induction of A1 in endothelial cells. In order to investigate the role of NF-κB transcription factor activation in monocyte induction of A1, NF-κB activation was inhibited using the proteasome inhibitor, calpain inhibitor I (CI).

The addition of calpain inhibitor I to serum starved endothelial cells blocked IL-1 induction of A1 at 4 hours by 85% and 76% in two experiments (Figure 4.17) Calpain inhibitor I also inhibited the TNF induction of A1 expression in endothelial cells in two experiments by 78% and 97% (Figure 4.17). When A1 expression was determined at 4 hours CI had little effect on the monocyte induction of A1 expression in one experiment while in the other experiment the inhibition was 63% (Figure 4.17). When A1 expression was analysed at 21 hours in one experiment, the addition of CI to endothelial cell/monocyte cocultures inhibited the monocyte induction of A1 by 92% (Data not shown). Whilst these results are only preliminary, they suggest that monocyte induction of A1 may be dependent on NF-κB, in a similar manner to IL-1 and TNF.

Effect of monocytes on expression of other apoptosis-related genes in endothelial cells

Bax mRNA was constitutively expressed in endothelial cells, and the levels were not altered by serum starvation, coculture with monocytes, or IL-1 stimulation, except for a small increase after serum starvation at 6 hours. It is unlikely, however that changes in bax levels are responsible for the protective effect of monocytes on survival of serum starved endothelial cells seen at 21 hours because bax expression at this time point was unchanged under all conditions (Figure 4.18). Bcl-x_L mRNA was also expressed by unmanipulated endothelial cells, but mRNA levels were not altered by serum starvation, nor by subsequent coculture with monocytes or by stimulation with IL-1 (Figure 4.19). Bcl-2 mRNA was expressed at low levels in endothelial cells and appeared to

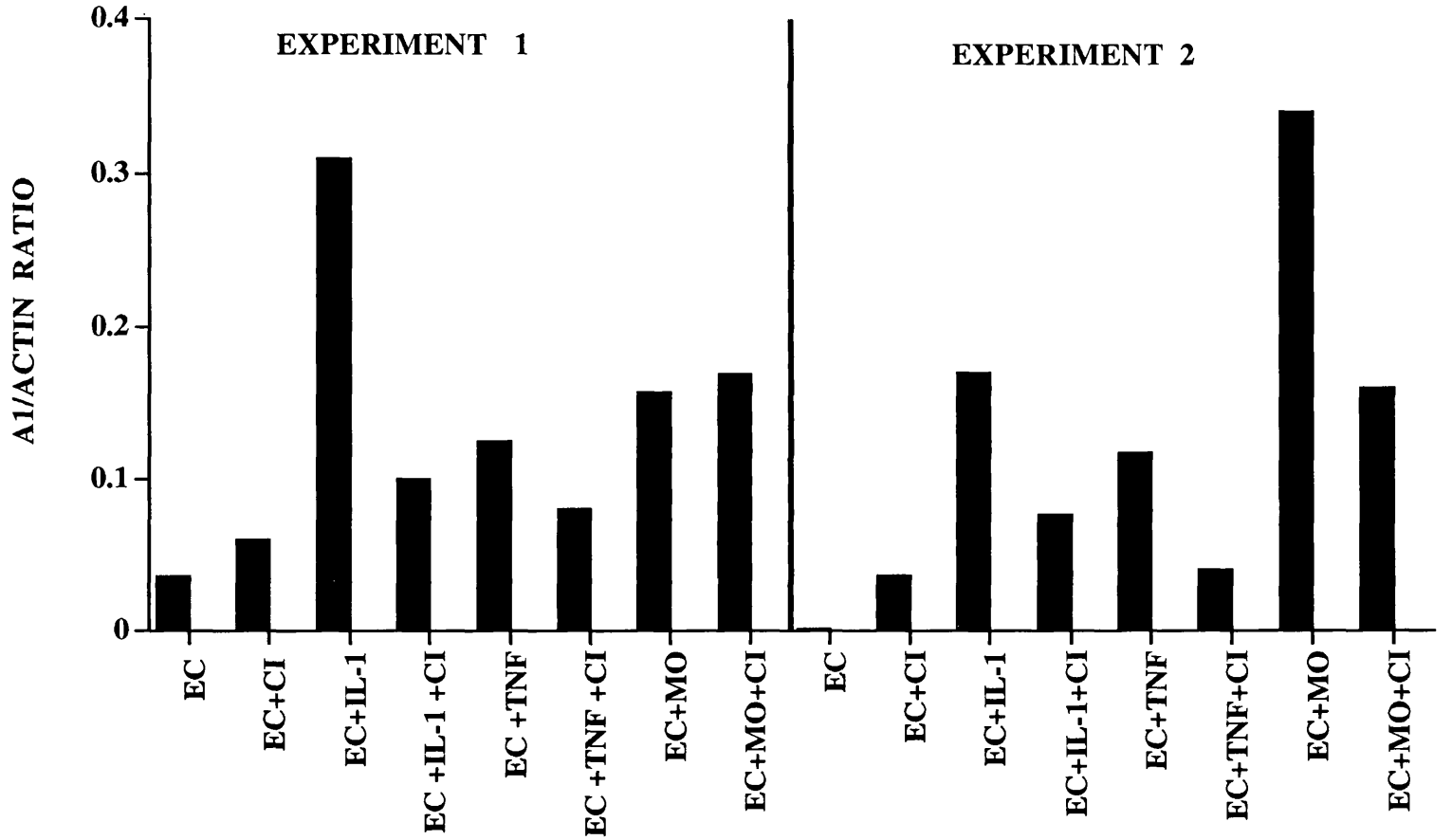

Figure 4.17 Role of NF-κB activation in monocyte stimulation of A1 mRNA in endothelial cells. Endothelial cells were serum starved as described in Figure 4.1a. and monocytes (1:1), TNF (100U/ml) or IL-1 (100U/ml) were added for 4 hours. Calpain inhibitor I (CI), 50μg/ml, was added at the start of the coculture and was present throughout the duration of the experiment. Control cultures included DMSO at the same concentration as was present in cultures containing CI. Data shown from two separate experiments.

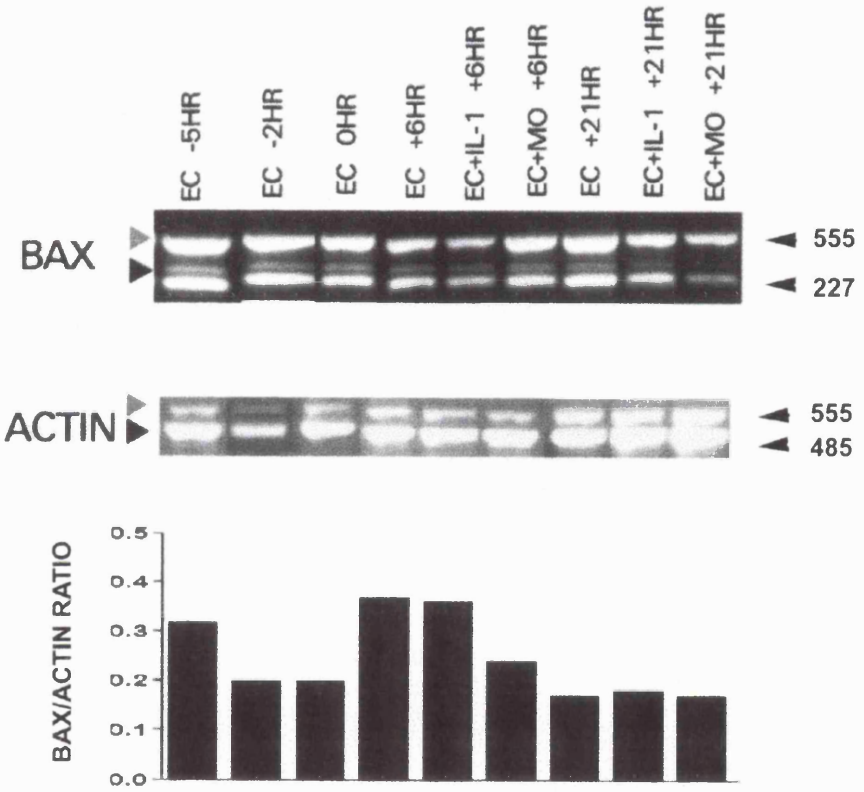

Figure 4.18 Effect of monocytes and IL-1 on expression of Bax mRNA in endothelial cells. Endothelial cells and monocytes were set up as described in Figure 4.1a and notations are the same as in Figure 4.1b. The bands shown are for one representative experiment, and the histogram displays bax mRNA levels relative to actin. The m.wts of the products are indicated

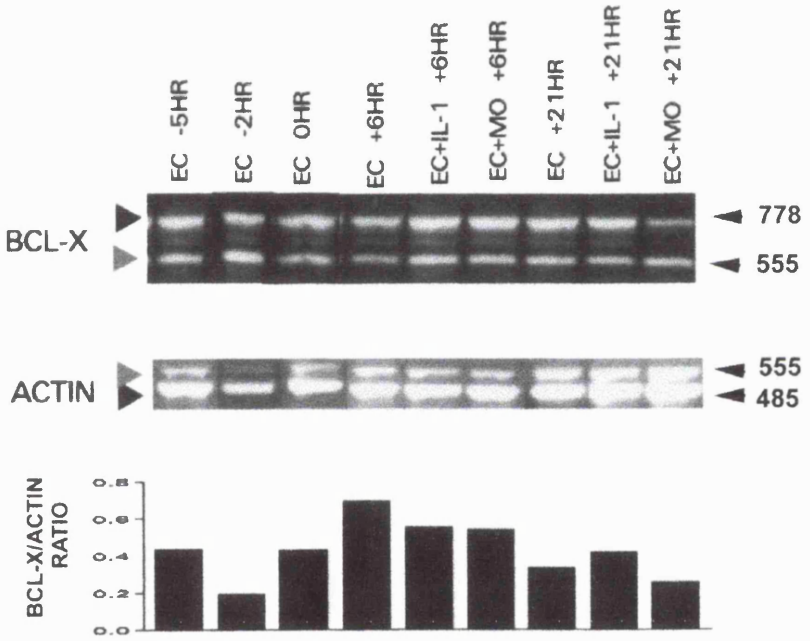

Figure 4.19 Effect of monocytes and IL-1 on Bcl-x mRNA expression in endothelial cells. Endothelial cells and monocytes were set up as described in Figure 4.1a and notations are the same as in Figure 4.1b. The bands shown are from one representative experiment, and the histogram displays bcl-x mRNA levels relative to actin. The m.wts of the products are indicated.

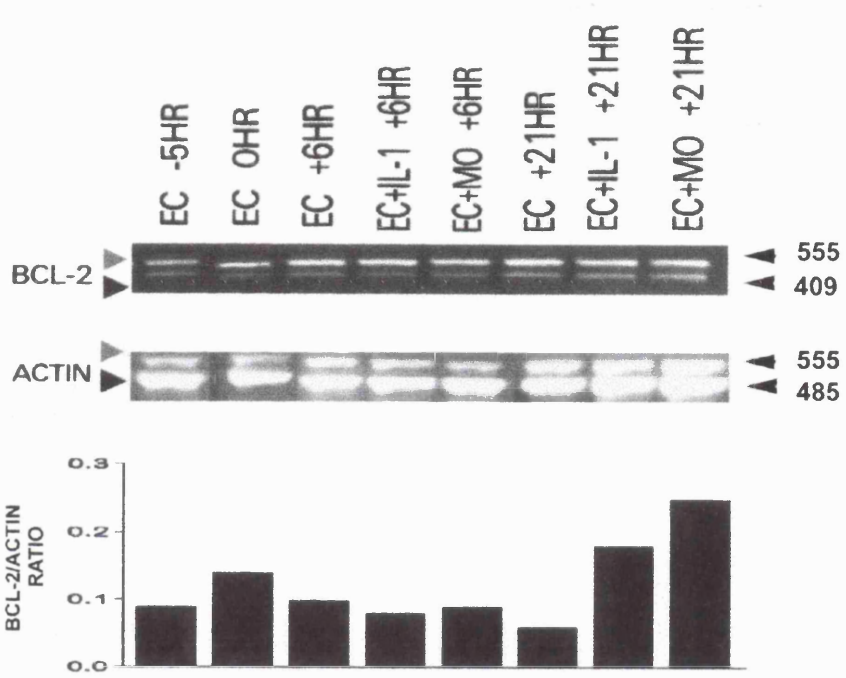

Figure 4.20 Effect of monocytes and IL-1 on Bcl-2 mRNA expression in endothelial cells. Endothelial cells and monocytes were set up as described in Figure 4.1a and notations are the same as in Figure 4.1b. The bands shown are from one representative experiment and the histogram displays bcl-2 mRNA levels relative to actin. The m.wts of the products are indicated.

be up-regulated in response to coculture with monocytes, but with delayed kinetics compared with A1 (Figure 4.20).

Discussion

Monocytes induce A1 mRNA expression in serum starved endothelial cells with a sustained expression at 21 hours compared to the transient response to IL-1. Monocytes also confer survival on serum starved endothelial cells, decreasing the percentages of apoptotic cells at 21 hours compared to the numbers in serum starved conditions. IL-1 offered no protection to serum starved cells. Contact between monocytes and endothelial cells is required to achieve optimal induction of A1 expression in endothelial cells, however the adhesion pathways involved in this induction were not fully elucidated. Monoclonal antibodies directed against PECAM-1 reduced the monocyte induction of A1 in endothelial cells with a concurrent decrease in cell survival. TNF appears to play a role in the A1 induction by monocytes and is more influential at the earlier time point of 6 hours as evidenced by the effects of anti-TNF antibody. Exogeneous TNF was more effective at inducing endothelial A1 expression after 6 hours than after 21 hours of stimulation. The addition of IL-10 to monocyte/endothelial cell cocultures partly inhibited the monocyte induction of A1 at 6 hours.

Endothelial cells provide an antithrombotic and anti-inflammatory barrier for the normal vessel wall. In normal arteries endothelial cells are remarkably quiescent but acceleration of the turnover rate of endothelial cells can lead to their dysfunction. Apoptosis is a physiological process that contributes to vessel homeostasis, by eliminating damaged cells from the vessel wall. Increased endothelial cell turnover mediated through accelerated apoptosis may however alter the function of the endothelium and therefore promote atherosclerosis.

Apoptotic endothelial cells can be detected on the luminal surface of atherosclerotic coronary vessels, but not in normal vessels (Alverez et al, 1997). Extracellular stimuli which lead to apoptosis fall into two groups: positive induction by ligand binding to a plasma membrane receptor or negative induction by loss of a suppressor signal. In cultured endothelial cells several treatments lead to the onset of apoptosis, including cholesterol oxides, derivatives of cholesterol (Lizard et al, 1996), and free fatty acids such as linoleic acid (Toborek et al, 1997) as well as alteration in haemodynamic forces (Kaiser et al, 1997). These conditions may be considered as molecular consequences of traumatic or pathogenic events whilst the withdrawal of serum such as in these experiments represents an artificial situation. Serum has been well documented as a specific inhibitor of apoptosis in human endothelial cells (Zoeliner et al, 1996) and serum starvation was chosen in these experiments to provide a pro-apoptotic situation for endothelial cells so that any pro-survival signals could be detected. Endothelial cells serum starved in this way exhibited classical characteristics of apoptosis, as described in other reports (Zoeliner et al, 1996) such as membrane blebbing, loss of focal adhesions and retraction from the substratum, nuclear condensation and fragmentation as detected morphologically and confirmed by FACS analysis .

Studies implicate integrin receptors in mediating survival of cultured endothelial cells and normal cells denied anchorage to an appropriate extracellular matrix undergo apoptosis, in fact serum does not protect cells also deprived of adhesion. Thus inducers and inhibitors of apoptosis have been identified for endothelial cells, but little is known of cell-cell interaction and its role in the modulation of apoptosis except that alveolar epithelial cells protect endothelial cells from apoptosis (Wendt et al, 1994) and that interaction of the proximal

part of the prostrate gland with underlying stromal cells prevents apoptosis when hormones are removed (Tenniswood, 1986).

The monocyte specificity of the A1 induction in serum starved endothelial cells could be related to the fact that neutrophils and unactivated lymphocytes do not release TNF and TNF has been shown to induce expression in endothelial cells (Karsan et al, 1996) and to play a large role in A1 induction by monocytes as evidenced in my studies. On the other hand, monocytes and TNF have both been implicated in causing endothelial cells apoptosis. Recently peripheral blood mononuclear cells were found to cause endothelial cell apoptosis when cocultured with the endothelial cells (Lindner et al, 1997), and this was cell-cell contact mediated via mTNF-α. In these experiments, however, the mononuclear cells were preactivated by ionising radiation or by LPS, and the authors do state that untreated PBMCs did not cause endothelial cell apoptosis. In addition lymphocytes, which formed a large percentage of the PBMC, were found to express TGF-β which has been shown to cause endothelial cell apoptosis by downregulating the expression of bcl-2 (Tsukada et al, 1995) but not to induce A1 in endothelial cells (Karsan et al, 1996).

While lymphocytes on their own did not stimulate A1 expression in endothelial cells, the possibility that the small number of contaminating lymphocytes may interact with monocytes/endothelial cells and contribute to the increase in A1 mRNA levels in endothelial cells cannot be excluded. Lymphocytes adhering to the endothelial cells may cause the release of platelet activating factor which would in turn stimulate the production of TNF. Thus, the presence of lymphocytes in the monocyte preparation might represent more closely the *in vivo* situation.

The addition of IL-10 to monocyte/endothelial cell cocultures inhibits the monocyte induction of A1 with greatest effect at 6 hours. This result lends support to the involvement of TNF in the monocyte induction of A1 since IL-10 is an immunomodulatory cytokine that inhibits TNF production in monocytes. It is possible that the inhibitory action of IL-10 on A1 expression in endothelial cells may be the result of suppression of TNF synthesis by monocytes.

TNF is synthesised by monocytes either as membrane bound or as a soluble protein and both forms may be important in the monocyte induction of A1 expression in endothelial cells. Evidence for a role for soluble TNF is provided by the finding that anti-TNF monoclonal antibody abolished A1 induction when monocytes were separated from the endothelium by 0.45 micron filters (data not shown).

TNF influences endothelial cell viability by altering the balance of regulatory molecules that either induce or suppress apoptosis. Whilst some papers state that TNF does induce endothelial cell apoptosis (Polunovsky et al, 1994; Robaye et al, 1992), endothelial cells are considered not to be susceptible to TNF induced apoptosis unless rendered sensitive by protein synthesis inhibitors. The requirement for protein synthesis suggests that TNF induces genes that confer protection on cells. Stimulation of cells with TNF generates two signals, one that initiates programmed cell death and one that leads to activation of the transcription factor, NF-κB. When NF-κB signalling is inhibited the cells become more sensistive to TNF cytotoxicity with an increase in apoptosis (Van Antwerp et al, 1996). NF-κB activates gene products such as TRAF-1, TRAF-2 and c-IAP that function cooperatively at the earliest check points to suppress TNF mediated apoptosis by suppressing initiation of caspase activation (Wang et al, 1998). These anti-apoptotic genes, including

A1, also act as inhibitors of NF-κB and thus may control expression of proinflammatory genes during endothelial cell activation (Soares et al, 1998). Similiarly, LPS has been shown to induce A1 expression (Hu et al, 1998) and thus not cause death in endothelial cells, however, when expression of new proteins was inhibited with cycloheximide endothelial cells became apoptotic (Karsan et al, 1996), suggesting that the anti-apoptotic pathway is dependent on synthesis of new proteins. When NF-κB activation was inhibited, the accumulation of A1 mRNA was blocked thus A1 induction requires activation of NF-κB (Karsan et al, 1998). The overall result in a specific cell type is dependent on the balance of the two signals.

In my studies, using a proteasomal inhibitor, ALLN (CI), that blocked NF-κB activity, TNF and IL-1 induced A1 expression was inhibited in endothelial cells at 6 hours suggesting that these cytokines activate NF-κB resulting in upregulation of A1. These results are supported by other work also using this inhibitor (Read et al, 1994; Karsan et al, 1998). The proteasomal inhibitor, ALLN, however, failed to conclusively inhibit the monocyte induced A1 expression at 6 hours suggesting that the monocyte induction of A1 may be NF-κB independent.

The reasons for sustained A1 expression in endothelial cells cocultured with monocytes remains unclear. Monocytes could influence the stability of A1 mRNA, increase the rate of transcription, or increase the stability of A1 protein. It has not been possible to analyse the rate of transcription of the A1 gene by nuclear run ons since the experiment requires a larger number of endothelial cells than can be cultured and the experiment is limited by the number of monocytes that can be isolated from peripheral blood using Nycoprep. In

addition A1 protein could not be studied due to the lack of a suitable antibody for use in Western blotting.

Monocytes not only induced A1 mRNA expression but also confered a survival signal to serum starved endothelial cells. This increase in cell viability could be related to the increase in A1 gene expression however I have not confirmed a direct relationship between the two. Evidence to support the hypothesis that increased A1 mRNA levels leads to increased survival include the fact that IL-1 did not provide protection to endothelial cells at 21 hours when the level of A1 in IL-1 treated endothelial cells had decreased to near basal levels. In another study retroviral-mediated transfer of A1 cDNA offered protection in human microvascular endothelial cells treated with TNF. When these cells were treated with TNF in the presence of cycloheximide for 12 hours, A1 protein was not detectable after 3 to 6 hours, and the level of protection was much reduced (Karsan et al, 1996).

Other anti-apoptotic molecules are also upregulated by TNF, including A20 which is a cytoprotective gene in endothelial cells that acts to protect endothelial cells from apoptosis (Ferran et al, 1998). This protein is also dependent on NF-κB for expression and can inhibit activation of NF-κB. Expression of other members of the bcl-2 family such as bax, bcl-x_L and bad can also be induced in endothelial cells. The bcl-2 family proteins regulate apoptosis through forming physical interactions with each other in a complicated network of homo- and heterodimers and the ratio between anti-apoptotic proteins such as A1 relative to pro-apoptotic proteins such as bax determines the ultimate sensitivity of cells to various apoptotic stimuli (Zha et al, 1996). For example LPS causes endothelial apoptosis by reducing expression of bcl-2 protein and and at the same time upregulating the expression of bax

protein (Haendeler et al, 1996). A1 can bind to bax and thus function like bcl-2 by inhibiting bax induced cytochrome C release from mitochondria hence reducing the activation of caspase 3 (Sedlak et al, 1995). Monocytes had little effect on bax expression and this suggests that monocytes confered protection through inducing A1 expression as opposed to altering the expression of the pro-apoptotic gene, bax. Bcl-x_L is constitutively expressed by endothelial cells but levels were not significantly altered by monocyte coculture. Bcl-2 mRNA levels in endothelial cells increased in response to coculture with monocytes, but with slower kinetics when compared with A1 mRNA. A possible role for bcl-2 protein in the protection of endothelial cells from apoptosis cannot be excluded.

The experiments suggest that cell-cell contact is required for maximal induction of A1 in endothelial cells by monocytes. This generation of a signal inducing A1 could be as a result of ligation of surface adhesion receptors. I was not, however, able to inhibit the induction of A1 by using monoclonal antibodies to CD11b/CD18 complex, despite the fact that these antibodies produced partial inhibition of monocyte adhesion to serum starved endothelial cells. These results suggest that the monocyte induction of A1 does not rely on only one adhesion pathway such as CD11b/CD18 but in fact on multiple receptor-ligand pathways including VLA-4/VCAM and E-selectin. Studies have shown that adherence of monocytes to TNF stimulated endothelium is not blocked by the use of antibodies to CD18, CD49d or E-selectin when used singly but combinations of these antibodies produce significant inhibition (Carlos et al, 1991; Beekhuizen et al, 1991).

The reduction in A1 stimulation by anti-PECAM-1 monoclonal antibodies in endothelial cell/monocyte cocultures raises the interesting possibility that

monocyte transmigration may be important in stimulating the increase in A1 mRNA in endothelial cells. It may be that the engagement of PECAM-1 on transmigrating leukocytes can lead to cell activation (Berman et al, 1995) which in turn releases signals to the endothelium to influence cell behaviour and survival. Release of cytokines from monocytes is induced when there is co-ligation of PECAM-1 and FCγRII (Chen et al, 1994). Whilst endothelial cell activation by ligation of PECAM-1 has yet to be confirmed there is data to suggest that PECAM-1 is tyrosine phosphorylated and is involved in signal transduction (Masuda et al, 1997).

The results presented in this chapter show that monocytes, in contact with endothelial cells, induce A1 mRNA expression in endothelial cells, and confer survival on serum starved endothelial cells, decreasing the percentage of apoptotic cells. Monocytes are only likely to come into contact with endothelial cells as part of an inflammatory response and thus these results suggest that endothelial cells receive survival signals during inflammation. This is supported by the evidence that TNF and IL-1, inflammatory cytokines also induce A1 expression in endothelial cells. Survival signals are important since the vascular endothelium plays a pivotal role in the regulation of the inflammatory response, thus the maintenance of endothelial cell integrity and survival is crucial to the effective development as well as the successful resolution of inflammation. Indeed, endothelial cell loss would expose the subendothelial matrix which would contribute to the generation of thrombosis. In addition, A1 inhibits NF-κB suggesting that A1 functions to protect the endothelial cells not only from apoptosis but also from the potentially untoward effects of unfettered activation, that can lead to altered endothelial cell function, leading to fluid leakage and thrombosis. Endothelial cell apoptosis and uncontrolled and ongoing activation

are both undesirable and the presence of A1 and other protective genes in endothelial cells may play a key role in the homeostatic regulation of the endothelium.

Chapter Five

The Role of Calpain in Neutrophil and Monocyte Transendothellal Migration

Introduction

A critical step in inflammation is migration of circulating leukocytes from the vascular compartment into surrounding tissue, via passage between adjacent endothelial cells. During the process of extravasation leukocytes transiently adhere to the endothelium and subsequently with a variety of extracellular matrix components. These interactions are likely to serve as modifiers of transcriptional activity and to prime the leukocyte and endothelium for altered gene expression. Firm adhesion and spreading of adherent leukocytes on endothelium is a prerequisite for transmigration and evidence suggests that in addition to the signalling events that lead to leukocyte transmigration, cellular interactions with transmigrating leukocytes may trigger further endothelial cell responses.

PECAM-1, a member of the immunoglobulin superfamily which is expressed on leukocytes and endothelium, is concentrated at the junctions between endothelial cells and is considered to have an important role in the transendothelial cell migration of leukocytes (Elias et al, 1998). In addition to mediating transmigration, PECAM-1 may also play a role in cell signalling. Ligation of PECAM-1 results in endothelial cell activation with a sustained increase in endothelial intracellular calcium (Gurubhagavatula et al J, 1998). In addition, anti-PECAM-1 antibody inhibits the induction and sustained upregulation of E-selectin (Chapter 3) and A1 mRNA (Chapter 4) expression in monocyte/endothelial cell cocultures. These results raise the question as to whether it is PECAM-1 engagement itself, during leukocyte transmigration, that is important for cell signalling resulting in induction of E-selectin and A1 genes. Monocytes co-ligated with PECAM-1 and FCγRII are induced to release TNF-α, IL-1β and IL-8 (Chen et al, 1994) and engagement of PECAM-1 on neutrophils

results in L-selectin shedding, upregulation of CD11b/CD18 and the release of oxygen radicals, while monocytes secrete TNF (Elias et al, 1998). Monocytes are also induced to form reactive oxygen intermediates when activated with PECAM-1 monoclonal antibody (Stockinger et al, 1990). Hence the engagement of PECAM-1 during monocyte transmigration may be important in cellular activation.

Alternatively, ligation of surface receptors by transmigrating leukocytes could lead to endothelial cell activation and the inhibitory effect of anti-PECAM-1 antibody on A1 and E-selectin induction is due to the prevention of leukocyte transmigration. Transmigrating leukocytes could also be activated to release cytokines or chemokines which would in turn act on the endothelial cells. It is possible that by blocking transmigration with anti-PECAM-1, induction of A1 and E-selectin genes is inhibited as a result of preventing leukocyte migration between endothelial cells, rather than by inhibiting PECAM-1 ligation. This has led me to investigate the mechanisms underlying monocyte and neutrophil transmigration.

Leukocytes migrate across adherens junctions and these junctions are transmembrane multimolecular complexes in which the actin based cytoskeleton is linked to the plasma membrane through a specialised submembrane plaque. Major elements in this structure are members of the cadherin family and a cytoplasmic anchoring system which binds these molecules to the microfilament network. Directly associated with the junctional cadherins are the anchor-protein catenins, α, β and plakoglobin (Lampugnani et al, 1995). Adhesion of resting neutrophils to TNF stimulated endothelium results in adherens junction disassembly (Del Maschio et al, 1996), with a

reduction in the junctional localisation of VE cadherin, α and β catenin and plakoglobin.

Cell migration requires a dynamic interaction between a cell, its substratum and the actin cytoskeleton. Integrin receptors, which are αβ heterodimers present on the cell surface play an important role during cell migration by mediating these actions and transmitting forces between the extracellular matrix and actin cytoskeleton. The mechanisms by which these linkages are regulated and released at the rear of the cell during migration are still unclear. Engagement of integrins with the appropriate ligands initiates intracellular signals in the leukocyte including tyrosine phosphorylation of cellular proteins and changes in cytoplasmic Ca^{2+} concentrations (Mondal et al, 1995). Such calcium transients have been implicated in the adhesive release in leukocytes migrating on certain substratums and $[Ca^{2+}]_i$ activates enzymes and mediators such as protein kinase C (PKC), MLCK and calpain which have been implicated in integrin function. An advancing cell needs to bring new receptors such as integrins, for substrate attachment to its front and as the cell advances receptors attached to the substratum move backwards with respect to the cell. The release of integrins from the substratum requires intracellular $[Ca^{2+}]_i$ transients, suggesting that calcium-dependent mechanisms are strongly involved in this recycling of integrins. Calpain, a calcium dependent protease is a possible regulator of this release of adhesions at the rear of the cell with subsequent detachment and retraction, since it localises to focal adhesions and cleaves many focal adhesion-related proteins such as integrin receptors and talin (Du et al, 1995).

In addition, the binding of neutrophils to the apical surface of endothelial cells increases the permeability of the endothelial monolayer (Del Maschio et al, 1996), and leads to an increase in endothelial Ca^{2+} levels (Huang et al, 1993).

The VE-cadherin/catenin complex is important for the junctional integrity of endothelial cell layers. Endothelial cell Ca^{2+} levels regulate transendothelial migration of neutrophils by participating in a signal cascade which stimulates endothelial cells to open their intercellular junctions to allow transendothelial passage of leukocytes.Therefore the concept of adhering leukocytes having an effect on the VE-cadherin junctional complex remains attractive and a role for calpain in this disassembly of adherens junctions is possible, although evidence is still lacking.

In this chapter the role of calpain in the transmigration of neutrophils and monocytes in response to cytokine stimulation of the endothelium and also chemokines is investigated through use of various cell permeable calpain inhibitors.

Results

Calpain Inhibitor I reduces neutrophil migration across cytokine stimulated endothelium

Neutrophils migrate spontaneously across unstimulated endothelium (8.7±1.4%,n=3, Figure 5.1). Pretreating the endothelium for 4 hours with TNF (100U/ml) or IL-1 (20U/ml) significantly enhances neutrophil transmigration by 214±125% and 336±91% respectively. Percent neutrophil migration increased to 23.9±4.9% with TNF ($p< 0.05$, n=3) and to 35.4±0.6% with IL-1 ($p<0.01$, n=3, Figure 5.1). In the presence of calpain inhibitor I (CI), neutrophil transmigration across TNF stimulated endothelium was reduced to baseline levels (5.4±1.7%, $p<0.05$, n=3, Figure 5.1) as was migration across IL-1 treated endothelium (8.2±2.7%, $p<0.01$, n=3, Figure 5.1). The effect of CI was dose dependent, with

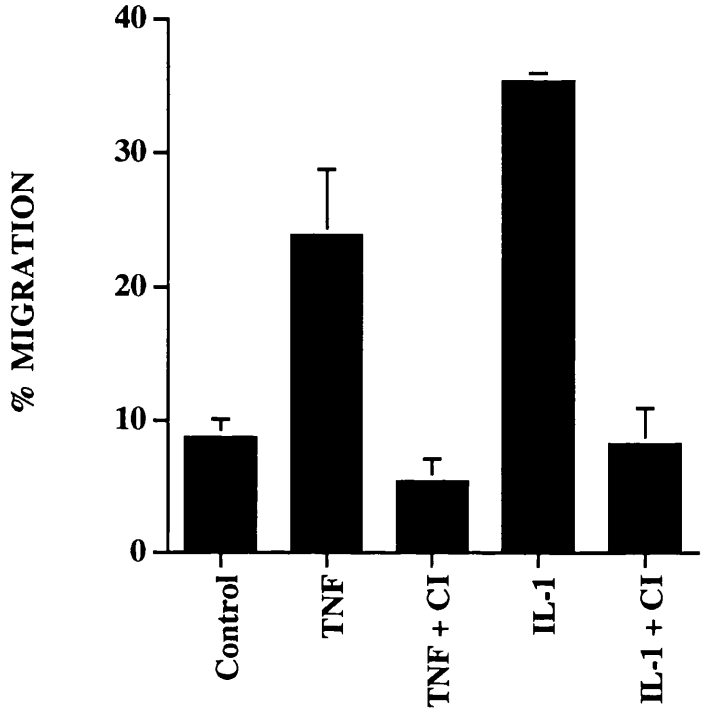

Figure 5.1 Calpain inhibitor I reduces neutrophil transmigration across cytokine activated endothelium. Endothelial cells were cultured and grown to confluence on 3 micron Transwell filters and neutrophils were isolated and ^{51}chromium-labelled as described in the Methods. Endothelial cells were stimulated with TNF (100U/ml) or IL-1 (20U/ml) for 4 hours, washed and neutrophils added at a concentration of $3x10^6$cells/ml in 100μl of complete growth medium. Calpain inhibitor I (CI), 50μg/ml was added concurrently with neutrophils and remained present throughout the duration of the experiment, 90 minutes. After this time, the migrated neutrophils were harvested and the percentage migration determined as detailed in the Methods. Data are mean ± s.e.m. of 3 experiments.

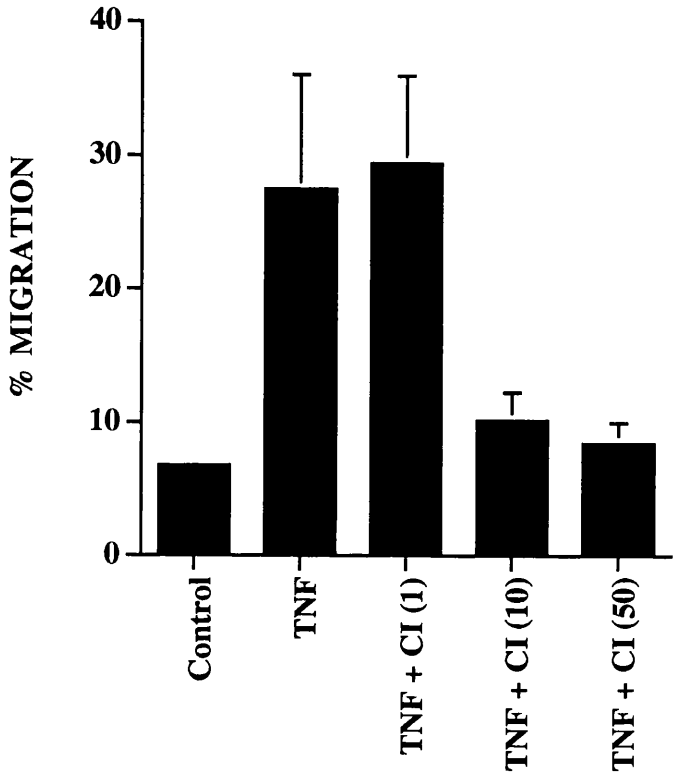

Figure 5.2 Dose response of calpain inhibitor I. Endothelial cells and neutrophils were prepared as described in Figure 5.1, except that calpain inhibitor I was added at three different concentrations. Data are mean ± s.e.m. of 3 experiments. Calpain inhibitor I used at 1-50μg/ml.

maximal inhibition achieved at 10-50μg/ml (Figure 5.2). Preliminary experiments showed that for optimal inhibition, calpain inhibitors had to be present throughout the assay, however, no preincubation of either endothelium or neutrophils was required.

Effects of other Calpain Inhibitors on neutrophil migration across TNF stimulated endothelium

The addition of calpain inhibitor II (260μM) also reduced neutrophil transmigration across TNF stimulated endothelium producing an inhibition of 68±10% ($p<0.05$, n=3, Figure 5.3a). Calpain inhibitors I and II both also block proteasome activity, hence to investigate the specificity of the inhibitory effect, the proteasome inhibitor, lactacystin was tested in the migration assays. Lactacystin had no effect on TNF-stimulated neutrophil migration (Figure 5.3b). E64D, another calpain inhibitor, reduced transmigration by 72±5%, from 11.62±2.4% across TNF stimulated endothelium to 3.8±0.72%, $p<0.05$, n=4 (Figure 5.3c). I was unable to assess the effects of calpeptin, also a calpain inhibitor, on neutrophil transmigration because this inhibitor caused loss of integrity of the endothelial monolayer, as determined by the passage of ^{51}Cr-EDTA ; the other inhibitors used did not compromise monolayer integrity (Table 5.1).

Calpain inhibition does not alter adhesion of neutrophils to TNF treated endothelium

The tight adhesion of leukocytes to the endothelium precedes leukocyte transendothelial migration, thus I investigated the effect of calpain inhibitors on neutrophil adhesion to TNF-stimulated endothelium. Neutrophils adhere poorly

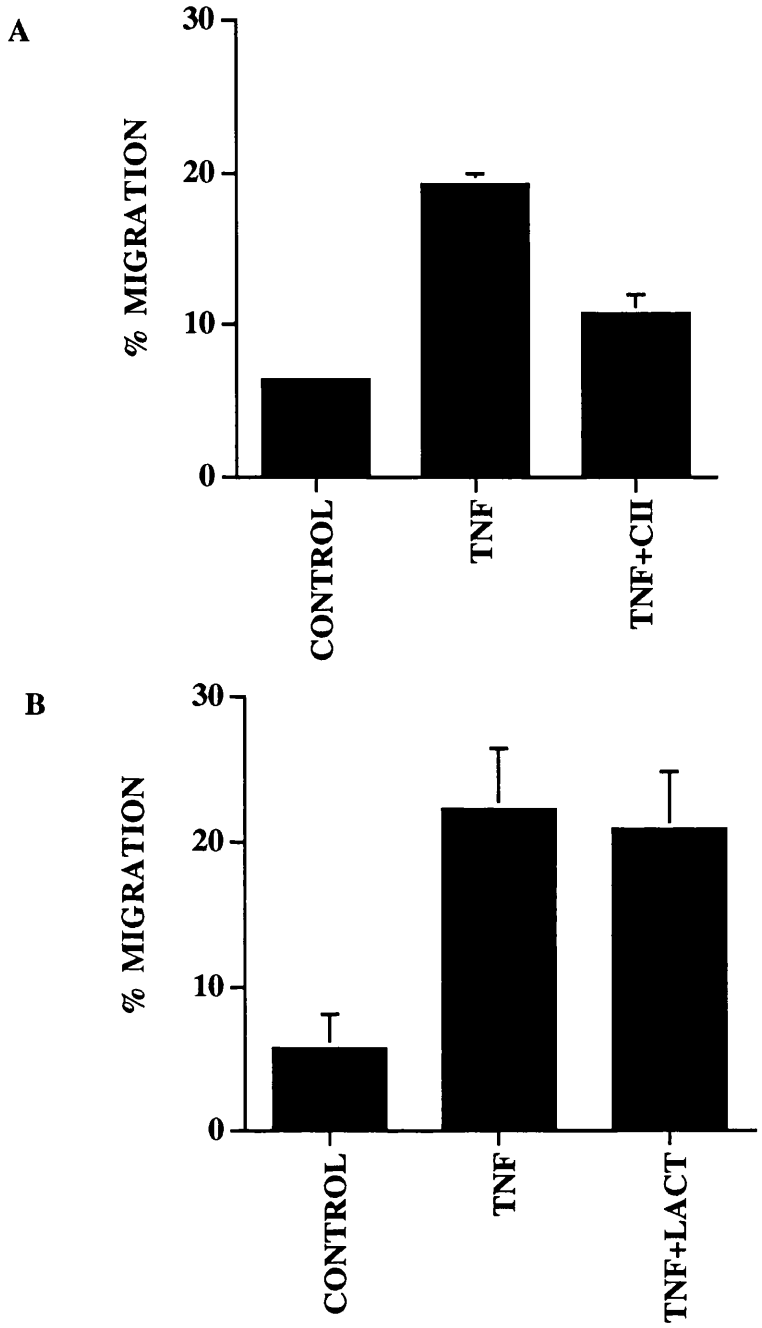

Figure 5.3 Effects of other calpain inhibitors on neutrophil transmigration across TNF stimulated endothelium. Endothelial cells and neutrophils were prepared as described in Figure 5.1 and A) calpain inhibitor II (CII), 260μM B) lactacystin (LACT), 50μM, and C) E64D, 100μg/ml were added concurrently with the neutrophils, and remained present throughout the 90 minute migration assay. Transmigrated neutrophils were harvested and the percentage migration determined as detailed in Methods. Data are mean ± s.e.m. of 3 experiments for CII and 4 experiments for lactacystin and E64D.

C

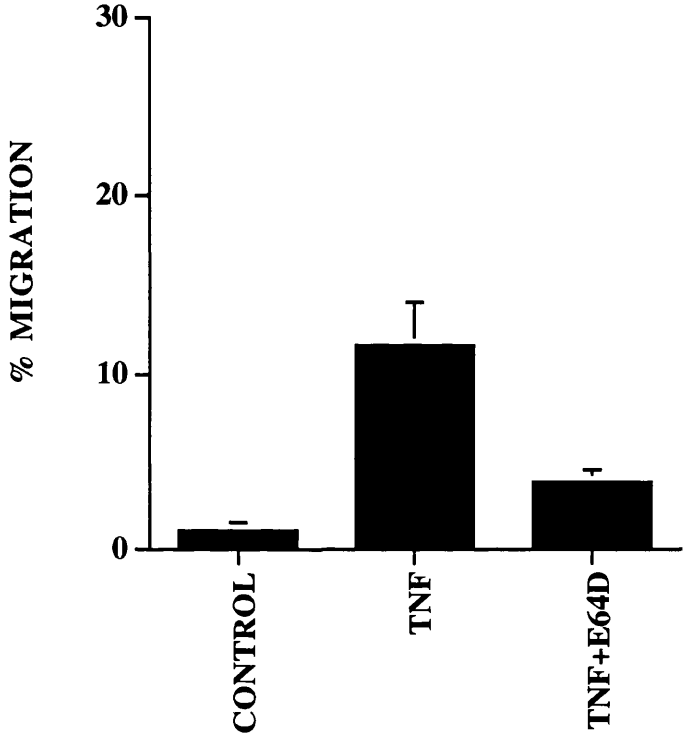

Figure 5.3 Effects of other calpain inhibitors on neutrophil transmigration across TNF stimulated endothelium. C) E64D, 100μg/ml.

inhibitor added	% equiilbration of 51Cr-EDTA in the bottom well
Nil	20
+ Calpain Inhibitor I	27
+ Calpain Inhibitor II	26
+ Lactacystin	21
+ E64D	26
+ Calpeptin	56

Table 5.1 Barrier function of endothelium treated with calpain inhibitors Endothelial cells were grown to confluence on 3 micron filters as described in the Methods. ^{51}Cr-EDTA was placed in the top chamber and after 30 minutes the distribution of the radioactivity between the upper and lower chambers counted. 100% equilibration of ^{51}Cr-EDTA in the bottom well would occur if the radioactivity distributed equally between the upper and lower chambers by 30 minutes (i.e. 85% of total ^{51}Cr-EDTA would be in the lower chamber since 600μl of medium is in the bottom well and 100μl in the top chamber).

to unstimulated endothelium, but stimulation of endothelium with TNF leads to a significant increase in adhesion from 1.05±0.6% to 17.86±5.2% (n=3, $p<0.05$, Figure 5.4). The level of adhesion is not affected by the addition of calpain inhibitor I, lactacystin or E64D (Figure 5.4). Calpain inhibitor II also had no effect on neutrophil adhesion to TNF stimulated endothelium (data not shown). Inhibition of neutrophil transendothelial migration by calpain inhibitors was thus not due to reduced neutrophil adhesion to endothelial cells.

Calpain inhibition reduces neutrophil transmigration in response to chemoattractants

Neutrophils migrate in response to chemoattractants such as IL-8. Migration of neutrophils across unstimulated endothelium increased 7-fold in response to IL-8, (7.02±1.5% migration across unstimulated endothelium in the absence of chemoattractant compared to 52.87±8.9% towards IL-8, $p<0.01$, n=4, Figure 5.5). Neutrophil migration due to cytokine activation of endothelium occurs in response to the production of endothelial derived chemokines and thus calpain inhibition of migration across TNF and IL-1 stimulated endothelium could result from inhibition of the neutrophil migratory response to chemokines. Calpain inhibitor I reduced neutrophil migration towards IL-8 (50ng/ml) by 37.3±4% (35.2±4.7% migration in the presence of CI I compared to 52.87±8.9% without, $p<0.05$, n=4, Figure 5.5). At lower doses of IL-8 the neutrophil transendothelial migratory response was lower (26.6% with 10ng/ml) and the inhibitory effect of CI was greater (83% inhibition, from 26.6% to 13.6%, one experiment, data not shown).

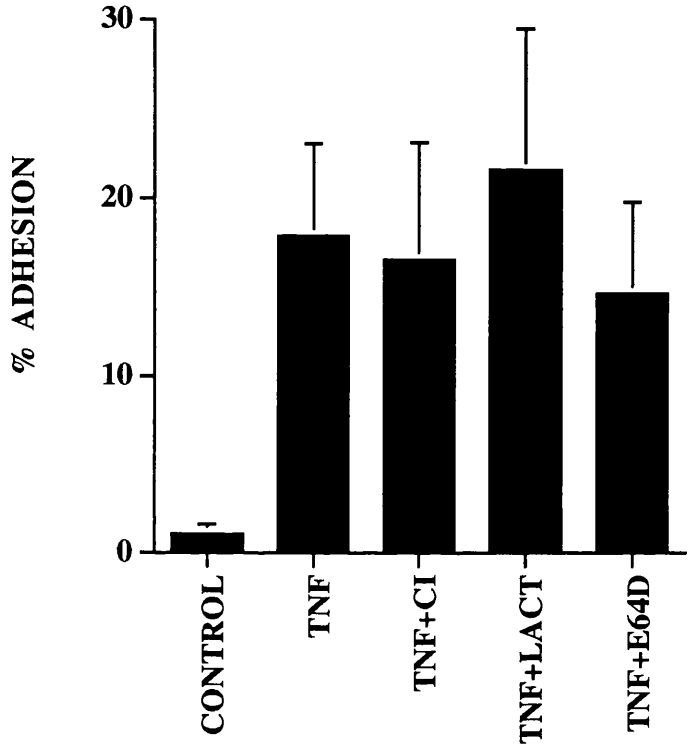

Figure 5.4 Effect of calpain inhibitors on neutrophil adhesion to TNF stimulated endothelium. Endothelial cells were grown to confluence in 48 well plates, stimulated with TNF (100U/ml) for 4 hours, washed and ^{51}Cr labelled neutrophils added at a concentration of $3x10^6$cells/ml in 200μl. Calpain inhibitor I (CI), 50μg/ml; lactacystin (LACT), 50μm; and E64D, 100μg/ml were added with the neutrophils and remained present throughout the experiment. After 90 minutes, non adherent neutrophils were washed off and adherent neutrophils harvested and the percentage adherence determined as detailed in the Methods. Data are mean ± s.e.m. of 3 experiments.

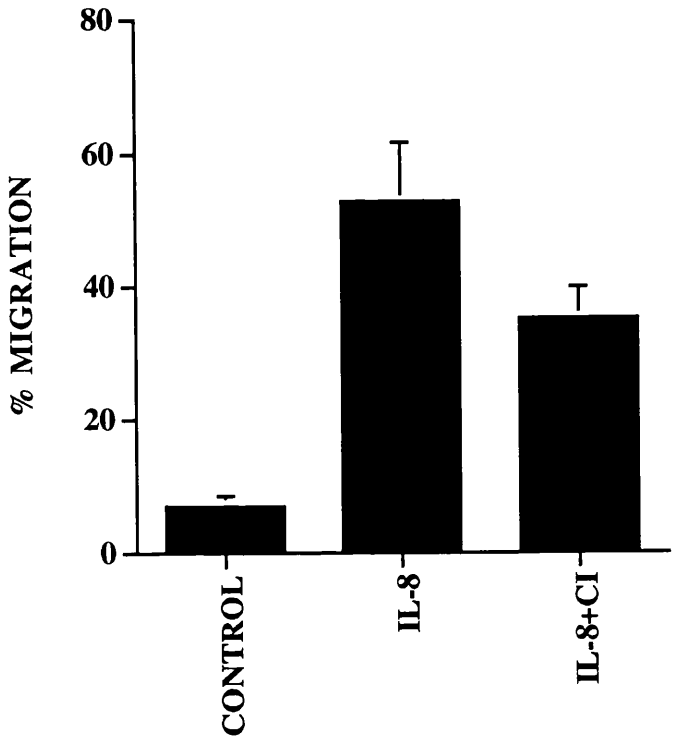

Figure 5.5 Calpain inhibitor I reduces IL-8 induced neutrophil transmigration across endothelium. Endothelial cells and neutrophils were prepared as described in the Methods. Calpain inhibitor I (CI), 50μg/ml was added concurrently with ^{51}Cr-labelled neutrophils to washed endothelial cells. IL-8, 50ng/ml, was added to the bottom chambers of the Transwells 10 minutes later. After 90 minutes, migrated neutrophils were harvested and the percentage of migration determined as detailed in the Methods. Data are mean ± s.e.m. of 4 experiments.

Calpain inhibition reduces transmigration by acting predominantly on the neutrophil

To investigate if the inhibitory effect of CI was directed at neutrophils or endothelial cells, neutrophils were transmigrated across filters alone (1 micron pore) towards IL-8. Neutrophil migration across filters alone over 90 minutes was 3.2±0.35% (n=4, Figure 5.6), increasing to 15.87±3.9% when IL-8 was placed in the lower chamber. The percent migration was reduced by 65±6% in the presence of CI (7.5±1.2%, n=4, $p<0.05$, Figure 5.6). This percentage of inhibition is similar to that achieved with the addition of calpain inhibitor I to neutrophils migrating across unstimulated endothelium towards IL-8, suggesting that calpain inhibition is directed at the neutrophil, although a contributory effect on the endothelium cannot be discounted. IL-8 was used at maximal concentrations of 50ng/ml. Other calpain inhibitors were also inhibitory; E64D (50μg/ml) reduced IL-8 stimulated migration by 28% and 66% and calpain inhibitor II by 18% and 27% in 2 separate experiments (data not shown). Calpeptin was used in this system since endothelium was not present and reduced IL-8 directed migration by 46% and 33% in two experiments (data not shown). Lactacystin had no effect on chemoattractant stimulated neutrophil migration across filters alone (data not shown).

Monocyte migration towards a chemoattractant is reduced by Calpain Inhibitor I

Monocytes are the predominant cell type present in atherosclerotic lesions and the mechanisms that enable monocytes to migrate from the blood into tissues are only now becoming elucidated. The spontaneous migration of monocytes through unstimulated endothelium (10.56±2.19% over 2.5 hours, n=4, Figure 5.7) occurs at a greater rate than neutrophils. Monocyte transmigration is

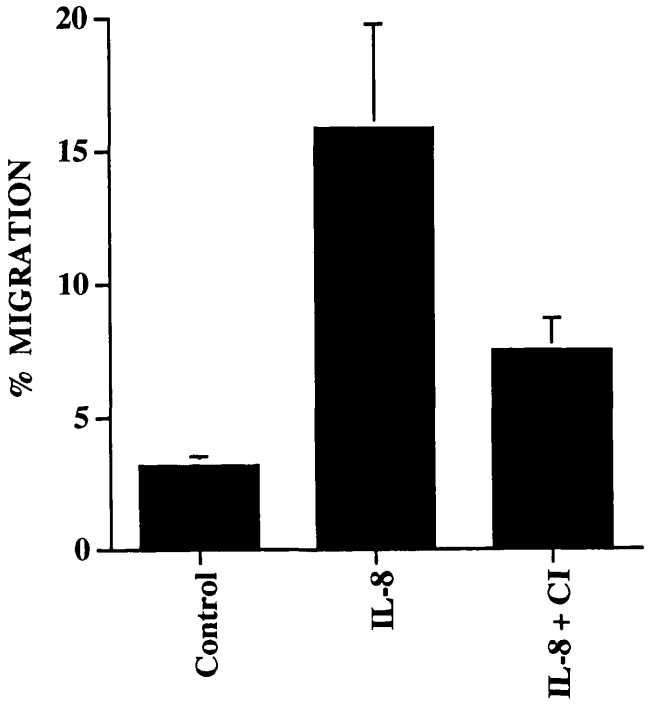

Figure 5.6 Calpain inhibitor I inhibits IL-8 induced neutrophil transmigration across filters alone. Neutrophils labelled with ^{51}Cr were added to 1 micron filters in the absence of endothelial cells at the same concentration as in Figure 5.1. Calpain inhibitor I (CI), 50μg/ml was added concurrently. IL-8, 50ng/ml, was added to the bottom chamber of the Transwells 10 minutes later. After 90 minutes migrated neutrophils were harvested and the percentage migration determined as detailed in the Methods. Data are mean ± s.e.m. of 4 experiments.

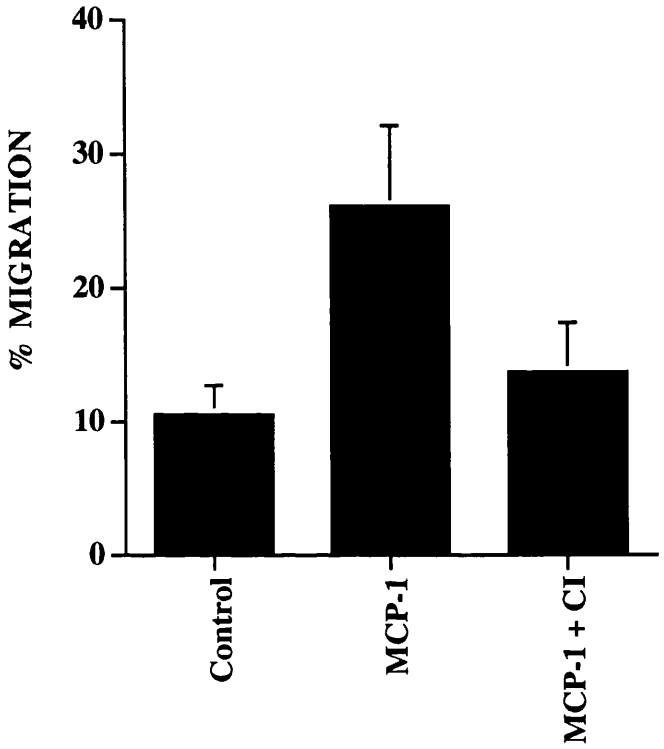

Figure 5.7 Calpain inhibitor I inhibits MCP-1 induced monocyte transmigration across endothelium. Endothelial cells were grown on 3 micron filters as detailed in Figure 5.1. Monocytes were labelled with ^{51}Cr as described in the Methods and were added to the washed endothelial cells ($3x10^6$cells/ml in 100μl) along with calpain inhibitor I (CI), 50μg/ml. MCP-1, 50ng/ml, was added to the bottom chamber of the Transwells 10 minutes later. After 2.5 hours migrated monocytes were harvested and the percentage migration determined as detailed in the Methods. Data are mean ± s.e.m. of 4 experiments.

significantly enhanced by MCP-1 (50ng/ml) to 26.13±5.9% ($p<0.05$, n=4 Figure 5.7). This is reduced in the presence of calpain inhibitor I to 13.7±3.7% ($p<0.05$, n=4,Figure 5.7). In experiments where calpain inhibitor I was included in the control, CI reduced baseline migration from 12.2±3.5% to 6.2±2% (n=3, data not shown).

Monocyte migration across cytokine stimulated endothelium

IL-1(20U/ml) stimulation of endothelium (4 hours pretreatment) does not increase the level of monocyte transmigration (21±4.5% migration across IL-1 stimulated endothelium compared to 24±5.8% migration across unstimulated, n=4, Figure 5.8a). Similiarly endothelial cell activation with TNF(100U/ml) does not increase monocyte migration (n=3, Figure 5.8b). In these experiments CI reduced migration across both unstimulated and stimulated endothelium. The addition of calpain inhibitor I to IL-1 stimulated endothelium reduced migration to 12.5±4.2% (n=4, Figure 5.8a) and addition of CI to TNF-stimulated endothelium reduced monocyte migration from 12.2±3.5% to 5.7±2% (n=3, Figure 5.8b).

Calpain inhibition does not alter upregulation of CD11b/CD18, or priming of the respiratory burst in IL-8 stimulated neutrophils

To characterise further the role of calpain in cytokine and chemokine stimulated leukocyte migration, I investigated whether other biological responses of leukocytes to chemokines were altered by calpain inhibition. Neutrophils were used for studying these effects since sufficient numbers of monocytes are not yielded from peripheral blood.

Chemokine stimulation leads to an increase in CD11b/Mac-1 expression and shedding of L-selectin; such alterations in surface adhesion receptors are

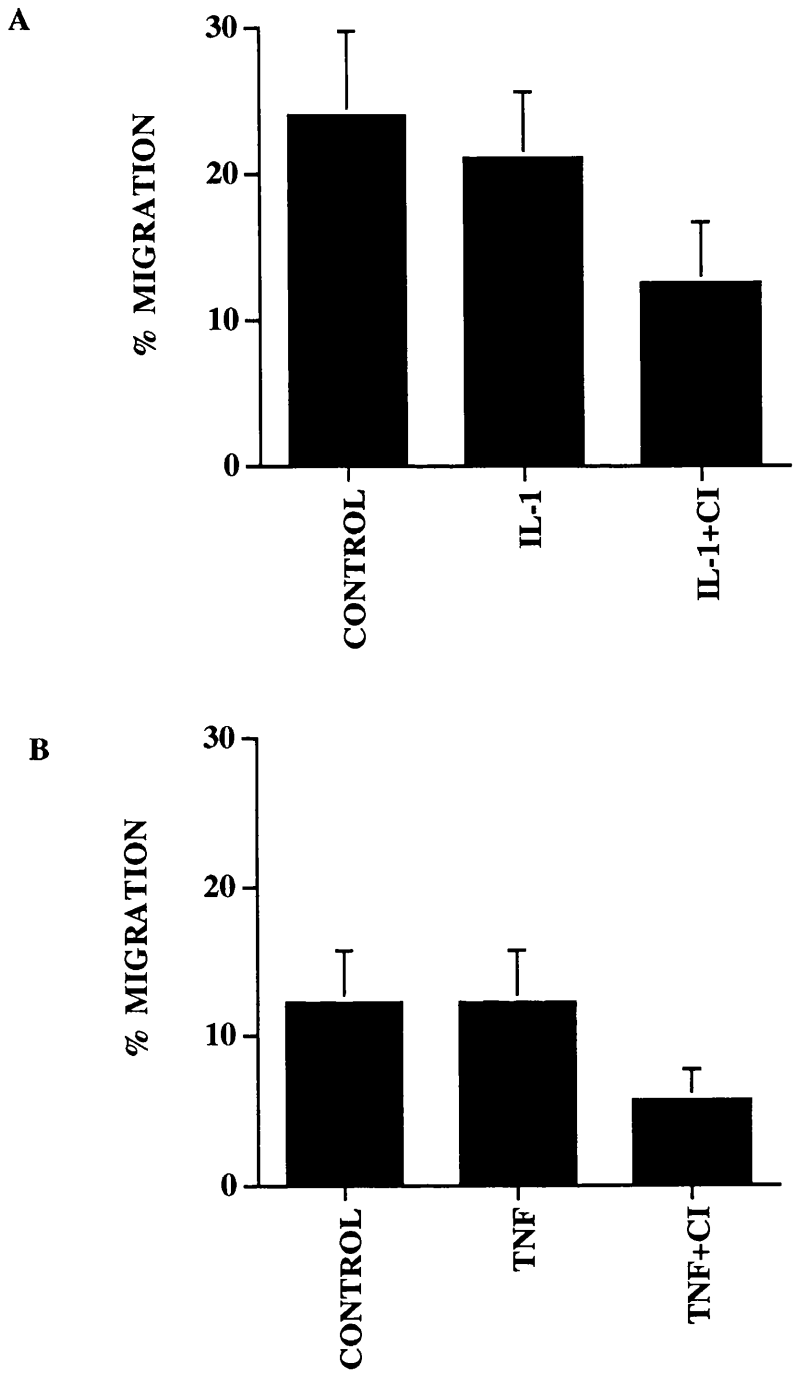

Figure 5.8 Monocyte transmigration across cytokine stimulated endothelium. Endothelial cells and monocytes were prepared as detailed in Figure 5.7. Endothelial cells were stimulated with A) IL-1 (20U/ml) or B) TNF (100U/ml) for 4 hours. ^{51}Cr-labelled monocytes were added to washed endothelial cells along with calpain inhibitor I (CI), 50μg/ml. After 2.5 hours migrated monocytes were harvested and the percentage migration determined as described in the Methods. Data are mean ± s.e.m. of 4 experiments for IL-1 and 3 experiments for TNF.

considered to be important for the progression from the initial stage of rolling to firm adhesion and transmigration of leukocytes. I therefore investigated the effect of calpain inhibition on chemokine-induced changes in neutrophil adhesion molecule expression.

IL-8 stimulation (50ng/ml for 45 minutes) increased surface CD11b levels to 187±32% of control (cells incubated at 37°C with medium alone) while fMLP increased levels to 282±123% of control (n=3, $p<0.001$ for both). In neither case did the presence of CI (up to 50μg/ml) have any effect on CD11b expression (Figure 5.9a). CI did not affect the reduction in surface L-selectin expression on neutrophils stimulated with IL-8 or fMLP (data not shown). Finally preincubation of neutrophils with IL-8 (100ng/ml) augmented the respiratory burst in response to fMLP (10^{-7}M). This priming of the oxidative response to fMLP was not affected by CI (Figure 5.9b). The dichlorofluorescein diacetate (DCF) based respiratory burst assay on neutrophils was carried out as previously described (Khwaja et al, 1992).

Discussion

In this study I used HUVEC cultured on 3 micron pore Transwell filters to investigate the role of calpain during neutrophil and monocyte transmigration across cytokine treated endothelium and in response to the chemoattractants IL-8 and MCP-1. Using this model system I have demonstrated that inhibition of calpain reduces neutrophil and monocyte transmigration across cytokine stimulated endothelium and in response to chemoattractants, without affecting adhesion. Migration assays using filters alone revealed that the inhibitory effect is directed predominantly on the leukocyte. Inhibiting calpain does not alter upregulation of CD11b/CD18, or priming of the respiratory burst in IL-8 stimulated neutrophils.

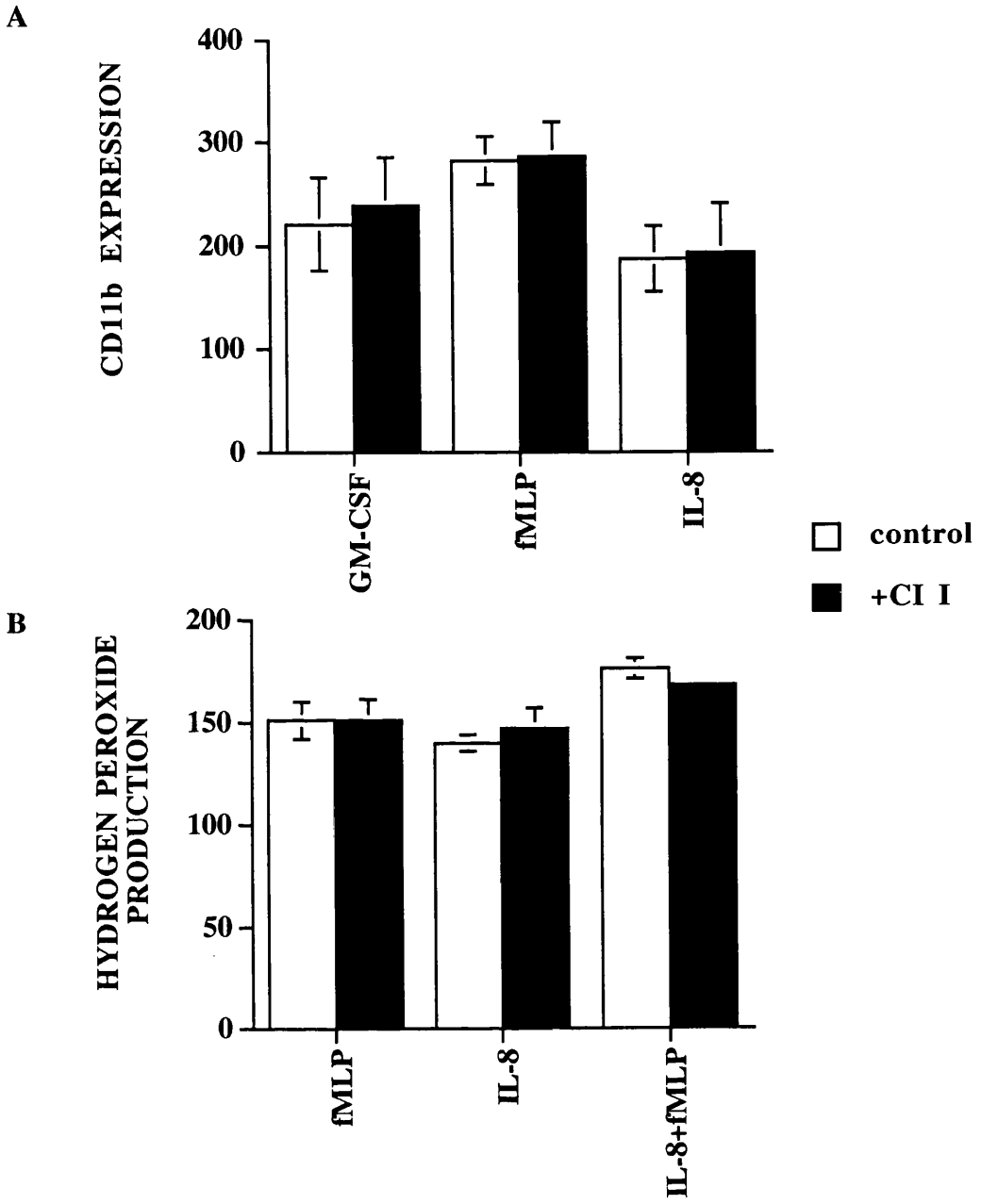

Figure 5.9 Calpain inhibitor I does not alter upregulation of A) CD11b/CD18, or B) priming of the respiratory burst in IL-8 stimulated neutrophils. A) Neutrophils were stimulated in whole blood with agonists (GM-CSF 20ng/ml; fMLP $5x10^{-7}$M; IL-8 50ng/ml) ± calpain inhibitor I (CI) for 45 minutes at 37°C and analysed for CD11b expression by flow cytometry. Data are mean ± s.e.m. of 3 experiments, and are expressed as percentage of levels on unstimulated cells. B) Neutrophils were loaded with 5mM dichlorofluorescein diacetate for 15 minutes at 37°C, then calpain inhibitor I (CI), 50μg/ml, was added for 30 minutes, followed by IL-8 (50ng/ml) and finally fMLP (10^{-7}M) was added for 10 minutes. The mean cell fluorescence of unstimulated cells was considered to be 100%. Data are mean ± s.e.m. of 4 experiments.

Calpain is a typical cytosolic cysteine protease that absolutely requires calcium for activity. The two forms, μ and m are expressed ubiquitously in animal tissues. Calpain cleaves target proteins in a restricted manner to modify their properties rather than digest the substrate proteins (Suzuki et al, 1998). Calpain exists as an inactive proenzyme, and pro-calpain is activated at the biological membrane in the presence of calcium and phospholipids such as PIP_2. This binding lowers the intracellular calcium concentration required for activation of calpain (Sorimachi et al, 1994). Activation requires intracellular calcium levels much higher than those found in normal resting cells, but similiar to those levels reached by calcium flux accompanying cell activation (Deshpande et al, 1995). Activation of calpain leads to irreversible proteolytic processing of substrate proteins (Saido et al, 1994). Such substrate proteins include talin which is degraded by calpain in platelets (Inomata et al, 1996) and α-actinin, where in T cells as a consequence of calpain induced cleavage of α-actinin, the actin cytoskeleton rearranges and pseudopod formation begins (Selliah et al, 1996).

In the Transwell system leukocyte migration was inhibited by several calpain inhibitors including calpain inhibitor II (ALLM) and E64D. I was also able to discount the possibility of proteasomal involvement by demonstrating that lactacystin was not inhibitory in this system. These results are in contrast to work showing that lactacystin, when added to 4 hour TNF stimulated endothelium reduced neutrophil migration by between 50-70% over 90 minutes, the same time period as employed in my studies (Allport et al, 1997). The conditions used did however differ from those employed in my experiments in that the neutrophil migrations were performed under flow where different adhesion molecules such as selectins are involved in the initial cell

attachments. The reduction in migration may have been due to the effects of lactacystin on other molecules dependent on the proteasome.

Using adhesion assays I confirmed that the reduced leukocyte migration was not due to altered adhesion. These observations are in agreement with a study where lactacystin affected migration without altering neutrophil adhesion to TNF stimulated endothelium (Allport et al, 1997). On the other hand, T cell adhesion to ICAM-1 induced by calcium mobilisers was inhibited by calpeptin (Stewert et al, 1998). This discrepancy could be due to the fact that these experiments employed a T cell line and studied adhesion to an immobilised ligand. In contrast, in my studies described here, multiple adhesive pathways are likely to be involved in neutrophil adhesion to activated endothelium. Secondly calpain activity may not be required for non-LFA-1-dependent pathways such as that mediated by E-selectin, which is induced on cytokine activated endothelium, and which mediates the binding of neutrophils.

Calpain inhibition does not alter CD11b upregulation by IL-8 or fMLP on neutrophils nor the shedding of L-selectin suggesting that calpain is not involved in the modulation of these surface adhesion molecules induced by cellular activation. The role of calpain in enhancing leukocyte migration may thus be directed at linkages between the cell and substratum. Regulation of the formation and dissolution of adhesive complexes, as well as the interactions with the actin cytoskeleton are pivotal to the control of cell migration. Calcium is proposed to play a role in cell motility and gradients of calcium with the highest concentrations at the rear of the cell are seen in migrating eosinophils, providing an assymetrical distribution of this putative regulatory molecule. Focal adhesions are adhesive complexes that form between the cell and its surrounding substratum, usually extracellular cell matrix. Cells that form strong

focal adhesions are less migratory whilst more mobile cells form less organised adhesive structures. However some sort of organised linkage connecting cell membrane and cytoskeleton is required for cell migration, linking the termini of actin filament bundles to ECM via integrins providing traction for migration. There is growing evidence that $pp125^{FAK}$, a 125 kDa form of focal adhesion kinase plays a role in regulating the assembly of the integrin rich cytoskeletal signalling complexes. $pp125^{FAK}$ phosphorylates proteins such as paxillin and tensin which contribute to the anchorage of integrins to contractile cytoskeleton. Calpain cleaves $pp125^{FAK}$ downregulating its kinase activity resulting in a relaxation of contacts in the focal adhesion (Cooray et al, 1996). The addition of monocytic cells to IL-1β stimulated endothelium induces a decrease in the amount of $pp125^{FAK}$ in endothelium and in parallel, there is a decrease in actin stress fibres resulting in decreased strength of attachment of the endothelial cell to extracellular matrix. This may allow monocyte migration to occur (Iwaki et al, 1997).

Calpain inhibition caused greatest reduction in transmigration when cytokine stimulated endothelium was employed as compared with the response to chemokines. Neutrophil migration across TNF stimulated endothelium was reduced to baseline compared to 37% inhibition of the migratory response to IL-8. This difference could be due to substratum adhesiveness. Cell-substratum adhesiveness is an important determinant of cell migration speed (Huttenlocher, 1997). Maximum cell migration speed is achieved on a substratum with intermediate adhesiveness. This is due to the fact that cells need a certain amount of contact in order to generate traction, if the connections are too strong then detachment is too difficult and the cell cannot move and if substratum concentration is low then insufficient attachments are

formed at the front of the cell. The reduction in migration caused by calpain inhibitors is greatest at high substratum concentrations since it affects the rate of detachment. CHO cells treated with calpain inhibitors exhibit lamellipodial projections but have inhibited release at the rear of the cell and when adhesions are released it occurs in a sudden snapping motion, with large pieces of membrane remaining on the substratum. Such observations suggest that calpain inhibitors block migration specifically by inhibiting cell-substratum detachment at the rear of the cell, by stabilising cytoskeletal linkages strengthening focal adhesions (Huttenlocher, 1997). Integrins are left on the substratum after cell detachment and the proportion remaining increases with the concentration of substratum. Calpain inhibitors reduce the amount of integrin debris since integrin-cytoskeletal linkages are strengthened. The role of calpain in integrin dependent migration is further supported by the observations that calpain inhibitors block both β1 and β3 integrin mediated cell migration, and alter the fate of these receptors at the rear of the cell. The β3 cytoplasmic domain subunit is cleaved by calpain, removing residues critical for the attachment of the integrin to the cytoskeleton (Du et al, 1995).

In addition fibroblast cell lines overexpressing calpastatin, a natural inhibitor of calpain, show inhibited cell spreading on fibronectin due to an absence of a lamellipod (Potter et al, 1998) and CHO cells expressing low levels of calpain show reduced migration rates (Huttenlocher et al, 1997).

The lesser effect of calpain inhibitors on chemokine dependent migration could be related to the ability of chemoattractants such as MCP-1 and IL-8 to induce β2 integrin expression on leukocytes and on endothelial cells (Vaddi et al, 1994). This family of integrins has not yet been found to be cleaved by calpain (Stewert et al, 1998) so migration in response to chemoattractants may not be

so reliant on calpain cleavage. During migration across cytokine stimulated endothelium monocytes employ β2, β1 and β3 integrins and the last two families of integrins are cleaved by calpain. In experiments where neutrophils were prevented from migrating across vitronectin by calcium buffering or inhibition of calcineurin, motility was restored by blocking binding of the cell to β3 integrins, suggesting that calcium is required for neutrophil migration using β3 integrins. The effects of blocking β3 are distinct from the effects of blocking β2 integrins where motility is not restored suggesting that migration using β2 integrins required more than just calcium to enable cell motility to occur (Hendey et al, 1996). The inability of neutrophils to migrate on adhesive substrates when $[Ca^{2+}]_i$ is blocked is due to ineffective recycling of β3 integrins from the rear of the cells to the front. The polarised distribution of β3 integrins in migrating cells is maintained by $[Ca^{2+}]_i$ dependent release of adhesion followed by endocytosis of these integrins and recycling to the leading edge (Lawson et al, 1995). These studies have largely focused on the random movement of cells on ECM substrates whereas leukocyte migration across endothelium is more directed and thus the involvement of calcium regulated proteolytic mechanisms may differ.

Monocyte migration across unactivated endothelium in response to chemoattractants is much more CD11/CD18 dependent and β1 functions as a less efficient alternative mechanism (Chuluyan et al, 1993). In neutrophils, blocking antibodies to β2 integrins inhibit chemokine induced migration by 90% (Furie et al, 1991). It is possible that neutrophil migration across cytokine endothelium uses VLA-5 and VLA6 (β3) integrins in addition to β2 integrins and thus inhibition of calpain will have a greater effect here. The release of β2 integrin attachments are independent of $[Ca^{2+}]_i$ signalling (Lawson et al, 1995).

Finally in this series of experiments using the Transwell system, monocyte migration was not enhanced by pre-stimulation of endothelium with IL-1 or TNF. These results are supported by other work that have noted that whilst these cytokines do increase monocyte adhesion and migration the migratory response across activated endothelium is much weaker than that displayed by neutrophils. An important factor appears to be the time period employed for the migration. In one study, at 20 minutes, the difference between monocyte migration across unstimulated and cytokine stimulated endothelium is greatest but after 2 hours there is no difference (Chuluyan et al, 1993; Meerschaert et al 1994). This monocyte behaviour *in vitro* contrasts with *in vivo* findings which show a strong monocyte recruitment to sites injected with these cytokines. The weak *in vitro* monocyte migration across cytokine stimulated endothelium has been considered to be due to the type of subendothelial matrix used for culturing endothelium (Hakkert et al, 1991). However attempts by the above group as well as by Chuluyan et al to optimise migration levels using different types of matrix showed no significant effect on migration across cytokine treated endothelium. Another factor that could contribute to the enhanced monocyte migration *in vivo* is the presence of tissue derived chemotactic factors which would stimulate monocyte recruitment.

In conclusion, calpain has been shown to be important in the directed transmigration of neutrophils and monocytes across cytokine activated endothelium, or in response to chemokines. The precise role of calpain in regulating cell motility, particularly during transendothelial movement is likely to be complex, involving more than one substrate. Calpain may function both to promote integrin clustering which is important for firm adhesion, as well as to cleave cytoskeletal linkages, thus helping in pseudopod formation allowing

forward movement, as well as in releasing attachments at the rear. Understanding the processes involved in leukocyte transmigration may lead to therapeutic strategies in the control of chronic inflammatory states and intervention into the development and progression of atherosclerotic lesions.

Chapter Six
The Role of IL-10 in
Endothelial Cell:Monocyte Interactions

Introduction

Inflammatory processes contribute to the development of atherosclerosis, while atherosclerosis itself provokes inflammatory processes within tissues. Inflammation is a classical cytokine-mediated phenomenon. Proinflammatory cytokines, such as IL-1 and TNFα are generated in areas of inflammation. TNFα is produced by monocytes and endothelial cells in coculture and has been identified in endothelial cells, smooth muscle cells and macrophages in atherosclerotic vessels. Anti inflammatory cytokines such as IL-4 and IL-10 are also produced. Evidence to date suggests that IL-10 may play a role in atherosclerosis. IL-10 mRNA has been found in atherosclerotic plaques and oxLDL can induce release of IL-10 from monocytes (Berg et al, 1995). Evidence for the anti-inflammatory effects of IL-10 comes from the observation that IL-10 deficient mice develop enterocolitis (Kuhn et al, 1993), produce enhanced amounts of TNF-α in response to LPS and are more sensitive to endotoxic shock (Berg et al, 1995). In fact, IL-10 inhibits monocyte production of proinflammatory cytokines, such as TNF-α, IL-1α and IL-6 (Gerard et al, 1993; Fiorentino et al, 1991). Thus, it can be considered that the balance between proinflammatory cytokines and negative cytokines contributes to the level of the immune-mediated injury in atherosclerosis.

Monocytes, which are the main source of IL-10, produce IL-10 in response to TNF-α (Donnelly et al, 1995). TNF-α is unique among the inflammatory cytokines such as IL-1α, β , IL-6, GM-CSF, IFNγ and TGFβ in its capacity to upregulate IL-10 expression in human peripheral blood monocytes, both at the mRNA synthesis and protein secretion levels (Wanidworanum and Strober, 1993).

Following stimulation with TNF-α or LPS, monocytes initially produce TNF-α and only after 24 hours is IL-10 mRNA detected (de Waal Malefyt et al, 1991). Thus TNF-α released from monocytes in contact with endothelial cells could cause induction of IL-10 mRNA synthesis and protein production. IL-10 could then in turn modulate the inflammatory responses by downregulating TNF-α synthesis (Ralph et al, 1991; Niho et al, 1998). As E-selectin induction is partly dependent on TNF-α, IL-10 could have an inhibitory effect on adhesion molecule expression in monocyte/endothelial cell cocultures.

In this chapter, the role of IL-10 in modulating endothelial cell:monocyte inflammatory responses has been investigated by adding exogenous IL-10 to endothelial cell:monocyte cocultures. In addition, I also investigated the induction of endogenous IL-10 in monocytes cocultured with endothelial cells.

Results

Addition of IL-10 to endothelial cell/monocyte cocultures inhibits E-selectin induction

Untreated endothelial cells do not express E-selectin. Coculture of monocytes with endothelial cells results in a significant induction of E-selectin protein expression at 4 hours (O.D. 0.29±0.06 compared to 0.024±0.009 on untreated endothelial cells, $p<0.05$, n=5, Figure 6.1) which is sustained over 21 hours (O.D. at 21 hours 0.28±0.1, $p<0.05$, n=5, Figure 6.1). When IL-10 is added at the start of the coculture there is a reduction in the level of E-selectin induction at both time points with 100ng/ml of IL-10 (at 4 hours, the O.D. of cocultures reduced from 0.29±0.06 to 0.13±0.04 while at 21 hours O.D. of cocultures was

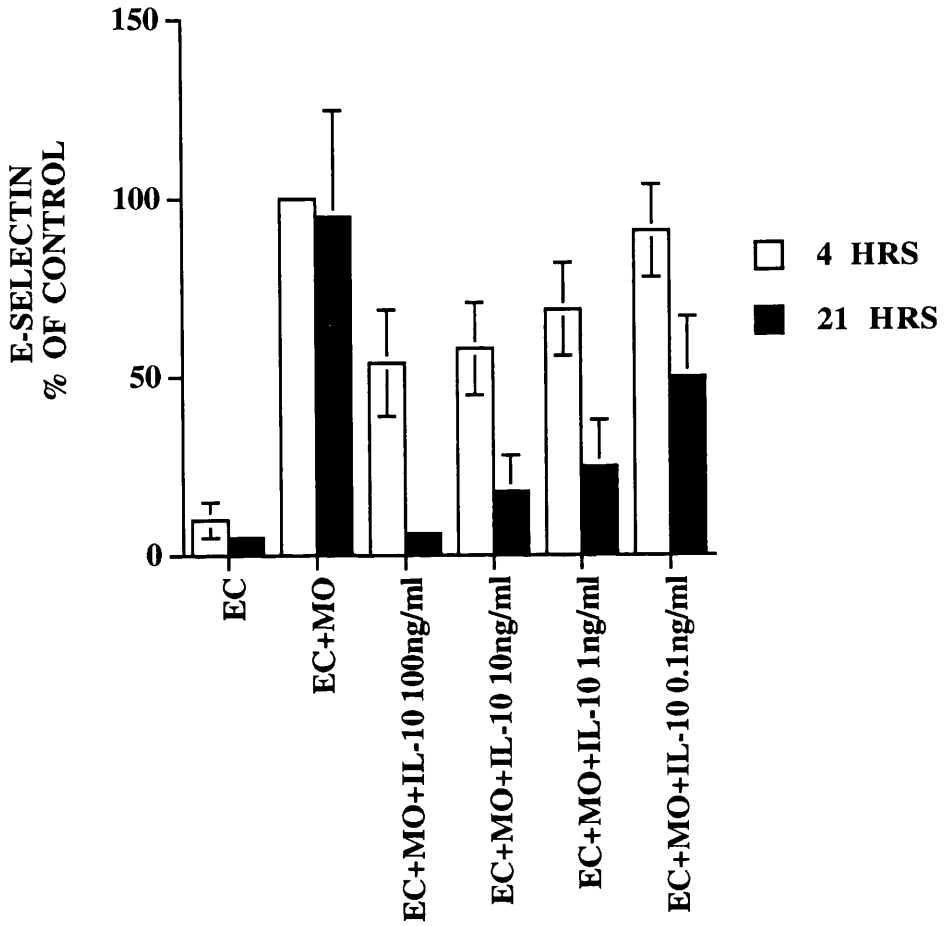

Figure 6.1 Effect of exogenous IL-10 on E-selectin expression on endothelial cells cocultured with monocytes. Endothelial cells were cultured to confluence on 96 well plates, and monocytes isolated, as described in the Methods. Monocytes and IL-10 were added concurrently to the washed endothelial cells for 4 and 21 hours. E-selectin protein expression was determined by ELISA as detailed in the Methods. Data has been normalised and is expressed as a percentage of E-selectin expression on endothelial cell cocultures at 4 hours. Mean ± s.e.m. of 5 experiments

reduced from 0.28±0.1 to 0.02±0.005, $p<0.05$, n=5 for both, Figure 6.1). This effect of IL-10 is concentration dependent (Figure 6.1). At 4 hours there is a maximal inhibition of 46±13% using 100ng/ml of IL-10 and a reduction of 31.5±13% using 1 ng/ml of IL-10 ($p<0.05$, n=5 for both). The level of inhibition achieved by IL-10 is greatest at 21 hours. With the maximal concentration of IL-10 (100ng/ml) there is 94±1.3% inhibition of E-selectin induction by monocytes ($p<0.05$) and IL-10 is still inhibitory at 0.1ng/ml reducing E-selectin expression by 50±17% ($p<0.05$). This inhibitory effect of IL-10 may be a direct effect on the response of endothelial cells or, alternatively IL-10 may be acting on monocytes to block the induction of E-selectin on endothelial cells. In order to distinguish between these possibilities, I next examined the effect of IL-10 on cytokine induction of E-selectin in endothelial cells.

Addition of IL-10 to IL-1 treated endothelial cells does not alter E-selectin expression

IL-1(10U/ml) upregulates E-selectin protein expression on endothelial cells with maximal effect at 4 hours after which the level of expression falls (O.D. 0.53±0.13 at 4 hours compared to 0.24±0.06 at 21 hours, Figure 6.2a). IL-10, at concentrations up to 100 ng/ml had no effect on E-selectin induction by IL-1 either at 4 or 21 hours (Figure 6.2a). The addition of TNF (100U/ml) to endothelial cells also induces E-selectin protein expression with the same kinetics as IL-1 (Figure 6.2b). The addition of IL-10 at 100ng/ml had no effect on the induction of E-selectin by TNF at either time point. This suggests that IL-10 acts directly on monocytes to inhibit the induction of E-selectin expression

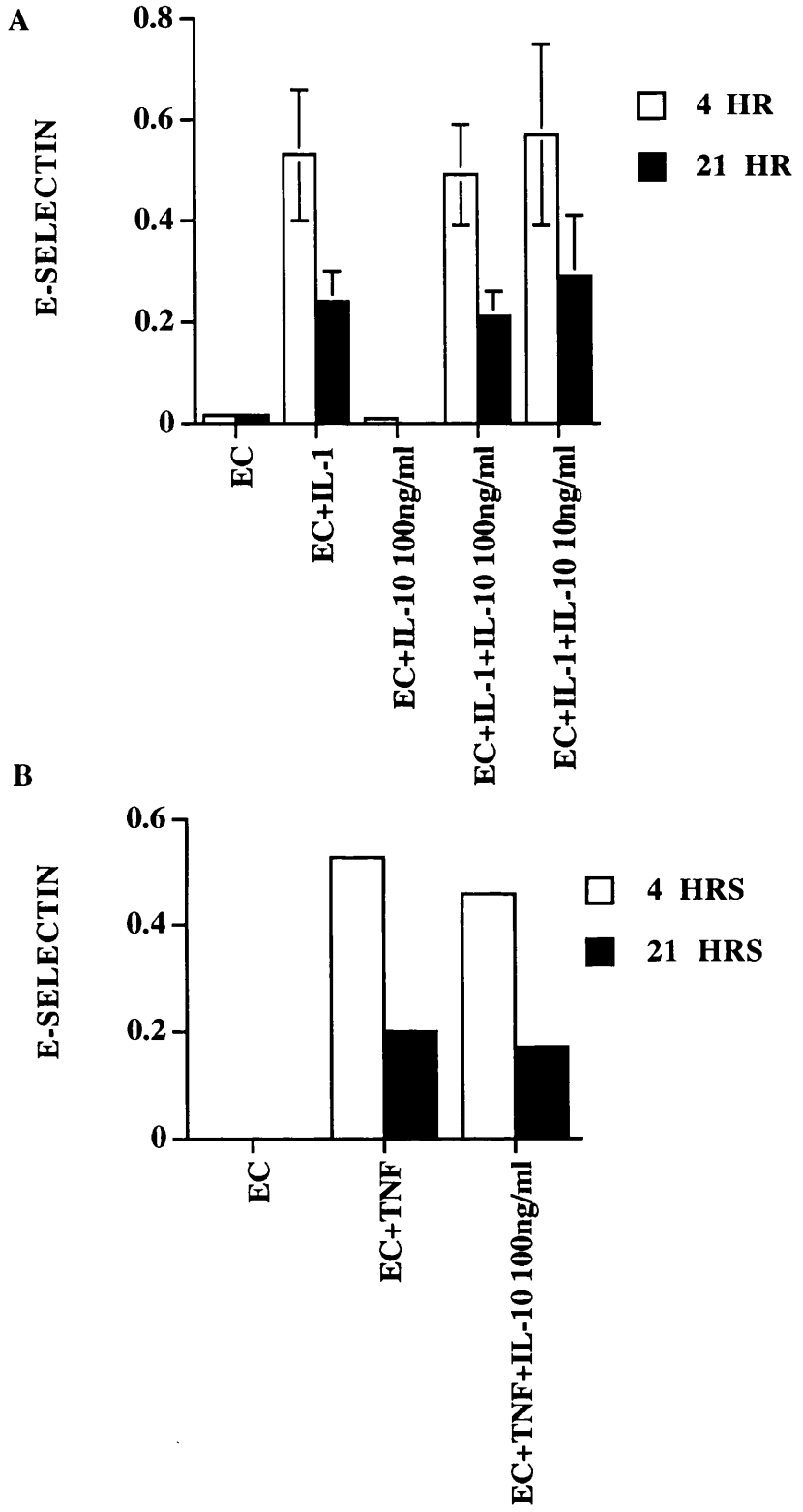

Figure 6.2 Effect of exogenous IL-10 on cytokine stimulated E-selectin induction on endothelial cells. Endothelial cells were grown to confluence in 96 well plates and A) IL-1 or B) TNF were added to the washed endothelial cells for 4 and 21 hours, concurrently with IL-10 (100 or 10 ng/ml). E-selectin protein expression was determined by ELISA as detailed in the Methods. Data are mean ± s.e.m. of 3 experiments for IL-1 and one representative experiment for TNF.

on endothelial cells and does not affect the ability of endothelial cells to respond to proinflammatory cytokines.

IL-10 does not induce E-selectin expression on endothelial cells

The possible direct effect of IL-10 on E-selectin expression was studied both at the protein and the mRNA level. IL-10 (100ng/ml) itself does not induce E-selectin protein expression on endothelial cells either at 4 or 21 hours as measured by ELISA (Figure 6.2a). To confirm these results the level of E-selectin protein on IL-10 treated endothelial cells was determined using FACS analysis as an ELISA may not be sensitive enough to detect a slight response in a small subset of endothelial cells. Unstimulated endothelial cells demonstrate little specific staining for E-selectin, which was not significantly different from the isotype control (Figure 6.3). Only a very small percentage of untreated endothelial cells stained positive for E-selectin (9.07±1.6%, Figure 6.3). Following stimulation with IL-1 (50U/ml) for 4 hours, the percentage of endothelial cells staining positive for E-selectin increased to 87.82±9.3% ($p<0.05$, $n=3$, Figure 6.3). In contrast endothelial cells exposed to IL-10 (up to 250ng/ml) for 4 hours showed no increase in E-selectin expression above baseline.

Untreated endothelial cells express low levels of E-selectin mRNA (E-selectin/actin ratio 0.26, Figure 6.4) which are markedly increased following treatment with IL-1(100U/ml) for 4 hours (E-selectin/actin ratio 2.99, Figure 6.4). The addition of IL-10 at concentrations up to 200ng/ml did not alter the levels of E-selectin mRNA at 4 hours compared to untreated cells (E-selectin/actin ratio 0.18 with 200ng/ml of IL-10).

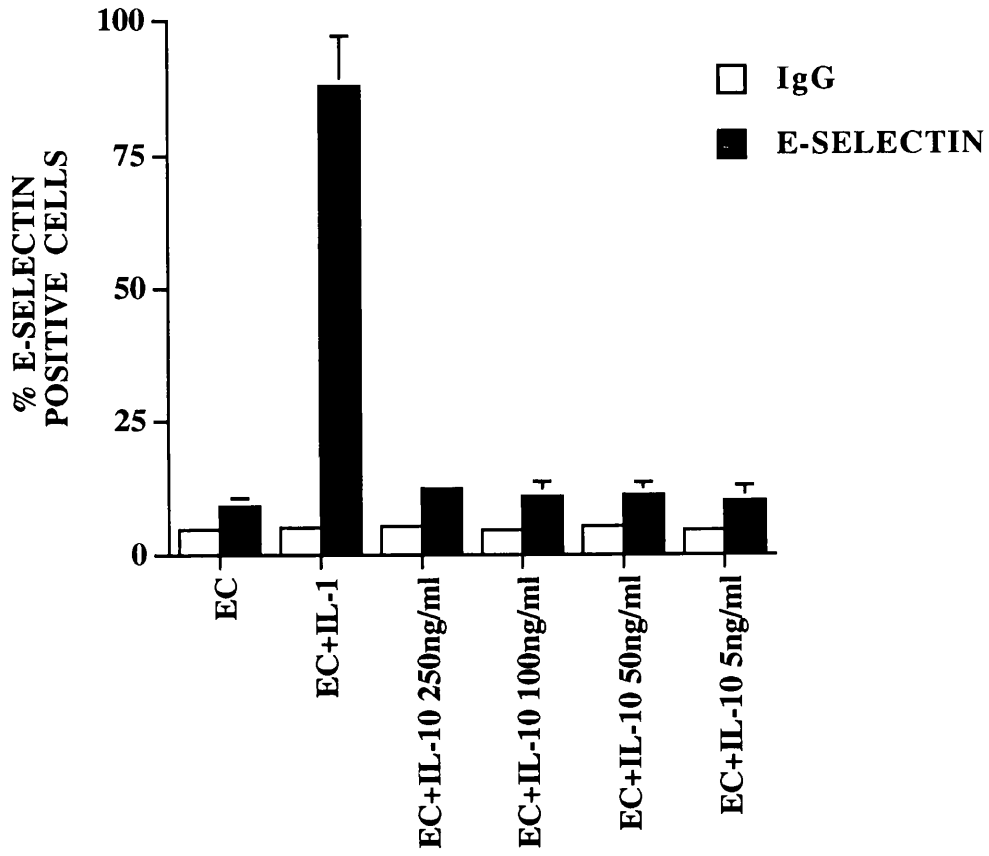

Figure 6.3 Exogenous IL-10 does not induce E-selectin protein expression on endothellai cells. Endothelial cells were grown to confluence in 6 well plates and IL-10 was added at various doses for 4 hours. IL-1 (50U/ml) was used as a positive control. E-selectin protein expression was determined by flow cytometry and the results are the mean ± s.e.m. of E-selectin positive endothelial cells from 3 experiments.

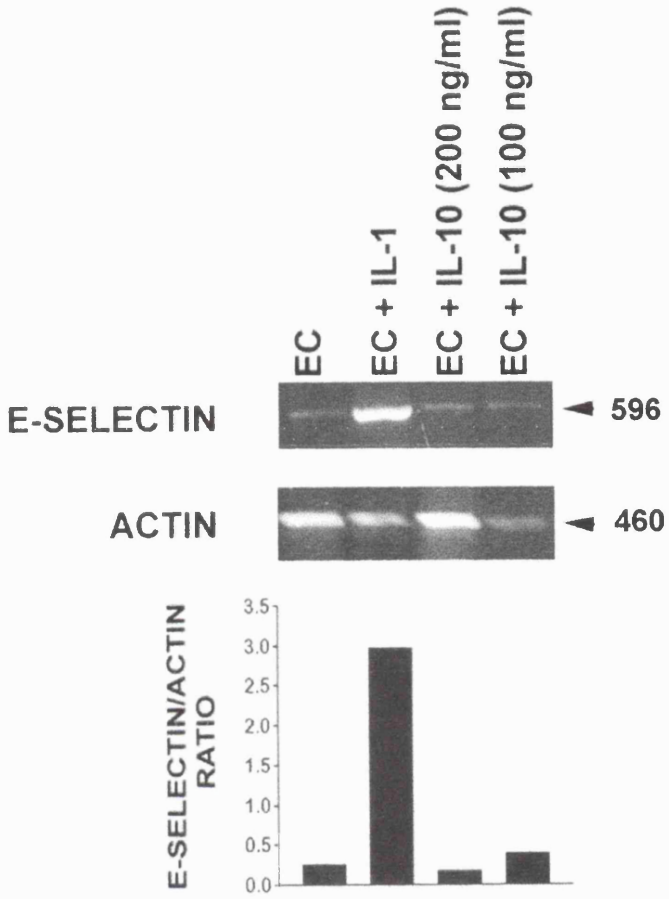

Figure 6.4 Effect of IL-10 on E-selectin mRNA expression in endothelial cells. Endothelial cells were cultured to confluence. IL-1 (100U/ml) and IL-10 were added to washed endothelial cells for 4 hours. Total RNA was then isolated and RT-PCR reactions performed as described in the Methods. EC=endothelial cells in medium alone; EC+IL-1=endothelial cells stimulated with IL-1; EC+IL-10=endothelial cells stimulated with IL-10. The bands shown are from one representative experiment and the histogram displays E-selectin mRNA levels relative to actin. The m.wt of the products are indicated.

IL-10 addition to monocyte/endothelial cell cocultures reduces monocyte adhesion to endothelial cells

The addition of IL-10 to monocyte/endothelial cell cocultures could be inhibiting E-selectin induction by altering monocyte adhesion to endothelial cells. To investigate whether IL-10 does affect monocyte adhesion to unstimulated endothelial cells, IL-10 was added to the endothelial cells concurrently with the monocytes. At 4 hours, 10.4±1.6% of monocytes added were adherent to the endothelium, and the addition of IL-10 (100ng/ml) to the cocultures reduced the percent of adhesion to 5.6±1.6 ($p< 0.01$, n=3, Figure 6.5). After 21 hours, the percent of monocyte adhesion to control endothelium had increased to 14.9±2.6% (n=3, Figure 6.5). In the presence of IL-10 (100ng/ml), monocyte adhesion was reduced to 12.6±3.2% (n=3, Figure 6.5). The addition of 10ng/ml of IL-10 caused a larger reduction, decreasing monocyte adhesion from 14.9±2.6% to 8.3±4.1% ($p<0.05$, n=3, Figure 6.5).

Anti-IL-10 monoclonal antibody does not alter E-selectin expression induced in monocyte/endothelial cells coculture

The preceding observations suggest that IL-10 acts on monocytes to reduce the induction of E-selectin on endothelial cells cocultured with monocytes. Monocytes themselves produce IL-10 following stimulation for example with LPS or TNFα, and this endogenous IL-10 acts in an autocrine manner to inhibit further TNFα production.

To investigate the role of endogenous IL-10 in monocyte/endothelial cell cocultures, an anti-IL-10 antibody was included in the coculture system and E-selectin protein expression was assessed by ELISA. Control cocultures were incubated with IgG_1 isotype control. Figures 6.6a and b show the levels of E-

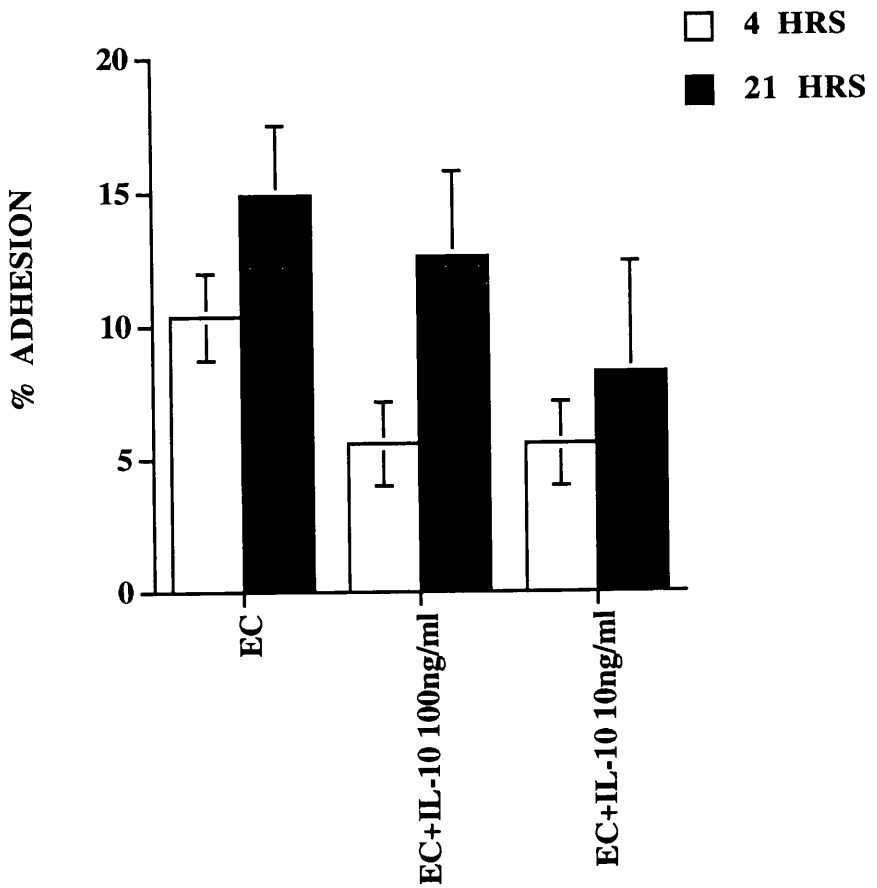

Figure 6.5 Effect of exogenous IL-10 on monocyte adhesion to unstimulated endothelium. Endothelial cells were cultured to confluence in 48 well plates. Chromium labelled monocytes, isolated and radiolabelled as detailed in the Methods, were added concurrently with IL-10 to the washed endothelial cells for 4 and 21 hours. Percentage adhesion was determined by adhesion assay, as described in the Methods. Data are mean ± s.e.m. monocyte adhesion of 3 experiments.

selectin on unstimulated endothelial cells or following coculture with monocytes for 4 and 21 hours with the addition of the anti-IL-10 antibody (20μg/ml) or an IgG isotype control. The addition of monocytes to endothelial cells in control cocultures with IgG isotype induced the expression of E-selectin on endothelial cells. Although E-selectin induced by monocytes is sustained for longer than that induced by IL-1, after 21 hours the level of E-selectin expression starts to decline. IL-10 inhibits E-selectin expression on endothelial cells cocultured with monocytes and it is possible that the reduction in E-selectin expression at 21 hours is due to the production of IL-10 in the system. If this hypothesis was correct, blocking IL-10 with anti-IL-10 monoclonal antibody, would result in continued elevation of E-selectin up to and after 21 hours. As seen in Figures 6.6a and b, anti-IL-10 antibody at up to 20μg/ml had no effect on the level of E-selectin expression on endothelial cells either at 4 or 21 hours.

IL-10 production by monocytes in contact with endothelial cells could not be detected by soluble ELISA

Supernatants were harvested from endothelial cell/monocyte cocultures at 4 time points and assayed for IL-10 protein using a soluble IL-10 ELISA. The results shown in Table 6.1 are from one representative experiment. Stimulation of monocytes with LPS (10ng/ml) induces IL-10 production by 7 hours (175pg/ml), which by 20 hours has increased to 1800pg/ml. At 30 hours the concentration of IL-10 produced by stimulated monocytes is still high at 1900pg/ml. Endothelial cells and monocytes cultured alone do not release any detectable IL-10 over the 30 hours and there was no detectable IL-10 in the supernatant of monocytes cocultured with endothelial cells up to 30 hours.

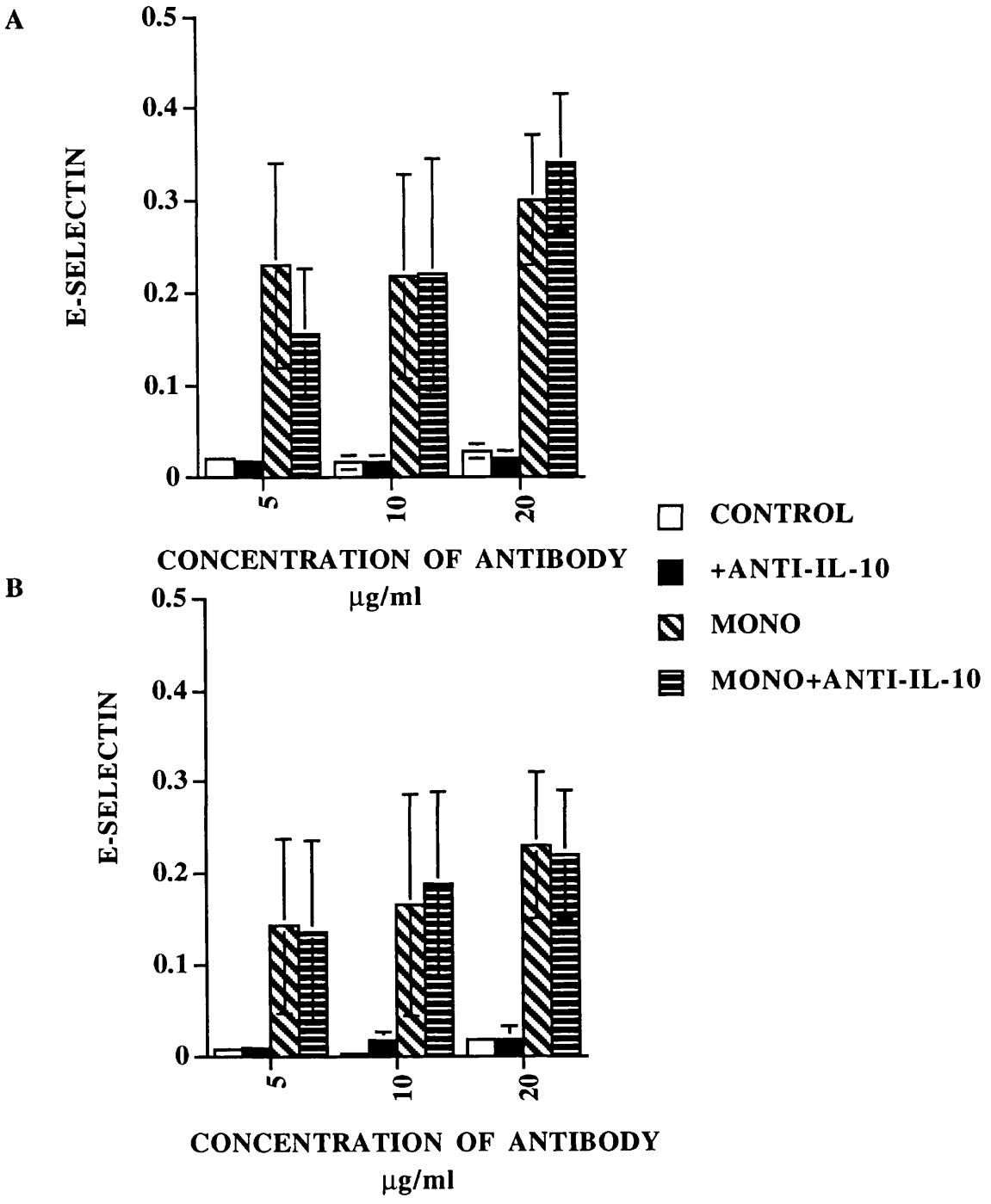

Figure 6.6 Effect of anti-IL-10 monoclonal antibody on the monocyte induction of E-selectin expression on endothelial cells. Endothelial cells were cultured to confluence on 96 well plates. Monocytes, isolated as detailed in the Methods, were added to the endothelial cells after 5 minutes preincubation with anti-IL-10 mAb for A) 4 and B) 21 hours. An IgG isotype control was included in control cocultures. The antibodies were present throughout the duration of the experiment. E-selectin protein expression was determined by ELISA as detailed in the Methods. Data are the mean ± s.e.m. of 4 experiments.

	4 HOURS	7 HOURS	20 HOURS	30 HOURS
ENDOTHELIAL CELLS	0	0	8	8
ENDOTHELIAL CELLS AND LPS	8	8	8	8
MONOCYTES	8	8	8	8
MONOCYTES AND LPS	8	175	1800	1900
ENDOTHELIAL CELLS AND MONOCYTES	8	8	8	8

Table 6.1 IL-10 production from monocytes cocultured with endothelial cells. Endothelial cells were cultured to confluence in 96 well plates and monocytes were added to washed endothelial cells was up to 30 hours. In addition LPS (10ng/ml) was added to endothelial cells and monocytes cultured alone in medium. At the time points indicated supernatants were harvested and assayed for IL-10 protein using a soluble IL-10 ELISA. Results are expressed as pg/ml of IL-10 protein and are from one representative experiment.

IL-10 mRNA is detectable in monocytes in contact with endothelial cells

IL-10 mRNA was not detectable in freshly isolated monocytes but after culturing in medium for 23 hours, IL-10 mRNA can be detected (IL-10/actin ratio 0.04, Figure 6.7). LPS (10ng/ml) stimulation for 23 hours increases IL-10 mRNA levels in monocytes (IL-10/actin ratio 0.09, Figure 6.7). Endothelial cells cultured alone or stimulated with LPS do not express IL-10 mRNA at any time point studied (Figure 6.7). In monocytes cocultured with endothelial cells IL-10 mRNA is detected initially at 6 hours (IL-10/actin ratio 0.02), increases over 23 hours (IL-10/actin ratio 0.2) and is still detectable at 30 hours (IL-10/actin ratio 0.11). To ensure that the induction of IL-10 mRNA in monocytes was not due to LPS contamination in the culture system, monocytes were cocultured with endothelial cells in the presence of polymixin B, a membrane-active antibiotic (Storm et al, 1977) at 10μg/ml. The addition of polymixin B to the coculture did not alter the induction of IL-10 mRNA in monocytes cocultured with endothelial cells (Figure 6.7). When polymixin B was added to endothelial cells stimulated with LPS (10ng/ml) for 6 hours the induction of E-selectin was inhibited by 78% (Figure 6.8). These observations suggest that the induction of mRNA in monocytes cocultured with endothelial cells was not due to LPS contamination.

Discussion

Work presented in chapter 3 showed that monocytes themselves can contribute to the sustained E-selectin expression on endothelial cells, however the expression does fall after 21 hours indicating that a mechanism exists to regulate E-selectin expression on endothelial cells. TNF-α contributes to the induction of E-selectin on endothelial cells cocultured with monocytes.

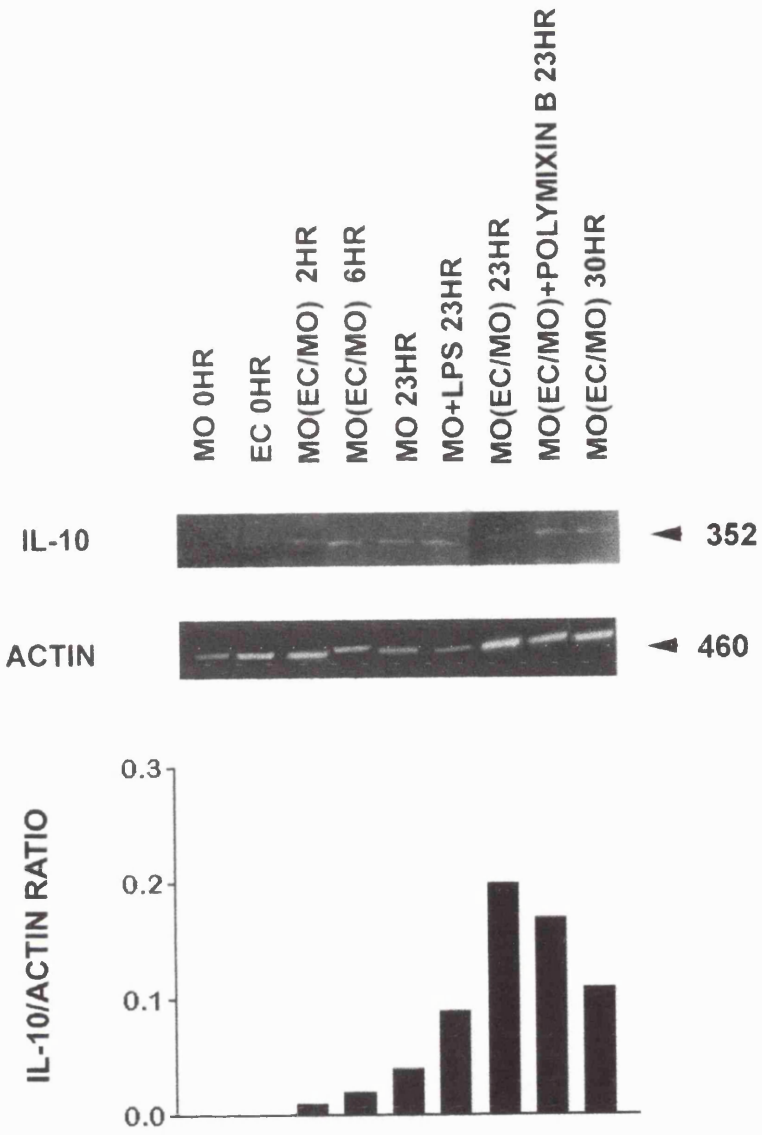

Figure 6.7 Expression of IL-10 mRNA in monocytes cocultured with endothelial cells. Endothelial cells and monocytes were isolated as described in the Methods. Monocytes were incubated with the endothelial cells for up to 30 hours, after which the monocytes were removed from the endothelial cells with 2mM EDTA. LPS (10ng/ml) was added to monocytes cultured in medium alone for 23 hours. Polymixin B (10μg/ml) was also included in one coculture for 23 hours. Total RNA was then isolated from the endothelial cells and RT-PCR reactions performed as described in the Methods. Times are given relative to the addition of monocytes or LPS. MO= monocytes cultured in medium alone; EC=endothelial cells; MO(EC/MO)=monocytes from endothelial cell/monocyte cocultures; MO+LPS=monocytes stimulated with LPS; MO(EC/MO) +POLYMIXIN B=monocytes from endothelial cell/monocyte cocultures in the presence of polymixin B. The bands shown are from one representative experiment and the histogram displays IL-10 mRNA levels relative to actin. The m.wt of the products are indicated.

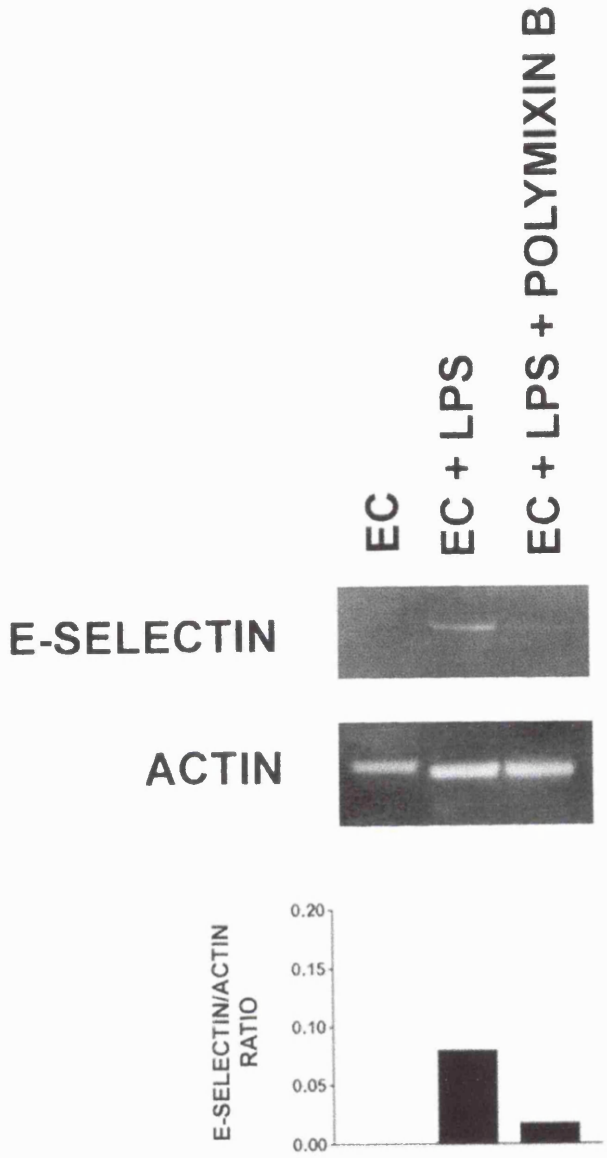

Figure 6.8 Effect of polymixin B on LPS induction of E-selectin mRNA in endothelial cells. Endothelial cells were cultured as described in the Methods. LPS (10ng/ml) was added to washed endothelial cells for 6 hours. Polymixin B (10μg/ml) was added concurrently with LPS. Total RNA was then isolated from the endothelial cells and RT-PCR reactions performed as described in the Methods. EC=endothelial cells alone; EC+LPS=endothelial cells stimulated with LPS; EC+LPS+POLYMIXIN B=endothelial cells stimulated with LPS in the presence of polymixin B. The bands shown are from one representative experiment and the histogram displays E-selectin mRNA levels relative to actin.

One possible mechanism for limiting E-selectin expression is via the production of IL-10 from TNF stimulated monocytes which in turn will reduce TNF production.

IL-10 inhibits E-selectin induction in monocyte/endothelial cell cocultures but not in response to IL-1 and TNF, suggesting that IL-10 acts directly on monocytes. IL-10 alone is not able to induce E-selectin protein or mRNA expression on endothelial cells. Although IL-10 protein was not detected in supernatants from monocyte/endothelial cell cocultures, IL-10 mRNA was increased in monocytes following coculture with endothelial cells.

It is proposed that IL-10 plays an immunosuppressive role in areas of inflammation and that this is achieved through multiple effects on monocyte/macrophage activites and inhibitory effects on cytokine elaboration.

Monocytes in contact with endothelial cells express functional TNF-α which is membrane bound prior to being secreted (Lidington et al, 1998; Rainger et al, 1996; Green et al, 1998). IL-10 inhibits TNF-α synthesis acting mainly at the level of cytokine gene transcription (Wang et al, 1994). The inhibition of mRNA accumulation and cytokine synthesis is time dependent (Donnelly et al, 1995), the addition of IL-10 to stimulated PBMC inhibits TNF-α release maximally at 4 hours. The inhibitory role of IL-10 on TNF-α synthesis is further supported by the observation that the addition of neutralising antibodies to IL-10 leads to an increase in TNF-α levels. The kinetics of IL-10 inhibition of TNF-α may help explain why when exogenous IL-10 was added to the cocultures the greatest reduction in E-selectin expression occurred at 21 hours and not at 4 hours. IL-10 at the earlier time point had not maximally inhibited TNF-α synthesis, and E-selectin transcription and protein production was already initiated. However by

21 hours, IL-10 had fully inhibited TNF-α induction with subsequent effects on E-selectin expression. A second explanation could be that between 4 and 21 hours E-selectin expression is largely dependent on TNF-α and that prior to 4 hours other mechanisms induce E-selectin expression.

A further action of IL-10 is to stimulate production and release of soluble TNF-RII (sTNF-RII or p75)(Dickensheets et al, 1997) from untreated and stimulated monocytes (Hart et al, 1996). Activation of monocytes by agents such as LPS and TNF induces rapid shedding of membrane TNF-RII molecules and internalisation of TNF-RI molecules that is enhanced by IL-10 (Joyce et al, 1994). Shed TNF-R molecules often function as TNF antagonists by competing for ligand with membrane-bound TNF-R, however under certain conditions, sTNF-R may enhance TNF activity by stabilising TNF molecules and prolonging their availability for binding to cell surface TNF receptors. This ability of IL-10 to concomitantly decrease production of TNFα and to enhance production of sTNF-RII by monocytes further confirms IL-10 as an anti-inflammatory cytokine. IL-10 would thus act in atherosclerotic plaques to reduce TNF availibility, hence limiting further inflammation.

Since activated monocytes release IL-10 in areas of inflammation it is important to exclude the possibilty that IL-10 is itself inducing expression of certain molecules that could contribute further to the progression of inflammation such as adhesion molecule expression on endothelial cells. E-selectin has been shown to be induced in human dermal microvascular endothelial cells (HDMEC) and HUVEC by IL-10 over 4 hours (Vora et al, 1996) and the induction was confirmed using immunofluorescence microscopy, FACS analysis and PCR analysis. In my experiments, however, I was not able to show

any induction of endothelial E-selectin either at the protein or mRNA level by IL-10.

An alternative, or additional method by which IL-10 could be inhibiting E-selectin induction in endothelial cell/monocyte coculture is by altering monocyte adhesion to endothelial cells. IL-10 inhibits adhesion of the monocytic cell line, THP-1 to IL-1 stimulated endothelial cells and IL-10 treated THP-1 cells do not adhere as well as untreated THP-1 cells to IL-1 stimulated endothelial cells (Krakauer, 1995). Also IL-10 can downregulate the expression of VCAM and ICAM on IL-1 activated endothelial cells (Krakauer, 1995).These results suggest that IL-10 counteracts the pro-inflammatory effects of IL-1 and regulates the adhesion of monocytic cells to endothelial cells. The IL-10 induced reduction in monocyte:endothelial cell contact could alter signalling pathways that either directly or indirectly regulate E-selectin induction on endothelial cells. Preliminary results have shown that the addition of IL-10 to monocyte:endothelial cell cocultures does reduce the adhesion of monocytes to unstimulated endothelium.

Synthesis of IL-10 by monocytes and by T cells is regulated by TNF-α. TNF-α is unique in its capacity to regulate IL-10 expression in human peripheral blood monocytes (Wang et al, 1994). Most studies have concentrated on the production of IL-10 from LPS stimulated monocytes, but recent work has shown that IL-10 is released by monocytes stimulated by TNF-α (Wanidworanum and Strober, 1993). IL-10 mRNA production and induction occurs between 8 and 24 hours post TNF-α addition. Small amounts of IL-10 mRNA have been detected in monocytes after 20 hours of TNF-α stimulation and detectable IL-10 protein measured in supernatants (Downing et al, 1998). These studies are in keeping with the findings in this chapter, showing IL-10 mRNA induction in monocytes

cocultured with endothelial cells. On the other hand, recent work has shown that IL-1α and β, and TNF-α in the absence of LPS do not induce IL-10 (Foey et al, 1998).

The lack of effect of anti-IL-10 antibodies in the coculture system may be due to the delayed kinetics of release of IL-10 protein by TNF stimulated monocytes. Thus endogenous IL-10 may not be important during the first 21 hours of monocyte:endothelial cell cocultures. In addition, surface E-selectin expression may not accurately reflect the inhibitory effect of IL-10 in the coculture, since E-selectin protein may remain on the surface of endothelial cells regardless of the cytokines present at that time, and the stability of the protein was not considered. Furthermore, TNF independent mechanisms may be regulating E-selectin levels at later time points.

The lack of detection of IL-10 in supernatants by ELISA despite the presence of mRNA may be due to the presence of cell surface biologically active IL-10 (Fleming and Campbell, 1996). Secondly, it is possible that IL-10 released by adherent monocytes binds to the cell surface receptors as it is secreted, and is thus not released into the culture supernatant.

IL-10 synthesis is tightly regulated. In the promoter region of its genomic structure are several motifs known to modulate transcription such as NF-κB-like recognition sites, an interferon inducibility sequence, a cAMP responsive element, and a glucocorticoid responsive element. There are suspected regulatory elements also in the 3' untranslated portion of the gene, which might play a role in IL-10 gene expression. Among these are AUUUA repeat sequences such as adenylate/uridylate-rich element (ARE), which have been implicated in regulation of stability and nucleo-cytoplasmic transport of other shortlived mRNA species (Le et al, 1997). Upregulation of IL-10 production and

gene expression may also be due to posttranscriptional mechanisms such as nuclear processing, nucleocytoplasmic transport, or mRNA stability. Differences arise in the level of IL-10 mRNA induction compared to the resultant protein production. TNF-α induced IL-10 protein secretion is considerably less than LPS-induced secretion, despite the fact that TNF-α induces higher levels of IL-10 mRNA than does LPS (Wanidworanum and Strober, 1993). The precise molecular mechanisms that account for TNF-α induction of IL-10 are largely unknown. It could be possible that TNF exerts a direct effect on the regulation of IL-10 mRNA transcription. Secondly, TNF could induce IL-10 mRNA synthesis indirectly by inducing other cytokines that then induce IL-10. Finally, it is possible that TNF-α affects IL-10 mRNA levels, not by up-regulation of transcription, but by stabilization of IL-10 mRNA. This then means that production of IL-10 protein is not always directly related to the amount of mRNA synthesised, and it is thus not possible to state with certainty that IL-10 protein is synthesised or released in this coculture system.

The results presented in this chapter suggest that IL-10 plays an immodulatory role in the endothelial cell/monocyte coculture, through inhibiting E-selectin expression on endothelial cells stimulated to synthesise E-selectin by monocyte derived factors and cell contact. Prolonged expression of E-selectin on endothelial cells would encourage the persistent recruitment of monocytes and neutrophils, with potentially damaging effects on surrounding tissues. The ability of endogenous IL-10 to downregulate endothelial cell activation may suggest a protective function for this cytokine guarding vessels against injury.

Chapter Seven

General Discussion

The cellular events underlying atherosclerosis include the accumulation of lipid-laden monocytes in the neointima. This process is associated with the expression of adhesion proteins and chemokines by the endothelium. In my studies I have examined the role of monocytes in regulating endothelial responses. Evidence is presented that monocytes cocultured with endothelial cells induce E-selectin expression on endothelial cells with sustained kinetics compared to that induced by proinflammatory cytokines. In addition, monocytes induce A1 mRNA expression and prevent endothelial cell apoptosis. The role that the immunomodulatory cytokine IL-10 plays in the monocyte modulation of endothelial cell activation has also been studied.

Apoptosis of vascular cells occurs within atherosclerotic lesions and is likely to contribute to tissue turnover and lesion development. Pro-apoptotic signals in these lesions include inflammatory cytokines produced by activated immune cells, mainly macrophages and T cells. Simultaneous exposure to TNF-α, IL-1 and IFN-γ, for example, may trigger apoptosis of vascular smooth muscle cells and endothelial cells.

Another source of pro-apoptotic signals are the oxidation products of lipids. Oxidised low density lipoproteins (OxLDL) cause chronic inflammatory responses in the vasculature that promotes the development of atherosclerotic plaques. In addition, OxLDL and its lipid constituents have several detrimental effects in endothelial cells function including the induction of apoptosis (Escargueil-Blanc et al, 1997; Dimmler et al; 1997; Harada-Shiba et al, 1998). OxLDL induces endothelial cell apoptosis through Fas-FasL interaction (Sata and Walsh, 1998). Under normal conditions, endothelial cells are resistant to Fas-mediated apoptosis, although they express detectable Fas on their cell surface (Richardson et al, 1994). OxLDL acts by sensitizing endothelial cells to

death signals from the Fas receptor, and upregulation of FasL alone is not sufficient for apoptosis to occur. Thus, endothelial cells become susceptible to apoptosis, mediated by Fas/FasL pathway, as a result of acute exposure to oxidised lipid. Vascular smooth muscle cells also exhibit characteristic features of apoptosis in advanced atherosclerotic plaques. Activation of the Fas death-signalling pathway contributes to the induction of SMC apoptosis, and expression of Fas on SMC is enhanced by IFN-γ, TNF-α and IL-1 (Geng et al, 1997).

Endothelial cells sensitized to death signals by oxidised lipids or cytokines may become damaged by continuing inflammation. Endothelial damage and denudation exposes the subendothelial matrix leading to platelet adhesion and aggregation and the consequent thrombosis. Activated platelets trigger an inflammatory reaction in endothelial cells via CD40. Platelets express CD40L within seconds of activation, and this activates endothelial cells, which express CD40 on their cell surface, leading to chemokine secretion, increased expression of adhesion molecules, such as E-selectin, VCAM-1 and ICAM-1, thereby generating signals for the further recruitment of leukocytes (Henn et al, 1998). Also, platelet CD40L is likely to trigger cells in the lesion, because monocytes secrete IL-1, TNF-α, IL-8 and MIP-1α upon engagement with CD40 (Alderson et al, 1993). Thus, the generation of inflammatory signals by platelets may play a critical role in the pathogenesis of atherosclerotic lesions. Monocytes, on the other hand, may exert a protective role on endothelial cells, as demonstrated in this thesis.

Anti-apoptotic genes such as Bcl-2, A20 and A1, as well as pro-apoptotic gene Bax have been found in the vascular cells and may participate in the regulation of vascular apoptosis during the development of atherosclerosis. The role of

Bcl-2 family members in preventing endothelial cell apoptosis induced by factors involved in atherogenesis has not been fully described. Bax is expressed throughout the full thickness of the vessel wall in human atherosclerotic lesions and Bcl-x_L is more abundantly expressed within intimal cells as compared with medial smooth muscle cells (Pollman et al, 1998). In addition, there is increased Bcl-x_L mRNA and protein expression in vascular lesions compared with normal vessels. While monocytes increased A1 and bcl-2 expression there was no evidence of any effect on Bcl-x_L and Bax in my experiments.

Toxic concentrations of OxLDL have been shown to induce apoptosis in endothelial cells expressing low levels of Bcl-2 protein (Meilhac et al, 1999). However, endothelial cells expressing high Bcl-2 levels undergo primary necrosis in the presence of OxLDL. Thus, it appears that Bcl-2 alters the balance between apoptosis and necrosis, but does not prevent cell death induced by OxLDL. OxLDL toxicity may be involved in the genesis of the necrotic core and of complicated atherosclerotic plaques prone to plaque rupture and thrombosis. If vascular cell death does occur then apoptosis may be less harmful since apoptotic cells are rapidly engulfed and cleared whereas necrotic cell debris may trigger a local inflammatory response, promote formation or enlargement of the necrotic core of atherosclerotic plaques, and therefore enhance the risk of plaque rupture and thrombosis. Whilst apoptosis of cells present in atherosclerotic lesions could be considered advantageous in so much that a reduction in the lesion size could occur, it really depends on which cell type undergoes apoptosis as to the consequences. When bcl-X_L expression was reduced by anti-sense technology in smooth muscle cells, the resulting apoptosis reduced intimal lesion dimensions inducing atheromatous

lesion regression (Pollman et al, 1998). However, if endothelial cells are encouraged to apoptose then there is subendothelial matrix exposure, platelet aggregration and monocyte recruitment which are all detrimental.

The Bcl-2 family members, Bcl-2 and Bcl-x_L appear to have a dual function in endothelial cells in that they are not only anti-apoptotic, but also act to inhibit activation of NF-κB, thus fulfilling an anti-inflammatory role. A1 is also considered to act as an inhibitor of NF-κB (Cooper et al, 1996). Bcl-2 and Bcl-x_L are involved in a complex regulatory network that serves to downregulate endothelial cell activation and its associated gene upregulation (Badrichani et al, 1999). The expression of these two genes inhibits the induction of E-selectin and IL-8 in endothelial cells by TNF, and is directly related to the inhibition of the transcription factor, NF-κB. This is further supported by work showing that Bcl-2 downregulates the activity of NF-κB induced upon apoptosis (Grimm et al, 1996). The expression of A20 in endothelial cells also inhibits gene upregulation associated with TNF and LPS-induced activation such as E-selectin, the mechanism of action is due to blockade of NF-κB, and the endothelial cells are still rescued from TNF-mediated apoptosis (Cooper et al, 1996, Ferren et al, 1998).

On the other hand, expression of A1 and A20 are considered to be NF-κB dependent. The suppression of the transcriptional activity of NF-κB, by overexpression of IκBα, sensitizes the endothelial cells to TNF mediated apoptosis and is accompanied by the lack of induction of the anti-apoptosis genes such as A1, and A20 (Soares et al, 1998). However, sensitization to TNF-mediated apoptosis is dependent on the method used to block activation of NF-κB, since suppression of NF-κB activation by a dominant negative mutant p65/RelA rendered endothelial cells resistant to TNF-mediated apoptosis, even

in the absence of A1 induction. These results suggest that the role of NF-κB in preventing TNF-induced apoptosis is very complex. To avoid the inflammatory response consequent upon monocyte interaction with endothelial cells, such as would occur in atherosclerosis, one would wish to inhibit NF-κB, which is a key to the induction of the proinflammatory genes in endothelial cells. However, the method used to suppress NF-κB must be chosen carefully to avoid sensitizing the endothelial cells to deleterious apoptosis.

Other studies have shown that TNF induction of NF-κB was not inhibited by Bcl-2, and that in fact suppression of apoptosis by Bcl-2 required activation of NF-κB (de Moissac et al, 1998). In addition, the expression of IEX-1L, a gene that protects cells from TNF-induced apoptosis, was decreased in cells with defective NF-κB activation, rendering them sensitive to TNF induced apoptosis (Wu et al, 1998). Recent work has shown that A1 does inhibit endothelial cell activation by TNF through inhibition of NF-κB. In addition, activation of NF-κB is essential for the expression of A1 (Stroka et al, 1999). The action of A1 to downregulate NF-κB activity in endothelial cells may account for the eventual fall in E-selectin expression on endothelial cells cocultured with monocytes. E-selectin expression is very dependent on NF-κB activity. Thus, at 21 hours when A1 expression is still sustained in endothelial cells cocultured with monocytes, E-selectin expression is starting to decline. Further work is required to determine whether A1 induction by monocytes and IL-1 as well as TNF is NF-κB dependent. Such studies are required in order that we may devise means to block aberrant activation of the endothelium, and thus induce plaque regression.

The adhesion of monocytes to endothelium is well established as a major early step in the development of atherosclerosis and atherosclerotic lesions develop

mainly in the arteries not in veins. Almost all the currently available data on leukocyte adhesion and adhesion molecule expression, however, have been obtained with venous cells, such as HUVEC. Furthermore, recent studies, suggest that there is a difference in leukocyte adhesiveness to arterial and venous endothelial cells under proinflammatory conditions. In work where monocyte adhesion was studied, TNF and LPS stimulated monocyte adhesion to HUVECs but not to human umbilical arterial endothelial cells (HUAEC), and there was no induction of VCAM-1 on HUAEC. In addition, there was no E-selectin induction by TNF on HUAEC despite TNF stimulating E-selectin on HUVEC. However IL-1 did stimulate monocyte cell adhesion to and VCAM-1 surface expression in both HUVEC and HUAEC (Kalogeris et al, 1999). Thus these studies show a striking insensitivity of arterial endothelium to the effects of TNF and LPS. Therefore there must be other factors present that encourage monocyte adhesion to endothelial cells.

In vivo endothelial cells are constantly exposed to haemodynamic forces, which include the shear stress, the tangential force due to blood flow, and the circumferential stress, the normal force due to transmural pressure. It is considered that shear stress and/or mechanical strain can modulate the expression of endothelial genes. Blood flow and shear stress in arteries are greater than in veins. Laminar shear stress downregulates the expression of VCAM-1, but in contrast, ICAM-1 expression is upregulated; E-selectin expression is not affected by shear stress (Chien et al, 1998). In addition there is a transient and rapid increase in MCP-1, and PDGF-B. The modulation of gene expression results from the binding of specific transcription factors to their target elements in the promoter region of the gene, and for example, shear stress increases the binding activities of NF-κB to its target sequences (Resnick

and Gimbrone, 1995). In addition, fluid shear forces promote leukocyte adhesion mediated by P-, L- or E-selectin (Lawrence et al, 1997). It appears that fluid shear-induced torque is crucial for the maintenance of leukocyte rolling, and that without such forces the leukocyte detaches from the substratum without forming additional bonds with selectins. Large enough fluid shear forces enable additional bonds to form as the cell experiences the torque into the vessel wall, and thus sustain and stabilise selectin-mediated adhesion (Lawrence et al, 1997). My studies were performed using a static model, and thus it is difficult to ascertain the full importance of selectins in the adhesion of monocytes to cultured endothelial cells. In addition, the involvement and role of endogenously produced cytokines and chemokines in monocyte adhesion are not considered properly as *in vitro* these proteins remain in contact with endothelial cells and monocytes whereas *in vivo* flow would remove them from their site of release.

The sustained expression of E-selectin on endothelial cells cocultured with monocytes mirrors the effect of endothelial cellular activation through ligation of ICAM-1. Crosslinking ICAM-1 on endothelial cells leads to the induction of ICAM-1 mRNA and protein (Clayton et al, 1998). In addition, a similar mechanism is triggered through the ligation of VCAM-1. Adhesion molecule upregulation by this mechanism may provide an important positive feedback pathway at atherosclerotic lesions, whereby transmigrated leukocytes remaining in close proximity with endothelial cells activate neighbouring cells. Thus the binding of inflammatory cells could promote further leukocyte binding, enhancing their recruitment to lesions. The signalling pathways involved in the induction and sustained expression of E-selectin on endothelial cells cocultured with monocytes have yet to be determined,however, their identification is

important for developing therapeutic strategies to combat prolonged activation of the endothelium.

It appears that monocytes in the coculture system play two contrasting roles. When cocultured with serum starved endothelial cells, monocytes prevent endothelial cell death, and thus have a protective role. However, monocyte coculture also causes persistent activation of endothelial cells, with sustained expression of E-selectin. IL-10 acts to counter both these actions of monocytes causing blockade of E-selectin induction as well as a reduction in A1 expression. In fact, IL-10 downregulates thrombus-associated inflammation and thrombosis (Downing et al, 1998).

IL-10 usually plays an anti-inflammatory role in monocyte/endothelial cell interactions. Monocytes treated with IL-10 do not adhere well to fibrinogen or to vitronectin. Vitronectin is an extracellular matrix protein, and fibrin and fibrinogen are present in the intima into which monocytes migrate. Reduced monocyte adhesion to fibrinogen is due to a decrease in CD11b/CD18 and monocyte associated ICAM-1 (Song et al, 1997), while decreased adhesion of monocytes to vitronectin by IL-10 is related to the decrease in uPA. uPA is important in the uPAR-dependent adhesion of cells to vitronectin (Paysant et al, 1998). A significant decrease in uPA and of uPAR on monocytes cultured with IL-10 can prevent the extracellular matrix degradation and plaque disruption which is the major complication of atherosclerosis.

When IL-10 is added to serum starved endothelial cells cocultured with monocytes, there is a reduction in A1 expression and a decrease in endothelial cell survival.Thus here, IL-10 is pro-inflammatory. The rational therapeutic use of IL-10 as an anti-inflammatory agent requires a more thorough understanding of its role in monocyte/endothelial cell interactions.

My studies have also examined monocyte transmigration, and shown that calpain plays a predominant role in leukocyte extravasation. Inhibitors of calpain reduced monocyte and neutrophil transmigration through cytokine stimulated endothelium as well as across endothelium towards chemoattractants. Calpain does not only play a part in leukocyte diapedesis, but can contribute to increased cytokine production. Calpain expression increases upon lymphoid cell stimulation and stimulated lymphoid cells release calpain into the extracellular environment (Deshpande et al, 1995). In monocytes calpain has been identified as a processing enzyme for IL-1α, allowing its secretion in a biologically active form. IFN-γ stimulation causes expression and secretion of calpain in monocytic cells over 18-24 hours. In atherosclerotic plaques T-cells secrete IFN-γ that could stimulate calpain production in monocytes allowing IL-1α to be secreted in a biologically active form. Thus calpain could play a role in inflammation through enhancing IL-1 production. Calpain inhibitors therefore could be used therapeutically to not only block excessive monocyte transmigration, but also to block processing of the proinflammatory cytokine, IL-1, thus decreasing the inflammatory state of the atherosclerotic lesion.

In conclusion, monocyte-endothelial cell interactions play a key role in the inflammatory response, thrombotic events and the development of atherosclerotic lesions. The effect of monocytes or their products influences endothelial gene expression, cell activation and cell survival. The finding that monocyte adhesion induces E-selectin and A1 on endothelial cells may have biological relevance. The present data support a hypothesis that monocyte adhesion to endothelial cells leads to monocyte and endothelial activation. The expression of E-selectin on endothelial cells may result in enhanced monocyte

recruitment, resulting in increased lesion size. Decreased endothelial cell apoptosis whilst reducing thrombotic complications by preventing subendothelial exposure will however enable lesions to increase in size. Thus the state of the endothelium and the cytokines present influence the outcome of these adhesive cellular interactions.

Chapter Eight

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Chapter Nine

Appendix

Tissue culture media, reagents, serum and enzymes

Product	Source
Bovine Serum Albumin (BSA)	Sigma
Collagen Type 1	Collaborative Biomedical Products
Collagenase A from Clostridium histolyticum	Boerhinger Mannheim
Dimethylsulfoxide (DMSO)	Sigma
Endothelial cell growth supplement (ECGS)	Sigma
Ethylenediaminetetra-acetic acid dipotassium salt (EDTA)	BDH
Fibronectin-from human plasma	Sigma
Foetal Calf Serum (FCS) - heat inactivated at 56°C for 20 minutes	Harlan Seralab
D(+)Glucose	BDH
E-Toxate	Sigma
Hanks Balanced Salt Solution	Sigma
Heparin-sodium preservative free	CP Pharmaceuticals
Hespan (Hetastarch Hydroxyethyl starch	Dupont Pharma
Histopaque 1119	Sigma

Insulin,Transferrin, Sodium Selenite	Sigma
Iscove's Modified Dulbecco's Medium (IMDM)	Sigma
Lipopolysaccharide (LPS)	Sigma
Lymphoprep	Nycomed
Nonidet P-40 (NP-40)	BDH
Nycoprep 1.068	Nycomed
O-phenylenediamine tablets (OPD)	Sigma
Paraformaldehyde	BDH
Penicillin/ Streptomycin	Sigma
Phosphate Buffered Saline Solution (PBS) without Ca^{2+} or Mg^{2+}	Sigma
RPMI-1640	Sigma
Saline solution 0.9% w/v	Maco Pharma
Trypan Blue	Sigma
Trypsin/EDTA (T/E)	Sigma

Plastics and Tissue Culture Materials

Product	Source
Bijou bottles 7 ml	Bibby Sterilin Ltd
Cannulae plugs	Vygon
Falcon Flasks- 25 and 75cm^2 tissue culture treated	Marathon
Filter units- 0.22 and 0.45 micron	Sartorius
Intravenous cannulae (14 gauge)	Abbotts
LP4 radioactivity tubes and caps	Sarstedt
Microcentrifuge Eppendorf tubes 0.5, 1.5ml	Sarstedt
Pipettes-5, 10, 25ml	Bibby Sterilin Ltd
Plates - tissue culture treated 6, 24, 48 and 96 well	Nunc
Quill filling tubes	Avon Medical
Sterile surgical swabs and towels	Rocialle
Surgical blade	Swann-Morton
Syringes- 1, 2, 5, 10,	Sherwood Medical

20. 50ml	
Transwell filter inserts- 1 and 3 micron	Costar
Tubes: 15ml polypropylene	Life Technology
30ml universal	Bibby Sterilin Ltd
50ml polypropylene	Greiner

RNA, RT and PCR Reagents

Product	Source
5x First strand buffer	Life Technologies
10xMg-free thermophilic DNA buffer	Promega
Absolute alcohol	BDN
Agarose	Life Technologies
β-mercaptoethanol	Sigma
Chloroform	BDH
Diethyl pyrocarbonate (DEPC)	Sigma
2'-Deoxynucleoside 5' triphosphates (dNTP)	Pharmacia Biotech
Dithiothreitol (DTT)	Life Technologies
Ethidium bromide	BDH
Guanidine thiocyanate (GITC)	Sigma
Isoamylalcohol (IAA)	BDH
Magnesium chloride ($MgCl_2$)	Promega
MMLV-reverse transcriptase	Life Technologies
Oligonucleotides	Pharmacia Biotech
Orange G	BDH
Phenol	Sigma

pGL2 vector	Promega
Random hexamer primers	Promega
RnasinRnase Inhibitor	Promega
Sarkosyl NL 30 (30% w/v aqueous solution)	BDH
Taq DNA polymerase	Promega

Antibodies, Inhibitors and Radioactive material

Product	Source
Calpain Inhibitors- Calpain Inhibitor I (ALLN) Calpain Inhibitor II (ALLM) E64D Calpeptin Lactacystin	Affinity Research Products Ltd
^{51}Chromium-sodium chromate in aqueous solution	Amersham
Dynabeads	Dynal
Goat anti mouse HRP-conjugated antibody	Dako
IgG1 isotype control	Sigma
Mouse anti human E-selectin	R&D
Mouse anti human IL-10	R&D
Mouse anti human IL-1β	Peprotech EC Ltd
Mouse anti human TNF	Kind Gift of M.Kaul
Mouse anti human IκBα	Santa Cruz
Mouse anti human IκBβ	Santa Cruz
Mouse anti human L-selectin (DREG- 56)	Kind Gift of J.Rothlein
Mouse anti human	Kind Gift of P.Newman

PECAM-1 (1.3)	
Mouse anti human CD18 (60.3)	Kind Gift of J.Harlan
Mouse anti human CD11b (7E3)	Kind Gift of B.Coller
Mouse anti human VCAM mAb	R & D
Rat anti human CD11b/CD18 (M170)	Boerhinger Mannheim
N-tosyl-L-phenylalanine chloromethylketone (TPCK)	Calbiochem

Cytokines and chemoattractants

Product	Source
N-formyl-methionyl-leucyl-phenylalanine (fMLP)	Sigma
Interleukin 1 beta (IL-1β)	Boerhinger Mannheim
Interleukin 8 (IL-8)	Peprotech EC Ltd
Interleukin 10 (IL-10)	R & D
Tumour necrosis factor alpha (TNF-α)	Genzyme Diagnostics
Monocyte chemoattractant protein-1 (MCP-1)	R & D

Equipment

Equipment	Model and Company
Light microscope	Labophot-2, Nikon
Phase contrast microscope	Olympus, Tokyo
Automated Gamma counter	1282 Compugamma Universal Gamma Counter, LKB Wallace, Finland
Laser Densitometric scanning	Molecular Dynamics, USA
Gel Tank	Electro-4 , Hybaid
FACS	Becton-Dickinson
Spectrophotometer	Unicam UV/Vis
PCR machine	Techne Genius
Plate Reader	MR700 Microplate Reader, Dynatech

Buffers and Solutions

ELISA

1) Citrate Phosphate Buffer, pH 5.0

0.1M citric acid (15ml) mixed with 0.1M Na_2HPO_4 (34ml)

RNA Isolation

1) GITC Solution

GITC	50g
Na Cit 0.75M pH 7.0	3.5ml
Sarkosyl 10%	5.3ml

Made up to 106ml with DEPC water, and stored at 4°C in the dark.

2) DEPC-treated Water

1ml of DEPC added to 1000ml of distilled water, mixed and after 24 hours autoclaved.

3) TBE Buffer 5x

Tris	27g
Boric acid	14g
EDTA	10ml of 0.5M

Made up to 500ml with distilled water.

Western Blotting

1) Laemmli SDS sample buffer(reducing)

Distilled water	4ml
0.5M Tris-HCl,pH 6.8	1ml
10% SDS	1.6ml

Glycerol	0.8ml
Dithiotreitol	0.1g
0.05% Bromophenol blue	0.2ml

2) Mammalian Cell Lysis Buffer

137mM NaCl	1mM β glycerophosphate
20mM Tris, pH 8	1mM NaF
1mM MgCl2	5mM Pyrophosphate
1mM CaCl2	2mM EDTA
1% NP-40	1mM PMSF
10% Glycerol	10μg/ml Aprotinin
1mM Na orthovanadate	10μg/ml Leupeptin
10μg/ml Pepstatin	

3) Running Buffer (x10)

Tris Base	29g
Glycine	144g
SDS	10g

Made up to 1 litre with dd water

4) Blotting Buffer

Glycine	2.93g
Tris	5.81g
SDS	0.375g
Methanol	200 mls

Made up to 1 litre with dd water

5) Blocking Buffer

TBS x20	50 mls
Powdered Milk	50g
FCS	10mls

Made up to 1 litre with dd water on day of use

6) Washing Buffer

TBS x20	50 mls
Powdered Milk	50g
Tween	0.1%

Made up to 1 litre with dd water on day of use

7) TBS x20

Tris Buffered Saline	48.4g
NaCl	160g
HCl	4mls

Made up to 1 litre, pH checked and then autoclaved

APAAP-Alkaline Phosphatase Substrate

Naphthol AS-MX phosphate	0.002g
Fast Red TR Salt	0.010g
0.1M Tris Buffer, pH 8.2	15ml
1M Levamisole	0.24g/ml

All reagents were mixed together and filtered

Publications arising from work presented in this thesis

Noble KE, Panayiotidis P, Collins PW, Hoffbrand AV, Yong KL. (1996). Monocytes induce E-selectin gene expression in endothelial cells:role of CD11/CD18 and extracellular matrix proteins. Eur J Immunol 26 p2944-2951

Noble KE, Wickremasinghe RG, DeCornet C, Panayiotidis P, Yong KL. (1999). Monocytes stimulate expression of the bcl-2 family member, A1, in endothelial cells and confer protection against apoptosis. J Immunol 162 p1376-1383