APOLIPOPROTEIN E AND EXPRESSION OF ENDOTHELIAL CELL ADHESION MOLECULES

by

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Abstract

I have hypothesized that: 'apolipoprotein E (apoE) has an anti-inflammatory/anti-atherogenic action by inhibiting cytokine-mediated upregulation of endothelial cell adhesion molecules (CAMs).' After rejecting ECV304 cells as a model of human vascular endothelium, I showed that primary cultures of human umbilical vein endothelial cells (HUVECs) were activated by cytokines and quantified CAM expression by ELISA and flow cytometry. Although HUVECs responded to known down-regulators of CAMs, a nitric oxide (NO) donor and 17β-estradiol, no inhibition of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) or E-selectin was observed with apoE complexed to phospholipid vesicles (apoE:DMPC). Additionally, apoE:DMPC did not affect HUVEC sub-populations by flow cytometry, nor did they suppress monocyte adhesion to activated HUVEC monolayers. ApoE-rich high density lipoprotein (HDL-E) was also benign. I concluded that plasma apoE is unlikely to limit endothelial activation in vivo.

By contrast, when HUVECs were transfected with an apoE expression plasmid, VCAM-1 induction was inversely correlated with the apoE secreted, while co-culturing HUVECs and recombinant CHO cells synthesizing apoE also suppressed VCAM-1. Incubating cell-conditioned media containing apoE with HUVECs reduced VCAM-1 in a dose-dependent manner. Characterization of this highly active apoE revealed similarities to the minimally-lipidated, spherical particles secreted by macrophages. ApoE most likely inhibits endothelial activation by stimulating NO synthase (NOS); apoE increased cGMP, as expected for NO release; a NOS inhibitor blocked its effect; and lipoprotein receptor-related protein 8 (LRP8), implicated in coupling apoE-NOS in platelets, was detected in HUVECs by RT-PCR and immunoprecipitation. Moreover, consistent with protein-protein module formation and cell signalling to activate NOS, a synthetic cytoplasmic motif within LRP8 bound [35S]proteins in HUVEC cytosol.

I propose, therefore, that apoE, secreted locally at lesion sites by macrophages, suppresses VCAM-1 expression on endothelium and that this anti-inflammatory action is mediated by activation of a LRP8-linked signalling cascade to generate intracellular NO.
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DEDICATION

I wish to dedicate this thesis to my parents June and Claude Stannard, my sister Linda, and in memory of Violet and Walter Tucker.
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<tr>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
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<td>diethylpyrocarbonate</td>
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<td>deoxynucleotide triphosphates</td>
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<td>1',1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
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<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
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<td>EDTA</td>
<td>ethylenediaminetetra-acetate</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ethyl-ITU</td>
<td>2-ethyl-isothiopseudourea</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitroso-L-glutathione</td>
</tr>
<tr>
<td>HCAEC</td>
<td>human coronary artery endothelial cell</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HEL</td>
<td>human erythroleukaemia</td>
</tr>
<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>heparin sulphate</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparin sulphate proteoglycan</td>
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</table>
HUVEC ............................................ human umbilical vein endothelial cell
ICAM-1 ............................................. intercellular adhesion molecule-1
IDL .................................................. intermediate density lipoprotein
IEF .................................................. isoelectric focusing
Ig .................................................... immunoglobulin
IgSF .................................................. immunoglobulin superfamily
I-κB .................................................. inhibitor of nuclear factor-kappa B
IL-1β ............................................... interleukin 1-beta
IP3 .................................................... inositol 1,4,5-trisphosphate
LCAT ................................................. lecithin-cholesterol acyltransferase
LDL .................................................. low density lipoprotein
LDL-R .............................................. low density lipoprotein receptor
LFA-1 ................................................ leukocyte function-associated antigen-1
Lp(a) .................................................. lipoprotein (a)
LPL ................................................... lipoprotein lipase
LPS .................................................. lipopolysaccharide
LRP ................................................... low density receptor-related protein
LRSF ............................................... low density lipoprotein receptor superfamily
MCP-1 ............................................... monocyte chemoattractant protein-1
M-CSF ............................................... macrophage-colony stimulating factor
NADPH ............................................ reduced nicotinamide adenine dinucleotide phosphate
NF-κB ............................................... nuclear factor-kappa B
NBT .................................................. nitro blue tetrazolium
NO .................................................... nitric oxide
NOS .................................................. nitric oxide synthase
OPD .................................................. O-phenylenediamine
oxLDL ............................................. oxidized LDL
PECAM-1 ......................................... platelet endothelial cell adhesion molecule-1
PBS .................................................. phosphate buffered saline
PCR .................................................. polymerase chain reaction
PSGL-1 .............................................. P-selectin glycoprotein ligand-1
PK .................................................... protein kinase
PMSF .............................................. phenylmethylsulphonylfluoride
PTB .................................................. phosphotyrosine-binding domain
PTK .................................................. protein tyrosine kinase
pY ...................................................... phosphotyrosine
RCT ............................................... reverse cholesterol transport
ROI .................................................. reactive oxygen intermediate
RT .................................................. reverse transcription
SDS-PAGE ....................................... sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM .................................................. scanning electron microscopy
sCAM .............................................. soluble cell adhesion molecule
SGC .................................................. soluble guanylate cyclase
SH2 .................................................. src-homology 2
SH3 .................................................. src-homology 3
sLex .................................................. sialyl Lewis X
sLea ................................................... sialyl Lewis A
SSC .................................................. saline sodium citrate
TBS .................................................. tris buffered saline
TEM .................................................. transmission electron microscopy
TEMED ............................................ N, N, N, N'-tetramethylethylenediamine
TM .................................................. thrombomodulin
TNF-α ............................................. tumor necrosis factor-alpha
TNFR1 .............................................. tumor necrosis factor receptor-1
VCAM-1 .......................................... vascular cell adhesion molecule-1
VLA-4 ............................................. very late antigen-4
VLDL .............................................. very low density lipoprotein
VLDL-R .......................................... very low density lipoprotein receptor
vWF ............................................... von Willebrand factor
Chapter 1
1. INTRODUCTION

1.1 Background.

The process of atherosclerotic lesion formation represents a complex interaction of a number of circulating blood cells, lipoproteins and cells that reside within the arterial wall. Understanding the cellular mechanisms involved in this process is vital for the development of novel therapeutic strategies for prevention and treatment of coronary heart disease (CHD). Over the past decade, there has been accumulating evidence that atherogenesis represents a chronic inflammatory process, in which a central feature is the recruitment of circulating leukocytes facilitated by the upregulation of endothelial cell surface ‘cell adhesion molecules’ (CAMs) in response to pathophysiological stimuli.

It has recently been demonstrated that the plasma protein, apolipoprotein E (apoE), is increasingly implicated in protection against early atherosclerotic development. In this thesis, I have sought to determine whether this protective molecule could influence endothelium CAM expression and therefore represent a novel target for therapeutic intervention in atherosclerosis.

1.2 Endothelial CAMs in Atherosclerosis.

1.2.1 Overview of Atherosclerosis.

Atherosclerosis is the main cause of CHD, cerebral ischaemia and gangrene of the extremities, and is responsible for 50% of mortality in the Western world [1]. The disease develops progressively over decades of life, starting in childhood [2], and involves the formation of increasingly complex vascular lesions, focused thickenings of the artery wall (the intima), which protrude into the lumen of the artery and impede blood flow. Lesions are associated with lipid accumulation, smooth muscle cell proliferation and migration, fibrous connective tissue growth and calcification [3,4]. Occlusive thrombosis and plaque rupture lead to the clinical events associated with complex lesions at the later stages of disease. Epidemiological studies have identified risk factors for atherosclerosis, including hypercholesterolaemia, cigarette smoking, hypertension, diabetes, obesity, age and hereditary factors [1,5]. Lipoproteins, particles that transport cholesterol and other essential fats complexed to proteins (apolipoproteins) in the blood, are intimately associated with atherosclerosis. Raised plasma cholesterol and its major plasma carrier, low density lipoprotein (LDL) are significant risk factors [6] whilst lowered plasma levels of high density
lipoprotein (HDL) cholesterol and apolipoprotein AI (apoAI), the major protein component of HDL, are powerful predictors of premature CHD [7-9]. Low apoE and HDL-E, a subclass of HDL containing apoE, are also important risk factors [10-12]. The mechanism of HDL atheroprotection may relate to both its role in 'reverse cholesterol transport' (RCT) whereby excess cholesterol from peripheral cells is transported to the liver for catabolism and secretion, and to a range of non-lipid transport functions of HDL [9,13,14] (Section 1.2.9.3).

1.2.2 A ROLE FOR 'DYSFUNCTIONAL' ENDOTHELium IN THE DEVELOPMENT OF Atherosclerosis.

In health, the vascular endothelium forms a multifunctional interface between the circulating blood and various tissues/organs of the body. It constitutes a selectively permeable barrier for macromolecules, as well as a nonthrombogenic and nonadhesive container that actively maintains the fluidity of blood and vessel tone. The endothelium is a dynamic and metabolically active organ, synthesizing a number of factors and mediators that influence the behaviour of other cells types [15].

Although there was a time when atherosclerosis was thought of as a natural consequence of ageing, it is emerging that it is a chronic inflammatory disease that develops as a cascade of events in 'response to injury', as initially proposed by Ross and Glomset in the 1970s [16,17]. Central to this theory is that atherosclerosis results from some form of injury to the endothelium in response to pathogenic stimuli. These stimuli lead to altered endothelial cell gene expression and dysfunctional responses; the functions that are important for vessel wall homeostasis are suppressed whilst the functions that initiate, maintain and progress atherosclerotic plaque formation are induced. Indeed, the vascular endothelium is an integrator of pathophysiological stimuli in atherogenesis [18]. Specific endothelial properties that may be directly relevant to atherosclerosis and its clinical sequelae include: the expression of leukocyte binding sites or CAMs; the production of pro-inflammatory cytokines; paracrine growth factors and chemoattractants; the ability to oxidize lipoproteins and to respond to lipoproteins and oxidized lipids; the ability to express pro- rather than anti-coagulant factors; the regulation of extracellular matrix formation; the modulation of plasma component levels within the vessel wall through changes in permeability function [19,20], and localized endothelial cell turnover indicative of demise and regeneration [21]. Endothelial dysfunction has often been defined as the decreased synthesis, release and/or activity of endothelial-derived nitric oxide (NO) [22], an important anti-atherogenic molecule crucial to the normal function of the endothelium (Section 1.4.4). Therefore, impaired
endothelial function is not simply one of the earliest markers of the atherosclerosis, but is an important initiator and contributor to the disease.

One area of debate is the source of the injury, and examples of potentially injurious atherogenic agents include hypercholesterolaemia, hyperhomocysteinaemia, by-products of cigarette smoking, glycosylated products secondary to diabetes or ageing, altered biomechanical forces, oxidative stress (including oxygen species, free radicals and oxidized lipids), and infection with viruses or bacteria [19]. Endothelial dysfunction also correlates with increasing age and with male gender. Importantly, there is a strong association between atherogenic risk factors and endothelial dysfunction. Interestingly, endothelial function improves with risk factor modification such as cholesterol reduction [22].

1.2.3 CAM Upregulation and Monocyte Recruitment - Early Cellular Events in Atherogenesis.

Endothelial dysfunction/activation triggers changes in endothelial adhesiveness by the localized upregulation of endothelial CAMs, membrane glycoproteins that control leukocyte adhesion via their specific interactions with the counter-receptors on the leukocyte surface. Endothelial CAM upregulation is a prerequisite for the selective and focal recruitment of circulating monocytes and T-lymphocytes into the subendothelial space [23-28], a characteristic early manifestation of the specialized immune mechanism in atherosclerosis [29]. It has been estimated that 80 % of cells in the early lesion are monocyte/macrophages, while 10-20 % are T-lymphocytes [30]. In the intima, monocytes undergo activation-differentiation to become tissue macrophages and ingest lipoprotein cholesterol via upregulated scavenger-receptors leading to the formation of 'foam' cells, the characteristic hallmark of the early fatty streak lesion [31]. The macrophage population can promote lesion formation through the local generation of cytokines, such as tumor necrosis factor-apha (TNF-α) and interleukin-1 beta (IL-1β), growth factors, procoagulant and fibrinolytic components and toxic oxygen products [32]. There is strong evidence that monocytes are involved in the initial stages of lesion formation; their intimal infiltration precedes the development of fatty streaks [33] and occurs in response to short-term atherogenic diet feeding in rabbits [23,24,26]. Suppressed monocyte recruitment, in certain genetically-altered mice, causes a profound reduction in atherosclerosis, suggesting that these cells also play a key role in the progression of the disease [34,35]. Macrophages also contribute to plaque stability and clinical events such as rupture [36]. Macrophages at lesion sites also secrete apoE [37,38] and the implications of this are discussed in Section 1.4.2. Accumulated intimal T-lymphocytes play a lesser role in the disease [39] but their secretion of different pro-
inflammatory cytokines adds further complexity to the local cytokine milieu and the potential activation states of the vessel wall cells.

1.2.4 THE ADHESION CASCADE.

Distinct adhesion molecules appear to regulate different stages of monocyte recruitment in a dynamic and coordinated multistep process known as the 'adhesion cascade' involving locally-generated chemokines that have direct chemotactic effects and regulate the affinity of leukocyte CAMs for their ligands (Figure 1.2-1). Most of these endothelial CAMs are either members of the immunoglobulin superfamily (IgSF), characterized by the presence of multiple Ig-like domains, or the selectin family which include an amino-terminal C-type lectin domain. Leukocyte coreceptors for the IgSF CAMs are the integrins, heterodimeric proteins consisting of noncovalently linked alpha and beta units. CAMs are also known by their cluster of differentiation (CD) antigen designation. The adhesion cascade, involving CAMs implicated in atherosclerosis, has been recently reviewed [40-43] and additional CAM information can be found in the Adhesion Molecule Facts Book [44].

1.2.4.1 Monocyte Tethering via Endothelial Selectins.

Monocyte adhesion to activated endothelium starts with the capture of cells from flow. The selectins mediate this initial attachment to the vessel wall through labile, transient adhesions which permit monocytes to roll in the direction of blood flow (Figure 1.2-1). P-selectin is the first endothelial CAM upregulated at the cell surface within minutes of endothelial activation and initiates the earliest phase of monocyte recruitment via interactions with P-selectin glycoprotein ligand-1 (PSGL-1/CD62P), its major leukocyte coreceptor. P-selectin is constitutively produced in a pre-formed state and is rapidly translocated from internal storage sites (Weibel-Palade bodies) to the membrane. P-selectin interacting with PSGL-1, acts in concert with monocyte L-selectin (CD62L) interacting with endothelial cell CD34 (a sialomucin). P-selectin is also transcriptionally regulated by cytokines. E-selectin (CD62E), also known as endothelial leukocyte adhesion molecule-1 (ELAM-1), is an endothelial-specific inducible molecule expressed rapidly in activated endothelium (Figure 1.2-2) that mediates a stronger interaction with carbohydrate ligands such as sialyl Lewis X and sialyl Lewis A (sLex and sLea) or PSGL-1 on monocytes [42].
Figure 1.2-1 Recruitment of monocytes to lesion-prone sites by the ‘adhesion cascade’.

In response to inflammation, circulating monocytes first roll on the endothelium lining the lumen of the vessel by transient interactions with upregulated endothelial CAMs of the selectin family (1). Monocyte integrins are then activated or ‘triggered’ by locally-secreted chemokines (2) to bind strongly to the IgSF endothelial CAMs to halt rolling and give firm adhesion (3) before the initiation of transendothelial migration (4).

1.2.4.2 Monocyte Integrin Activation by Chemokines.

The rolling monocyte becomes activated by local factors generated by the endothelium (Figure 1.2-1). Constitutively expressed monocyte integrins do not bind their counter ligands unless activated. This activation process involves integrins undergoing a conformational change in response to chemokines [45]. Chemokines are small secreted chemotactic peptides, the largest subfamilies designated C-C or C-X-C (according to the spacing of the
first 2 of 4 conserved cysteine residues) and mediate leukocyte activation, migration and growth [46]. MCP-1 (monocyte chemoattractant protein-1) is a chemokine that can activate monocyte integrins. MCP-1, secreted by vessel wall cells including endothelial cells, is upregulated in macrophage-rich areas of human and animal lesions [47]. Recent experiments with genetically-altered mice have confirmed its crucial role in initiating atherosclerosis [35,48,49]. MCP-1 is a chemoattractant for both monocytes and T-lymphocytes, which possibly explains why these cells are located together in lesions [50].

1.2.4.3 Firm Monocyte Adhesion and Transendothelial Migration via IgSF Members of Endothelial CAMs.

Selectin-mediated adhesion does not lead to firm adhesion and transmigration unless members of the IgSF are involved. Following integrin activation, monocytes adhere strongly to these endothelial CAMs and then monocytes flatten before extravasation can take place.

Intercellular adhesion molecule-1 (ICAM-1, CD54), has 5 Ig domains and is weakly expressed under resting conditions in vivo but upon endothelial activation expression is induced (Figure 1.2-2). ICAM-1 binds β2 integrins: αLβ2 also known as leukocyte function associated antigen-1 (LFA-1, CD11a/CD18) via Ig domains 1 and 2, and αMβ2 also known as Mac-1 (CD11b/CD18) via Ig domain 3. ICAM-2 (CD102), consisting of only Ig domains 1 and 2, mediates constitutive transendothelial leukocyte traffic and is not augmented by endothelial activation. Vascular CAM-1 (CD106) is absent on resting endothelial cells, but is induced by endothelial activation. VCAM-1 (CD106) is absent on resting endothelial cells, but is induced by endothelial activation. VCAM-1 is predominantly found in a 7 Ig domain splice variant form in human endothelium [51] and binds mainly to very late antigen-4 (VLA-4, CD49d/CD29, α4β1) via Ig domains 1 and 4 [52] (Figure 1.2-2). Platelet endothelial CAM-1 (PECAM-1, CD31), expressed constitutively at endothelial intercellular junctions, can bind both homophilically, participating in vascular permeability [53], or heterophilically to αvβ3 mediating monocyte transmigration. Transmigration is dependent on a chemotactic gradient and the activation of PECAM-1. PECAM-1 also has a striking ability to activate integrin adhesiveness. The latter stages of monocyte extravasation have been recently reviewed [54].

After endothelial penetration, monocytes must be retained in the intima to exert their pathological role in atherogenesis. In addition to their interaction with extracellular matrix components, monocytes may adhere to activated intimal smooth muscle cells expressing VCAM-1 [55] which may encourage a more permanent residence in the plaque [56].
Figure 1.2-2 Structures of E-selectin, ICAM-1 and VCAM-1.

Figure adapted from Imhof and Dunon [42] and Chia [40]. E-selectin, a 95-115 kDa glycoprotein, is a member of the selectin family of CAMs characterized by a common mosaic structure consisting of an amino-terminal C-type lectin (sugar-binding) domain, a single epidermal growth factor (EGF)-like domain, several short consensus repeats similar to those found in regulatory proteins that bind complement, a transmembrane domain and a short carboxy-terminal cytoplasmic domain. E-selectin binds to carbohydrate ligands (sLex, sLea and PSGL-1) via the lectin domain. ICAM-1 and VCAM-1 are members of the IgSF characterized by repeated domains, similar to those found in immunoglobulins (Ig), in the extracellular, amino-terminal portion of the molecule. Each 'Ig-like domain' is usually encoded by a discrete exon and consists of a primary sequence of 70-110 amino acids residues arranged in a β-barrel stabilized with a disulphide bridge to form an 'Ig fold'. The extracellular portions are glycosylated (not shown). IgSF CAMs have a transmembrane region and a short cytoplasmic tail. ICAM-1, a 90-115 kDa glycoprotein has five Ig domains with domains 1 and 2 able to bind ligand (LFA-1 and Mac-1). VCAM-1, a 90-110 kDa glycoprotein, is found in humans with 7 Ig domains as the major form, of which domains 1-3 are homologous to domains 4-6 (indicating an intergenic duplication event in the evolutionary history of the gene). Domains 1 and 4 bind ligand (VLA-4). Alternatively spliced variants of the same VCAM-1 gene have been described (not shown in figure [51]). In humans there is a biologically active minor variant lacking the 4th domain, whilst in rabbits both 7 and 8 domain forms exist [51]. In mice there is an additional and unique truncated form with only 3 Ig domains, which is bound to the cell membrane by a phosphatidylinositol linkage at its carboxy-terminus, but still binds VLA-4 [57].
1.2.5 **Endothelial CAMs Are Upregulated in Atherosclerotic Plaques.**

Levels of certain endothelial CAMs, in particular VCAM-1, ICAM-1, E-selectin and P-selectin, are elevated in human atherosclerotic tissue [58-62]. These molecules, especially VCAM-1, are also prevalent in the neovasculature (small vessels arising from the adventitia vasa vasorum), suggesting that monocytes may also be recruited to sites located deep within the atherosclerotic plaque via neovessels [63]. VCAM-1 is also expressed by human plaque smooth muscle cells and macrophages [59]. Endothelial CAM upregulation occurs in lesion prone areas of the arterial tree, where flow is slow or disturbed [64], both in humans [65] and in animal models of atherosclerosis [25,26,33,66]. This upregulation is an indicator of inflammation in early atherosclerosis [23-25,25-28] that persists as the disease progresses [27,58-62].

1.2.6 **The Importance of VCAM-1 in Atherosclerosis.**

Animal models of atherosclerosis have highlighted the importance of VCAM-1 as a requisite for monocyte adhesion and recruitment [23-25,25-28,67]. In addition, the expression of VCAM-1 by normal arterial endothelium may represent a phenotypic marker of predisposition to atherogenesis [66]. Different subsets of integrins are expressed by different populations of leukocytes allowing them to bind selectively to the vascular endothelium. The absence of VLA-4, a ligand for VCAM-1, on neutrophils may partly explain their exclusion from the atherosclerotic lesion, whilst the accumulation of only monocytes and T-lymphocytes [30] is also suggestive of VCAM-1 playing an important role in selective leukocyte recruitment.

Furthermore, the predominance of monocytes in atherosclerotic lesions may be because VCAM-1 is more effective at mediating the attachment of monocytes than lymphocytes [68]. Although CAMs are sometimes categorized as mediating only one kind of adhesive interaction, VCAM-1 can mediate monocyte rolling, firm adhesion and transmigration in cell culture studies under flow conditions [68], increasing VCAM-1's potential as a key molecule in the recruitment of monocytes.

Expression of VCAM-1 is modulated by many pro- and anti-atherogenic stimuli (Section 1.2.9). In particular, the regulation of VCAM-1 is coupled to oxidative stress, another early feature in the pathogenesis of atherosclerosis, by a reduction-oxidation (redox)-sensitive transcriptional regulatory mechanism [69] (Section 1.2.8).
1.2.7 SOLUBLE CAMs AS IN VIVO MARKERS OF ENDOTHELIAL DYSFUNCTION, INFLAMMATION AND ATHEROSCLEROSIS.

Although cellular expression of endothelial CAMs in vivo is difficult to assess clinically, the presence of soluble CAMs (sCAMs) in the circulation are markers of CAM expression [70]. Although the origins, metabolism and functional significance of sCAMs are not fully understood, their levels are known to be elevated in numerous pathological conditions, including atherosclerosis [71]. Soluble VCAM-1 (95-110 kDa) lacks the membrane-spanning and cytoplasmic domains and is generated by proteolytic cleavage of cell surface VCAM-1 at a site close to the point of membrane insertion, by a poorly understood 'shedding' process involving a metalloprotease [72]. The discovery of soluble forms of E-selectin, ICAM-1 and VCAM-1 present in the supernatants of cytokine-activated cultured endothelial cells [73] has facilitated in vitro research in this field. It is thought that shedding is not just a regulatory mechanism for reducing the amount of VCAM-1 at the cell surface; the shed portion of VCAM-1 is functional and, under flow conditions in vitro, negatively regulates monocyte adhesion to endothelial cells by competitively inhibiting monocyte integrin binding [74].

Interestingly, the circulating levels of sVCAM-1 are a good in vivo surrogate marker of the presence of endothelial activation/damage in patients with atherosclerosis [70]. Indeed, its concentration correlates well with VCAM-1 expression in the human atherosclerotic lesion [75]. Importantly, sVCAM-1 appears a better predictor of the extent and severity of the disease, than other sCAMs or plasma markers, with its concentration correlating strongly with intima thickness [70]. Therefore, sVCAM-1 maybe a dynamic surrogate marker for both risk assessment and for monitoring the effectiveness of therapeutic interventions.

1.2.8 TRANSCRIPTIONAL REGULATION OF ENDOTHELIAL CAM EXPRESSION.

Although E-selectin, ICAM-1 and VCAM-1 are structurally, and functionally, distinct, they do share a coordinated transcriptional response to pro-inflammatory cytokines. Cytokines are small secreted protein mediators that function as a communication system between cells involved in the inflammatory reaction. They act through high affinity receptors [76]. TNF-α and IL-1β, both 17 kDa pro-inflammatory cytokines that contribute to the pathogenesis of atherosclerosis [77,78], are found in human atherosclerotic plaques and are secreted locally by lesion macrophages, smooth muscle cells and endothelial cells [79,80]. These potent, structurally unrelated cytokines have distinct cell surface receptors but are capable of triggering biologically similar effects through the convergence of intracellular signalling pathways which cause the activation of the same set of transcription factors.
In cultured endothelial cells, E-selectin, ICAM-1 and VCAM-1 are all upregulated concurrently by TNF-α, IL-1β or cytokine-like stimuli, such as lipopolysaccharide (LPS) [81-84]. The endothelial NF-κB/1-κB system (nuclear factor-kappa B/inhibitor of kappa B system) plays a pivotal role in regulating cytokine-induced endothelial CAM expression [85].

1.2.8.1 Cytokine-Activation of NF-κB.

The signalling cascades which link the numerous cell surface signals with activation of NF-κB are still incompletely understood, however, NF-κB activation by TNF-α binding to its receptor (TNFR-1) is partly characterized (reviewed in [86]) (Figure 1.2-3). NF-κB consists of dimeric complexes of members of the Rel protein family of which the heterodimer p50/p65 is the best characterized. In resting cells, NF-κB is sequestered in the cytoplasm by the binding of an inhibitor termed I-κB to the p65 subunit of NF-κB. Upon activation of the cell by cytokines (Figure 1.2-3), NIK (NF-κB inducing kinase) phosphorylates the I-κB kinase complex which in turn phosphorylates I-κB to signal its ubiquitination and degradation. Dissociation of I-κB causes a conformational change in NF-κB which unmask both a nuclear localization signal (allowing nuclear translocation) and a CREB-binding protein (CBP) interacting domain on p65, which binds to a specific promotional DNA sequence and thereby influences the transcription of NF-κB-dependent genes.

Diverse stimuli activate NF-κB through distinct signalling pathways although the underlying mechanisms are unclear. LPS, a surface component of Gram-negative bacteria released following host infection, mediates pro-inflammatory gene expression (including E-selectin, ICAM-1 and VCAM-1 induction) by activating NF-κB [87], via a process thought to involve the synthesis of reactive oxygen intermediates (ROI) [88] (Figure 1.2-3).
Figure 1.2-3 Proposed intracellular signalling leading to NF-κB activation.

Figure adapted from Thurberg and Collins [86]. Ligand-induced trimerization of TNFR-1 leads to the association of the receptor with a series of adaptor proteins/transducers including TRADD (TNFR-1 associated death domain protein) and RIP (receptor interacting protein) which then interact with TRAF2 (TNFR-associated factor 2). TRAF2 serves as a docking protein for NIK (NF-κB inducing kinase) which stimulates NF-κB activation. Interestingly, TRAF6, which binds the IL-1 receptor (IL-1R), also interacts with NIK to stimulate NF-κB activity, suggesting that the two different cytokine signalling pathways converge at this point enabling them to elicit similar transcriptional activation [86].
1.2.8.2 NF-κB/IκB Dysfunction in Atherosclerosis.

NF-κB and IκB act in an autoregulatory mechanism. After removal of the stimulus, the IκB pool is replenished because NF-κB mediates activation of the IκB gene. IκB translocates to the nucleus and displaces the transactivating form of NF-κB before the inactive NF-κB/IκB complex is transported back to the cytoplasm, diminishing gene expression and returning the activated cell to the quiescent state [86]. In atherosclerosis, dysfunction of this control mechanism may contribute to prolonged NF-κB activation and thus contribute to the changes in gene expression during the disease. Indeed, activated NF-κB can be detected in endothelial cells of human atherosclerotic lesions [89]. There is a remarkable correlation between pro-atherogenic agents, such as inflammatory cytokines and oxidative stress, and NF-κB activation [86]. The dramatic changes in endothelial function early in atherogenesis may reflect the expression of many genes regulated at the transcriptional level by NF-κB. As well as CAM expression, endothelial NF-κB-dependent gene expression of secreted inflammatory cytokines [90], MCP-1 [91] and M-CSF (macrophage-colony stimulating factor), which drives monocyte differentiation and regulates foam cell formation [92], also contribute to the pro-inflammatory process in atherogenesis.

1.2.8.3 NF-κB as a Redox Monitor for Oxidative Stress in the Vessel Wall.

Oxidative stress, produced during inflammatory processes in response to various types of pro-atherogenic agents and characterized by an excess of oxygen free radicals or reactive lipid species, correlates with activation of NF-κB [93], whilst antioxidants suppress NF-κB. ROI in the cell activate redox-sensitive transcription via NF-κB [88], however, at the present time this pathway is undefined (Figure 1.2-3).

1.2.8.4 Other Transcription Factors Implicated in CAM Induction.

Although NF-κB is an important transcription factor, other transcription factors must co-ordinate with NF-κB to generate unique transcriptional activating complexes for endothelial CAM expression. For example, the VCAM-1 promoter has two closely linked NF-κB binding sites (with both sites necessary for activation of the VCAM-1 gene), as well as consensus binding sites for the GATA family of zinc finger transcription factors and an AP-1 (activating protein-1 or c-Fos/c-Jun) site [51]. AP-1 may modulate NF-κB’s regulation of VCAM-1 expression by TNF-α in endothelial cells [94]. Variation in the number of NF-κB molecules and interaction with other transcription factors, may provide the basis for
differential regulation of the different CAMs. Furthermore, the role of NF-κB in E-selectin and ICAM-1 gene expression is currently under debate [69,95,96].

1.2.9 ENDOTHELIAL CAM-MODULATORY AGENTS RELEVANT TO ATHEROSCLEROSIS.

Numerous endogenous mediators that either induce or augment cytokine-mediated effects, or downregulate endothelial CAM expression in culture, have been discovered in recent years. The following CAM-modulatory agents are relevant to atherosclerosis research, some acting via the NF-κB/I-κB system and others by, as yet, unknown mechanisms. Their effects are summarized, however, it should be emphasized that there are discrepancies between some reports that may reflect the organ-specific functions of the endothelial cells and be dependent on the vascular bed from which the cells were isolated [97]. For example, there are differences in CAM expression in cultured arterial and venous endothelial cells exposed to cytokines or LPS [98], which may explain differences in response to extracellular oxidant signals [99].

1.2.9.1 Oxidative Stress, Antioxidants and Nitric Oxide (NO).

The most striking evidence for the atherogenic role of oxidative stress and lipoprotein oxidation in early atherogenesis has been provided by the observation that powerful lipophilic antioxidants, such as probucol, significantly reduce progression of atherosclerosis, in part by the downregulation of VCAM-1 gene expression in vivo [27]. Oxidative stress is an important intracellular regulatory signal that activates NF-κB and mediates the selective expression of VCAM-1 [69], whilst antioxidants suppress NF-κB activation and induction of VCAM-1 in cultured endothelial cells exposed to oxidizing species [100] by preventing I-κB phosphorylation [101]. Advanced glycated endproducts (AGE), irreversibly glycated proteins found in diabetic patients, can induce oxidant stress and endothelial dysfunction by binding to endothelial AGE receptors (RAGE) [102]. The subsequent activation of NF-κB and upregulation of VCAM-1 may contribute to the accelerated vascular disease observed with diabetes [103].

NO, a potent anti-atherogenic molecule (Section 1.4.4), downregulates NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase activity [104], modifies the reactivity of oxygen intermediates in the vascular endothelium [105] and reduces oxidative stress [106]. NO can also directly inhibit NF-κB activation to downregulate cytokine-induced VCAM-1 [105,107-109], primarily through the induction of I-κB expression [109,110].
Estrogen is atheroprotective, in part by its direct effects on the vascular wall and its ability to upregulate endothelial NO production [111]. 17β-estradiol downregulates endothelial VCAM-1 [112,113], ICAM-1 and E-selectin expression [113]. However, NO-independent mechanisms may contribute to the potent CAM-modulatory effect of estradiol; it could act as an antioxidant to inhibit NF-κB, or estradiol-receptor complexes could interact with components of the NF-κB pathway to negatively affect transcription [113].

Fluid flow is a potent stimulus for the release of endothelial NO that serves to downregulate endothelial VCAM-1 (and monocyte binding) by NF-κB suppression [114], presumably explaining atheroprotection at sites of high shear stress. This is in contrast to the predisposition to increased CAM expression [26,33,64,66,115] and lesion formation in areas of the arterial tree exposed to low shear stress or disturbed blood flow [64,65].

1.2.9.2 LDL

LDL is a spherical particle consisting of a core of cholesteryl ester and triglyceride surrounded by a monolayer of phospholipid and cholesterol, with one 513 kDa integral apoB-100 molecule as the only apolipoprotein. It has a role in the delivery of dietary cholesterol to extrahepatic organs [116]. Elevated plasma levels of LDL constitute a major risk factor for atherosclerotic disease, although the mechanisms whereby it exerts its atherogenic effects remain poorly understood [6]. LDL upregulates both VCAM-1 and E-selectin expression in cultured human arterial endothelial cells [117]. A similar effect is observed with lipoprotein(a) [Lp(a)] [118], a potent atherogenic particle resembling LDL but with an additional apolipoprotein, apo(a), which has similarity to plasminogen and is covalently bound to apoB [119].

Modifications to LDL, in particular the oxidation of LDL trapped in the subendothelial space by vessel wall cells, make it more atherogenic than native LDL [3]. Oxidized LDL (oxLDL) may modify all stages of monocyte recruitment [120], presumably by increasing endothelial CAM expression and acting as a chemoattractant for monocytes. OxLDL can influence gene expression by causing oxidative stress and activating NF-κB in endothelial cells [121] (Figure 1.2-3). In cultured endothelial cells, highly-oxidized copper-treated LDL (oxLDL) induces P-selectin [122] and augments TNF-α-induced VCAM-1 expression [99]. However, exposing endothelial cells to minimally-modified LDL (MM-LDL) upregulates monocyte adhesion but not by inducing VCAM-1, ICAM-1 or E-selectin, indicating that MMLDL and cytokines activate distinct signal transduction pathways [123]. Furthermore, enzymatically-modified LDL (E-LDL), prepared by treating LDL with
lysosomal enzymes to resemble particles found in early human lesions, potently stimulates monocyte adhesion and transmigration in vitro by inducing ICAM-1 and P-selectin [124].

1.2.9.3 HDL

HDL particles are the smallest and densest of the plasma lipoproteins. They represent a structurally and functionally heterogeneous class of lipoproteins, most of which are lipid-rich, exhibit electrophoretic α-mobility when subjected to agarose gel electrophoresis and contain apoAI as the predominant protein constituent [125] (Figure 1.2-4). Total HDL in human plasma can be subfractionated into distinct populations on the basis of density by ultracentrifugation (to give a less dense subfraction designated HDL₂ and a more dense HDL₃ subfraction), particle size by non-denaturing gel electrophoresis, and apolipoprotein composition [126]. Quantitively minor HDL subgroups are lipid-poor or even lipid-free, exhibit preβ- or γ-mobilities (due to variations in surface charge) and contain apoAI, apoAIV or apoE as the only protein constituents [125,127,128].

As mentioned previously, epidemiological studies have established that plasma HDL-cholesterol concentrations are inversely related to the development of CHD [7], however, from human studies it is unclear whether HDL is directly protective or whether this relationship simply reflects some other factor which is the true cause. Recently, studies in transgenic mice overexpressing human apoAI, the apolipoprotein present in most subfractions of HDL, have confirmed the anti-atherogenic role of HDL; the animals have increased HDL-cholesterol and resist atherosclerosis induced by diet [129], by apoE-deficiency [130,131] or by the human Lp(a) transgene [132]. A direct protective role of HDL was also shown in cholesterol-fed rabbits when infusions of HDL regressed atherosclerotic lesions [133]. This protective effect of HDL may be associated with several functions, including a central role in RCT whereby excess cholesterol from peripheral tissues is transferred to HDL for subsequent delivery to the liver for catabolism [125]. HDL also has anti-oxidant [135,136] and anti-thrombotic properties [137,138]. Both mature HDL and lipid-free/lipid-poor apoAI particles as preβ-HDL can sequester cholesterol from peripheral cells, with esterification by LCAT (lecithin-cholesterol acyltransferase) reducing the possibility of cholesterol molecules diffusing back to the cell, thus promoting the RCT pathway [139]. Plasma-derived apoAI can enter atherosclerotic lesions [140,141] and participates with macrophage-derived apoE to reduce the cholesterol content of lipid-rich lesions [142]. Furthermore, HDL increases apoE secretion by cholesterol-loaded macrophages in vitro, and this increased metabolic pool of apoE may lead to more effective RCT [143].
Figure 1.2-4 Generalised structure of HDL.

Like other lipoproteins, HDL particles consist of a hydrophobic core (mainly cholesteryl esters plus a small amount of triglyceride) surrounded by a surface monolayer of phospholipids, unesterified cholesterol and apolipoproteins. ApoA-I is the principal protein of HDL and, as such, is a major anti-atherogenic protein; other apolipoprotein constituents include apoAII, apoCI and apoCII.

HDL and Modulation of Endothelial CAM Expression.

HDL has diverse anti-atherogenic effects on various cell types including endothelial cells [9]. In cultured endothelial cells, HDL stimulates synthesis of prostacyclin [144,145], a potent inhibitor of platelet and leukocyte functions. HDL also attenuates the upregulation of endothelial CAM expression in vitro [83,146-148], an effect that is mediated at the transcriptional level [83] but may not involve inhibition of the NF-κB pathway [144]. The CAM-modulatory effect of HDL has been largely attributed to its apoAII and apoAIII content [83].

1.2.9.4 Interactions with Other Cell Types.

Co-culturing monocytes with endothelial cells leads to cell-cell cross talk that directly stimulates endothelial CAM expression [149-151] and also enhances endothelial response to exogenous cytokines [152] or LPS [153]. Platelets may directly initiate an inflammatory response in the vessel wall and contribute to endothelial adheresiveness in atherosclerosis. When activated platelets are co-cultured with endothelial cells, ICAM-1 is induced via an NF-κB-dependent mechanism [154]. Interestingly, CD40 ligand (CD40L), a transmembrane protein structurally related to the cytokine TNF-α, is expressed on activated platelets and induces endothelial cells to secrete chemokines and upregulate VCAM-1, ICAM-1 and E-selectin expression via interaction with endothelial CD40 [155].
1.2.10 **OTHER FUNCTIONAL ROLES FOR VCAM-1 AND sVCAM-1.**

It is premature to conclude that VCAM-1 plays an exclusive role as an adhesion molecule in inflammation. Other functions include VCAM-1/VLA-4-induced T cell activation and proliferation [156-158]. In addition, VCAM-1 has several roles unrelated to atherosclerosis: in cardiovascular and placental development (VCAM-1-deficient mice are not viable and embryos fail to develop normal placentas and hearts) [159]; in inflammatory diseases of the nervous system (VCAM-1 is induced in activated neurons and cerebral endothelial cells) [160,161]; in TNF-α-enhanced metastasis [162]; and in receptor signalling [163]. Soluble VCAM-1 may also act as an angiogenic factor for new vessel formation; sVCAM-1 is chemotactic, but not mitogenic, for endothelial cells [115]. Thus, it is evident that regulation of VCAM-1 expression is a fundamental process in a wide array of disease pathologies.

1.3 **Apolipoprotein E.**

Plasma apolipoproteins serve to regulate lipoprotein metabolism and to control the transport and redistribution of lipids among tissues and cells. Apolipoproteins achieve this by performing at least one of three major roles (reviewed in [164,165]). Firstly, because of their ability to bind lipid, apolipoproteins stabilize the structure of hydophobic lipoprotein particles in an aqueous environment. Secondly, apolipoproteins can act as cofactors or activators of various enzymes or lipid transfer proteins that participate in the metabolism or 'remodelling' of lipoproteins as they circulate in plasma. Finally, some specific apolipoproteins serve as ligands for cell surface lipoprotein receptors and can direct, therefore, the delivery and redistribution of lipids to cells.

Of the 14 plasma apolipoproteins that have been described, apoE is one of the best characterized in terms of its structural and functional properties (reviewed in [166-168]). ApoE was initially characterized in several animal species after it was realised that dietary cholesterol altered its distribution in plasma; apoE becomes a major protein constituent of several cholesterol-enriched lipoproteins that accumulate in the plasma of animals fed high levels of fat and cholesterol [168]. ApoE is a 34.2 kDa protein in the surface of very low density lipoprotein (VLDL) and chylomicrons, or their remnants (referred to collectively as β-VLDL), and certain minor subfractions of HDL particles (HDL-E and γ-LpE). The normal human plasma concentration of apoE is 3-7 mg/dl [167,168] and its major physiological role is to mediate hepatic clearance of lipoproteins through two receptors, the
LDL receptor (LDL-R) and the LDL-R-related protein-1 (LRP1). In addition, HDL-E and γ-LpE participate in RCT by sequestering cellular cholesterol [127,169,170]. ApoE is protective against atherosclerosis, in part due to its function in lipid transport and metabolism, and also due to local protective effects within lesions restricting development of atherosclerosis (discussed in Section 1.4).

1.3.1 **Gene Regulation and Biosynthesis of Human ApoE.**

Human apoE consists of a single 299 amino acid polypeptide chain. The primary structure was first determined by direct amino acid sequencing of the protein purified from human VLDL [171] and later confirmed by nucleic acid sequencing of a full-length cDNA [172]. ApoE is encoded by the 3.7 kilobase \( APOE \) gene located on chromosome 19, which contains four exons and three introns [173,174]. The \( APOE \) promoter sequence TATAATT occurs approximately 30 base pairs (bp) upstream from the transcriptional initiation site. Other promoter and enhancer elements important in regulating apoE biosynthesis have also been identified [175]. ApoE mRNA is 1,163 bp in length and encodes a precursor protein containing an 18 amino acid signal peptide that is removed co-translationally during the translocation of the protein through the endoplasmic reticulum [172]. In humans, apoE is secreted as an O-glycosylated protein due to a single glycosylation site at Thr^ç^ [176]. In plasma, 90% of apoE is desialylated, although the relevance of this phenomenon for its function and metabolism is not understood at present [166]. Sialic acid variations of apoE often appear as multiple bands on polyacrylamide gels.

1.3.2 **Sites of Synthesis.**

Human apoE has a wide tissue distribution with a role related generally to inter- or intra-organ cholesterol transport. ApoE is produced in most organs and significant quantities of apoE mRNA are detected in the liver, brain, spleen, lung, adrenal, ovary, kidney and muscle in several different species [167,168]. The liver synthesizes >90% of plasma apoE. Hepatic parenchymal cells, which secrete apoE as a component of VLDL, are largely responsible for this apoE production [177]. However, it is possible that liver-synthesized apoE is released independently of VLDL, as discoidal particles that contain mainly phospholipids [127]. The second largest concentration of apoE mRNA is found in the brain (about one-third the amount in liver) [166,168]. In the brain, astrocytes are responsible for producing the majority of apoE [179,180], although glial cells and neurones have also been reported to produce apoE under certain conditions [180,181]. It is noteworthy that apoE is also a major apolipoprotein of cerebrospinal fluid (CSF) in humans [166,167,182].
ApoE is also synthesized by a wide variety of peripheral cells including macrophages, raising questions regarding the potential physiological roles of apoE made in peripheral tissues [168]. Macrophages are ubiquitous and a significant source of apoE at many tissue sites. Cholesterol-loaded macrophages secrete an abundance of apoE at atherosclerotic lesion sites [37,38]. Macrophage-derived apoE is secreted in combination with phospholipid [127] and has roles in vessel wall and cellular cholesterol homeostasis [183] (Section 1.4.2) as well as other potential anti-atherogenic roles at the lesion site (Sections 1.4.3 and 1.4.4).

1.3.3 ApoE Polymorphism.

The polymorphic nature of apoE was first established using isoelectric focusing (IEF)[184], and further clarified using two-dimensional electrophoresis [185]. The three major isoforms of apoE, referred to as apoE2, E3 and E4, are products of three alleles (ε2, ε3, ε4) at the APOE locus. Three homozygous phenotypes (apoE2/2, E3/3 and E4/4) and three heterozygous phenotypes (apoE3/2, E4/3 and E4/2) arise from expression of these alleles. The most common phenotype is apoE3/3 and the most common allele is ε3, therefore, apoE3 is considered the parent or 'wild-type' form of the protein, with apoE4 and E2 as variants [166-168]. Charge differences among the three isoforms detected by IEF are explained by single amino acid substitutions. ApoE2, the rarest mutant, differs from apoE3 by an Arg158Cys substitution and is associated with recessive forms of type III hyperlipoproteinaemia due to defective receptor binding [166,186]. ApoE4 (Cys112Arg) displays normal receptor binding but produces a dominant hyperlipidaemia, is a risk factor for restenosis [187] and is implicated in the pathogenesis of Alzheimer’s disease (for review, see [166,187,188]).

The three major isoforms differ from each other with respect to their association with lipoproteins, their binding affinity for the LDL-R, and their interaction with heparin [166-168,189-191]. ApoE3 displays a preference for HDL, as does apoE2, whereas apoE4 interacts with large lipoproteins such as VLDL [189]. Additionally, apoE3 and apoE4 bind equally well to the LDL-R, while apoE2 displays only approximately 1 % of their binding activity [168,190]. Besides a reduced affinity for the LDL-R, apoE2 also shows reduced binding to heparin sulphate proteoglycan (HSPG) [191], unlike apoE3 and apoE4 which bind HSPG with a high affinity. This heparin-binding property of apoE has been utilized for purifying apoE polypeptide or apoE-containing lipoproteins by affinity chromatography (Sections 3.2.5 and 3.2.7).
1.3.4 **Structure of ApoE.**

Many aspects of the structure-function relationships of apoE have recently been reviewed in some detail [167]. ApoE differs from other apolipoproteins in its tertiary structure. It is predicted to be highly helical and segregated into two independent structural domains separated by a large section whose structure is predicted to be random (Figure 1.3-1).

The amino-terminal domain includes both the receptor-binding and heparin-binding sites of apoE [167,168]. The recent determination of the crystalline structure showed its organisation into an anti-parallel four-helix bundle with the packing of hydrophobic side chains in the interior of the bundle contributing to stability of the tertiary structure [192]. The 4th helix has an area of positive charge due to basic lysine and arginine residues in the vicinity of amino acids 136-158, which is thought to mediate interaction between apoE and its receptors (for review, see [167,168,193]).

![Figure 1.3-1 The complete amino acid sequence of human apoE3.](image)

*Figure adapted from Rall et al [197]. Thr*<sub>394</sub> contains a variably sialylated carbohydrate moiety. Thrombin cleaves at the carboxyl terminal side of Arg<sub>271</sub> and Arg<sub>271</sub> [167]. The amino-terminal 22 kDa fragment (amino acids 1 to 191) is boxed in yellow, while the carboxyl-terminal 10 kDa fragment (amino acids 216 to 299) is boxed in blue. The LDL-R binding domain is highlighted in red. The basic amino acids within the LDL-R binding domain that are important for LDL-R binding are printed in blue bold. These two fragments have been used as models for the two structural domains of apoE.*
The carboxyl-terminal beyond amino acid residue 191 contains three predicted helices. In the absence of lipids, apoE self-associates as a tetramer over a wide concentration range [194,195], however, in contrast, self-association does not occur on lipid surfaces, implying that the self association and lipid binding moieties of apoE are structurally related. Residues 263-286 are critical for both lipid binding and tetramerization of apoE [196].

1.3.5 ApoE Receptors.

ApoE is recognized by a family of related receptors, the LDL receptor superfamily (LRSF) [193,198-200]. In mammals, the members of the LRSF include the LDL-R itself, the VLDL receptor (VLDL-R), the multifunctional LRP1 (α₂-macroglobulin receptor) and the newly characterized brain receptor LRP8 (previously known as apolipoprotein E receptor 2 or apoER2) (Figure 1.3-2).

The LRSF members are defined by common structural elements that show high degrees (50-100 %) of sequence identity, not only between each family member but also across a wide range of species [198-201]. Such sequence conservation is thought to have evolved by duplication and/or exon shuffling events from an ancestral gene [201]. The presence of extracellular LDL-R “class A” repeats, also known as LDL-R ligand binding repeats, is obligatory for membership of this gene family. Each class A repeat consists of approximately 40 amino acids, each containing six cysteine residues that are disulfide bonded in the pattern one to three, two to five and four to six. Reduction of these disulfide bridges destroys the structure and abolishes ligand binding [202,203]. Additionally, each of these repeats forms a complex with a single Ca²⁺ ion, which also stabilizes the ligand binding structure [203,204]. The class A repeats are arranged in head to tail fashion and are preceded and/or followed by epidermal growth factor-precursor repeats (EGF), each also with six cysteines. Other common elements are the “YWTD-repeats”, characterized by a length of approximately 50 residues containing a consensus tetrapeptide sequence F/YWXD. Typically, these are present in a group of five, flanked by EGF repeats. The LDL-R, VLDL-R and LRP8 contain a juxtamembrane O-linked sugar domain of approximately 60 amino acids that is enriched in clusters of serine and threonine [198]. There is a single membrane spanning stretch. The intracellular domain of all LRSF members identified so far contain one or more tyrosine containing hexapeptides (FxNPyY) that serve as an internalization signal to direct the receptors to clathrin-coated pits.

1.3.5.1 LDL-R.

The LDL-R was the first characterized member of this family (reviewed in [193]). It contains one cluster of seven class A repeats and one cluster of three EGF repeats, the latter
separated by a cysteine-poor spacer that contains five copies of the YWTD sequence (Figure 1.3-2). In particular, the LDL-R plays an important role in liver and steroidogenic tissues. The LDL-R binds plasma lipoproteins that contain apoB-100 or apoE, and it is responsible for the removal of most intermediate density lipoproteins (IDL) and LDL from plasma. As such, it plays an essential role in cholesterol homeostasis. Both IDL and LDL accumulate in plasma of patients with familial hypercholesterolaemia, who have mutations in the LDL-R gene.

1.3.5.2 LRP1.

The second member of the LDL-R gene family to be characterized was the LRP1, also designated the \( \alpha_2 \)-macroglobulin receptor (reviewed in [206]). This protein is much larger than the LDL-R (4525 versus 839 amino acids). It contains 31 class A repeats and 22 EGF repeats that are separated by eight spacer regions, each containing multiple YWTD repeats [207] (Figure 1.3-2). The juxtamembrane O-linked sugar domain is not present. The carboxyl-terminal cytoplasmic domain is 100 amino acids long, twice that of the LDL-R, and contains two copies of the FxNPxY internalization motif.

LRP1 is a multifunctional endocytotic receptor present on a variety of cell types and tissues; it is expressed predominantly in the liver where it has been proposed to act as a receptor for chylomicron remnants that become enriched with apoE during passage through hepatic sinusoids. Although it does not bind LDL, it can bind other ligands including lipoprotein lipase (LPL), an enzyme that is normally bound to the surface of endothelial cells, and \( \alpha_2 \)-macroglobulin-protease complexes.

1.3.5.3 VLDL-R.

The VLDL receptor (VLDL-R) is very similar in structure to the LDL-R (Figure 1.3-2), but contains eight rather than seven class A repeats in its ligand-binding domain and cannot bind LDL with high affinity (reviewed in [198]). Unlike the LDL-R, the VLDL-R is not expressed in the liver but predominantly in heart, adipose tissue and brain [208, 209], i.e. all tissues that metabolize fatty acids as an energy source. The ligand specificity of the VLDL-R is similar to LRP1. The proposed physiological role of VLDL-R in the delivery of triglyceride-rich lipoproteins to peripheral tissues is uncertain as mice lacking the VLDL-R show no abnormalities in their lipoprotein profile [210].
1.3.5.4 LRP8.

LRP8 is a newly described receptor consisting of five domains that resemble those of the LDL-R and the VLDL-R [198,200,211,212]. Although LRP8 and LDL-R contain the same number of class A repeat sequences, the ligand-binding domain structure of LRP8 is
much more closely related to that of VLDL-R; LRP8 and VLDL-R contain a short linker sequence between repeats 5 and 6, whereas that of LDL-R is located between repeats 4 and 5 (Figure 1.3-2). Due to high similarity with the VLDL-R, LRP8 is predicted to have a similar ligand specificity as this receptor, however, binding of β-VLDL to LRP8 does not result in its degradation [213]. Unlike the other LRSF members, LRP8 contains a unique 59 amino acid insert within its otherwise LDL-R-/VLDL-R-like cytoplasmic tail [211]. In human tissues, LRP8 mRNA is abundant in brain and placenta and undetectable in other tissues [211,212]. More recently, LRP8 has been identified as a signalling receptor, intimately involved both in neuronal development [205] and platelet function [214-217].

Recent studies have revealed that several splice variants of LRP8 are expressed in brain tissues, both at the transcript [212,218] and protein level [219]. These include variants of LRP8 lacking repeats 4-6 (LRP8Δ4-6) or 4-7 (LRP8Δ4-7) in the ligand binding domain, and LRP8 without the cytoplasmic insertion (Δinsert). The functional significance of alternative splicing and complete ligand specificity of LRP8 have yet to be determined.

1.3.5.5 Endothelial ApoE Receptor Expression.

The arterial wall is a special microenvironment that has specific characteristics distinct from the lipoprotein metabolism in tissues such as the liver [220,221]. Vascular endothelial cells, both in vivo and in culture, express some members of the LRSF involved in cellular lipid uptake. The LDL-R is not detected in endothelial cells of normal (or atherosclerotic) human arteries [222], possibly due to down-regulation of LDL-R by high LDL cholesterol concentration in the arterial extracellular fluid. However, the VLDL-R is expressed by vascular wall cells, including endothelial cells, and may be important for the binding and uptake of triglyceride-rich apoE-containing lipoproteins into the arterial wall [223]. Endothelial cells do not usually express LRPl [224] and although LRPl mRNA is present in normal human arterial endothelium, the cells are devoid of protein for the receptor [225]. In contrast to endothelium in vivo, endothelial cells isolated from human umbilical veins (HUVECs), the most popular model for the study of human vascular endothelium in vitro, express both LDL-R and VLDL-R, whereas only trace amounts of LRPl have been demonstrated [226-229]. However, the level of LDL-R (but not VLDL-R) expression is reduced in the presence of serum in the culture media [229]. The lack of a readily available antibody has hindered protein detection of LRP8 in tissues. However, preliminary reports identified LRP8 gene expression in rabbit arterial endothelium in response to vascular injury (although not apparent in controls [230]) and multiple LRP8 mRNA transcripts were detected in cultured HUVECs [231].
1.4 ApoE and Atherosclerosis.

It has been known for many years that defective expression of apoE (either null expression or expression of variant forms) was associated with an increased risk for atherosclerotic vascular disease [168,232,233]. Low apoE and HDL-E are important risk factors for CHD [10-12], and severe hyperlipidaemia and atherosclerosis ensues in humans or animals if apoE is dysfunctional or absent [168,234,235]. ApoE infusion into hyperlipidaemic rabbits reduces plasma cholesterol and regresses lesions [236], while transgenic mice overexpressing apoE rapidly clear VLDL/LDL-cholesterol and resist diet-induced hyperlipidaemia [237]. By contrast, expression of apoE3Leiden or apoE4Cysnaice, both natural mutants, produces a Type III hyperlipidaemia phenocopy [234,238], whereas apoE-deficient mice have marked hypercholesterolaemia and spontaneously develop atherosclerosis [239-241]. Furthermore, adenovirus-mediated replacement of the hepatic gene in apoE-deficient mice prevents atherosclerosis [242,243].

Until recently, the increased risk of CHD associated with defective apoE was largely ascribed to abnormalities in global lipoprotein transport and metabolism, highlighting the importance of apoE in facilitating these functions (Section 1.4.1). However, in addition to being synthesized by hepatocytes and intestinal cells, apoE is also synthesized by a wide variety of extrahepatic cells including macrophages. In particular, there is an emerging concept that macrophage-secreted apoE at the lesion site is protective against atherosclerosis (Section 1.4.2).

1.4.1 Global Lipid Transport.

One role for apoE in lipoprotein metabolism is cholesterol-delivery due to high-affinity binding of apoE-containing lipoproteins to the LDL-R. ApoE binding initiates the cellular uptake and degradation of lipoproteins, releasing cholesterol which ultimately regulates intracellular cholesterol metabolism. ApoE shares this delivery function with apoB-100, the protein constituent of plasma LDL.

ApoE also mediates the hepatic clearance of lipoproteins via the LDL-R and LRP1 (simplified in Figure 1.4-1). Chylomicrons are synthesized in the intestine and transport dietary triglycerides and cholesterol. During their circulation, the core triglycerides of chylomicrons are hydrolyzed by LPL, to produce cholesterol-enriched remnant particles. ApoE, acquired from HDL, becomes a significant protein constituent of these remnant particles which are then rapidly removed from plasma by two apoE-mediated mechanisms [167,168]. In vivo evidence suggests that LRP1 and HSPGs act together as the so-called 'remnant receptor' [199]. In addition, the LDL-R also appears to play a role in uptake [244].

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ApoE is a constituent of liver-synthesized, triglyceride-rich VLDL, which functions primarily to transport triglyceride from the liver to peripheral tissues. In a manner similar to that of chylomicrons, VLDL particles pass through a lipolytic cascade producing a spectrum of particles progressively decreasing in size [167,245] (Figure 1.4-1). These include VLDL remnants and IDL. Cholesterol-rich LDL represents the final stage of this process [245]. Although both VLDL and IDL contain apoE and apoB-100, these particles are cleared through apoE interactions with the LDL-R and LRP1, however, not all IDL is cleared by the liver [167]. In healthy humans, nearly all of the IDL is converted to LDL, a process that involves hepatic lipase (HL). During LDL maturation the apoE is lost from the surface, leaving apoB-100 as the sole apolipoprotein. The clearance of the LDL is then via apoB-100 through the LDL-R [245]. In addition to mediating hepatic clearance of lipoproteins, it has been suggested that apoE is involved directly in the lipolytic cascade by serving as a modulator of HL, LPL and LCAT [246-249].

**Figure 1.4-1 The role of apoE and its receptors in lipoprotein metabolism.**

### 1.4.2 MACROPHAGE-SECRETED APOE IS ANTI-ATHEROGENIC.

#### 1.4.2.1 Evidence from ‘ApoE Knockout Mouse’ Studies.

Although apoE can enter the artery wall from the periphery, the abundance of apoE found in atherosclerotic lesions is locally synthesized by resident cholesterol-loaded macrophages that represent an important hallmark of early atherogenesis [37,38]. ApoE, a
major secretory protein of macrophages, is secreted in association with phospholipid [127]. Macrophage apoE production is regulated transcriptionally by cholesterol, highlighting a potential role for apoE in arterial wall cholesterol homeostasis [250]. Macrophage apoE secretion is also stimulated by other factors found in the atherosclerotic vessel wall; physiologically-modified forms of LDL and proinflammatory cytokines [251,252].

Although mice are normally highly resistant to atherosclerosis, targeted disruption, deletion, or insertion of specific genes that regulate lipoprotein metabolism has resulted in the generation of a variety of murine models of hypercholesterolaemia and atherosclerosis [253,254]. In particular, studies with apoE-deficient (APOE\(^{-/-}\)) mice generated by 'knockout technology' [240,255] have helped to elucidate the role of macrophage secreted apoE in atherosclerosis. These mice, fed on low-fat/cholesterol diet, have delayed clearance of lipoproteins and, at lesion-prone sites, spontaneously develop the entire spectrum of lesions observed in humans. The exacerbation of atherosclerosis by high-fat/cholesterol (atherogenic) diets is also mimicked in these mice [33]. The susceptibility of apoE-deficient mice to atherosclerosis reflects not only their marked hyperlipidaemia, but also the inability of their arterial macrophages to make and secrete apoE [235].

Studies performed in apoE-deficient mice have provided the most convincing evidence for a prominent role of macrophage-derived apoE in tissue and cellular cholesterol homeostasis (reviewed in [183,256]). Bone marrow transplant from normal (APOE\(^{+/+}\)) mice to apoE-deficient (APOE\(^{-/-}\)) recipients, allows apoE production in the transplanted macrophages under the native promoter and hence physiologically relevant amounts of macrophage-derived apoE are produced [235,257]. As a result, these mice, with only \(\sim 10\%\) of normal circulating levels of apoE, have a normalized lipoprotein profile and are resistant to diet-induced atherosclerosis. Therefore, macrophage apoE is functionally active and sufficient to correct the metabolic defect in these mice, most probably by associating with circulating lipoproteins and inducing their hepatic clearance, although macrophage apoE may also have contributed to the protective effect at the vessel wall.

Other studies have demonstrated that local apoE production in the vessel wall can provide protection against atherosclerosis independently from its role in lipoprotein clearance and plasma lipid-lowering. Expression of human apoE in vascular endothelial and smooth muscle cells (which do not normally express apoE) in normal mice reduces atherosclerosis, without altering plasma lipid levels, by enhancing efflux of vessel wall cholesterol [237]. Macrophage-specific expression of apoE, under the control of a virus long terminal repeat in apoE-deficient mice, has a direct anti-atherogenic effect even in the presence of high levels of
atherogenic lipoproteins [258]. More recently, the use of retroviral gene therapy in apoE-deficient mice has shown that arterial macrophage-secreted apoE at levels as low as 0.5 - 1% of normal circulating levels can delay atherogenesis if expressed during foam cell formation, without effecting plasma cholesterol levels [259]. Conversely, when C57BL/6 mice which are susceptible to atherosclerosis are reconstituted with apoE-deficient macrophages and fed an atherogenic diet, they develop 10-fold more atherosclerosis with no significant change in lipoprotein profile [260]. Thus these investigations conclude that in addition to clearing atherogenic lipoproteins, local apoE production by macrophages in the arterial wall plays a special role in the prevention of atherosclerosis.

1.4.2.2 Macrophage-Secreted ApoE, Local Cholesterol Efflux and RCT.

The apoE released from macrophages is different from apoE isolated from circulating lipoproteins. It has a higher degree of sialylation [261] and is secreted in association phospholipid [127]. Both these factors affect the size and charge of the apoE-containing particles and influence their interaction with cellular and matrix components of the vessel wall [183]. These spherical lipoprotein particles contain apoE as their sole protein component and sphingomyelin as the major phospholipid [127]. Recent studies infer that locally-synthesized apoE particles have anti-atherogenic actions at lesion sites including cholesterol homeostasis of the surrounding cells of the vessel wall (see above).

As well as facilitating the hepatic clearance of lipoproteins, apoE also sequesters excess cellular cholesterol from the periphery. This local action of apoE is mediated by macrophage-secreted γ-LpE, which constitutes a significant part of the normal cholesterol efflux capacity of plasma [127,170,262]. Macrophage apoE may prevent foam cell formation by stimulating efflux of excess free cholesterol from lesion macrophages [127,170,263,264] and facilitate RCT of excess vessel wall cholesterol [237,262] perhaps in concert with HDL [265]. Although endogenous macrophage apoE is able to efflux cellular cholesterol in the absence [264] or presence of added cholesterol acceptors such as HDL₃ [266], macrophage apoE may also be transferred onto HDL to promote cholesterol efflux from the vessel [267]. HDL devoid of apoE is less able to efflux macrophage cholesterol as compared with HDL-E [265]. Interestingly, endogenous apoE expression also enhances HDL₃ binding to macrophages, and although this may not contribute to cholesterol efflux [268] this interaction may have other functions.
1.4.3 APOE HAS PROTECTIVE ROLES UNRELATED TO LIPID METABOLISM.

In addition to regulating cholesterol metabolism in the arterial wall, macrophage-derived apoE may impact vessel wall biology in other protective ways [183]. ApoE is an allele-specific antioxidant [269] that may protect the vessel wall from oxidative cytotoxicity and cellular dysfunction in vivo. Human atherosclerotic lesions contain regional accumulations of T lymphocytes [30], which may participate in the disease process [39], however, apoE can alter lymphocyte function by potently inhibiting their activation and proliferation, and by suppressing their production of cytokines [270-272].

ApoE produced by macrophages is bound by the subendothelial matrix of the vessel wall [38] and such interactions could influence the ability of lipoproteins to interact with cell surface receptors, the ability of LPL in the matrix to promote retention of atherogenic lipoproteins [273], the bioavailability of cytokines and growth factors retained in the matrix, and the regulation of arterial smooth muscle cell growth by matrix components [274]. In addition, apoE also stimulates endothelial production of heparan sulfate (HS), a major class of proteoglycan in the extracellular matrix, which has anti-atherogenic activity by inhibiting smooth muscle cell proliferation and also preventing monocytes from associating with the matrix [275].

ApoE inhibits platelet aggregation [276]. Activated platelets may directly initiate an inflammatory response in the vessel wall and contribute to endothelial adhesiveness in atherosclerosis [154,155], and therefore apoE-mediated anti-platelet action provides another anti-atherogenic function in the vicinity of the vascular wall [216,276]. ApoE may play a role in maintaining the normal mitogenic state of the vessel wall by inhibiting both endothelial [277] and smooth muscle cell proliferation [278], processes that are implicated in atherogenesis [1,21]. Interestingly, the ability of apoE to inhibit platelet aggregation and smooth muscle cell generation is due to apoE-induced release of anti-atherogenic NO by these cell types (see below).

1.4.4 APOE AND REGULATION OF NITRIC OXIDE (NO) PRODUCTION.

1.4.4.1 NO and NO Synthase (NOS) Isoforms.

As a free radical gas, NO is a uniquely diffusible and reactive molecular messenger with diverse biological actions throughout the body, including the vascular, immune and nervous systems (reviewed extensively in [279-283]). Formation of NO from arginine, catalyzed by NOS, is by the five-electron oxidation of the terminal guanidinium nitrogen of
the amino acid, L-arginine, yielding L-citrulline as the coproduct. The mechanism is a complex two-stage stereo-specific reaction, involving molecular oxygen and NADPH as cosubstrates, with numerous other redox cofactors.

Early studies indicated that based on several criteria including cellular location, regulation of activity and substrate/inhibitor profiles, there were three distinct NOS isoforms, as seen in Figure 1.4-2 (reviewed in [279,281,286]). A constitutive form, whose activity is regulated by Ca^{2+} and calmodulin (Ca-CaM), is found in vascular endothelium (termed eNOS or NOS-III) where the continuous generation of NO is involved in the regulation of blood pressure and blood flow [281]. Another Ca-CaM-requiring constitutive enzyme is present in neurones and skeletal muscle (nNOS or NOS-I). The NO produced in the neurones of the brain can link blood flow to neuronal activity, modulate neurotransmitter release, and influence brain development and function [283]. There is a Ca^{2+}-independent isoform (iNOS or NOS-II) found in macrophages, vascular smooth muscle cells and hepatocytes following induction by specific cytokines. Macrophage iNOS expression requires immunological or inflammatory stimuli, implicating NO release as an important means of controlling infection [287,288]. However, (and reflecting the seemingly ubiquitous role of NO in cell biology) it is now recognized that distribution of the three isoforms overlap in many tissues and cell types, their subcellular location is variable, the same isoform in different cells may evoke different biological effects, and that eNOS and nNOS are also inducible [285].

Each of the NOS isoforms requires CaM for activity. However, the constitutive isoforms are dependent on Ca^{2+}, which interacts with CaM to facilitate binding of Ca-CaM to NOS [281], whereas the inducible isoform has CaM permanently bound, and hence its activity is independent of Ca^{2+} levels [280,285]. Unlike the other isoforms, eNOS undergoes cotranslational N-myristoylation at its amino-terminal (Gly^5) and hence is predominantly membrane-associated [281] (Figure 1.4-2). It is also targeted to caveolae(-like) domains in the cell surface by post-translational cysteine palmitoylation (Cys^{35} and Cys^{36}) [289] and this reversible process, along with phosphorylation and dephosphorylation at multiple sites [290], may be an important means of regulating NOS activity and NO release [281].
Figure 1.4-2 The primary structure of the NOS isoenzymes.

Figure adapted from Riddell and Owen [216]. Molecular cloning of the three NOS isoforms established that the human eNOS, nNOS and iNOS isoenzymes have 1203, 1433 and 1153 amino acids (133, 161 and 131 kDa), respectively. Each NOS isoform has the same layout of catalytic domains: a carboxyl-terminal 'reductase domain' with one consensus binding site for each of the co-factors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH, and an amino-terminal 'oxygenase domain' that functions as a heme-, tetrahydrobiopterin- and substrate-binding site. Spanning these two regions is the CaM-binding site, an important regulatory unit permitting the transfer of electrons, which are donated by NADPH, via the flavins, to the heme catalytic site. nNOS contains an N-terminal PDZ domain to target NOS activity to postsynaptic sites. eNOS is targeted to the cell membrane by co-translational acylation (myristoylation). Normally, NOS exists as a homodimer (not shown) and only these dimeric forms exhibit catalytic activity [279].
1.4.4.2 Anti-Atherogenic Actions of Endothelial-Derived NO.

Continuous generation of NO by the endothelium is crucial for the regulation of blood pressure and blood flow, and for normal vessel wall homoestasis [281]. In vitro, NO can inhibit many of the key processes involved in the early pathogenesis of vascular injury and atherosclerosis [291] including the oxidation of LDL [292]. In the endothelium, NO can suppress the expression of many pathophysiologically relevant effector molecules including endothelial CAMs [90]. It plays an important role in prevention against early endothelial dysfunction [293] and stimulates the process of endothelial wound repair [294]. NO also helps to maintain the appropriate level of permeability in the endothelial layer [295,296].

Endothelial-derived NO is a potent countervailing force in the vessel wall that opposes atherogenesis, not only by regulating endothelial function, but also by its ability to diffuse across cell membranes [297] away from its site of synthesis, and affect other cell types in a paracrine fashion. Endothelial-derived NO can diffuse to the subjacent vascular smooth muscle cells, where it regulates vascular tone [298] and modulates their expression of secretory factors, such as MCP-1 [299]. It also can diffuse to the luminal surface of the endothelium, where it exerts a number of important physiological effects including the inhibition of platelet adherence and aggregation [300], the scavenging of superoxide radicals [301], and inhibition of leukocyte function by modulating monocyte adhesion to endothelium [302]. The last is dependent, in part, on the downregulation of monocyte adherence glycoprotein CD11/CD18 [303].

1.4.4.3 ApoE Stimulates NO Release in Cells of the Vasculature.

Recent evidence suggests that apoE may protect against atherosclerosis, in part, by upregulating eNOS activity. ApoE, via an apoE receptor-dependent mechanism, induces activation of eNOS in platelets with the production of NO inhibiting platelet aggregation [215,215,216,216,217,217,276]. In macrophages [304,305] and vascular smooth muscle cells [278,306,307], apoE causes activation of iNOS. Although not clear whether the effects of iNOS are pro- or anti-atherogenic, it is evident that apoE-mediated NO release in smooth muscle cells is associated with apoE's cytostatic functions of inhibiting both cell proliferation and migration, which may protect against atherosclerosis [278]. In macrophages, NO synthesized by iNOS is thought to be an important autoregulatory inhibitor of vascular inflammation, in part by inhibiting monocyte-endothelial adhesion [308].
1.5 Aims of Thesis.

ApoE is involved in atheroprotective mechanisms (Section 1.4). I have hypothesized that: ‘apoE has an anti-inflammatory/anti-atherogenic action by inhibiting cytokine-mediated upregulation of CAMs.’ There is some indirect evidence to support this proposal. Endothelial CAM expression is markedly elevated in the absence of apoE in a mouse model that develops lesions similar to those in humans \[25,309,310\]. Gene therapy to replace arterial macrophage apoE in these apoE-deficient mice is protective against the early stages of the disease, thus reducing monocyte recruitment and subsequent foam cell formation \[259\]. Furthermore, overexpression of apoE in co-culture models of aortic wall cells results in a marked reduction in monocyte adhesion and transmigration \[311\].

The aims of my thesis were, therefore:

**Aim 1:** to establish primary endothelial cell culture as an *in vitro* model of human endothelium in which to study CAM upregulation by pro-inflammatory cytokines.

**Aim 2:** to test the ability of plasma-purified apoE to modulate endothelial CAM expression.

**Aim 3:** to decipher whether locally-secreted, cell-derived apoE can suppress endothelial activation, in part, by designing cell culture models to mimic endothelial exposure to arterial macrophage apoE.
Chapter 2
2. CHARACTERIZATION OF HUMAN ENDOTHELIAL CELL MODELS

2.1 Introduction.

A model of human vascular endothelium was required for my study into the effects of apoE on endothelial CAM induction. In recent years, the quest for greater understanding of the vascular endothelial biology has led to methods for the isolation and in vitro culture of micro- and macro-vascular endothelial cells from a wide range of vessels from several species, including human [97]. Human umbilical vein endothelial cells (HUVECs) isolated from umbilical cords are a readily available and popular source, and early passages of cultured HUVECs constitute a well-established in vitro model system for studying human endothelial cells of large vessels. They have been used extensively for CAM research, especially in the field of atherosclerosis. In particular, HUVECs are the most widely used model in which to study the regulation of endothelial CAM expression by agents relevant to atherogenesis, including inflammatory cytokines [82,84], lipoproteins [83,99,122], estrogen [112,113] and NO [105,108].

There are disadvantages of HUVEC culture: time-consuming isolation and limited cell yield, slow growth, fastidious exogenous growth factor and high serum concentration requirements for optimal propagation, special culture substrata needs, relatively short life span, batch to batch variability of cells, heterogeneity within the same culture, the possibility of non-endothelial cell contamination, plus the risks associated with using material possibly infected with HIV or hepatitis B. For these reasons, a model endothelial cell line could overcome some of the difficulties encountered with primary endothelial cell culture. However, there is a lack of immortalized human endothelial cell lines available that have retained their endothelial nature. The ECV304 cell line, derived from a spontaneously transformed HUVEC culture [312], has been used to study various aspects of endothelial biology including angiogenesis [313], interferon-γ receptor expression [314] and plasminogen activator secretion [315].

This Chapter describes the establishment of HUVEC cultures using my optimized isolation protocols and immunophenotyping methods with which to characterize the cells. A detailed characterization of ECV304 cells was also performed to determine whether this transformed human endothelial cell line is a biologically relevant model system suitable for the investigation of CAM regulation by apoE.
2.2 Specialized Materials and Methods.

2.2.1 MATERIALS.

O-Phenylenediamine (OPD) substrate tablets, IgG\(_1\) negative control antibodies, von Willebrand factor (vWF) antibodies (clone F8/86) and StreptABComplex/HRP Duet kit were from DAKO Ltd (High Wycombe, UK). CAM monoclonal antibodies were from R&D Systems (Abingdon, UK): VCAM-1 (CD106; clone BBIG-V1), ICAM-1 (CD54; clone BBIG-11), E-selectin (CD62E; clone BBIG-E4) and PECAM-1 (CD31; clone 9G11). Other antibodies used for endothelial cell characterization were thrombomodulin (TM; clone QB/End/40) and CD34 (clone QB/End/10) both from Quantum Biosystems Ltd (Waterbeach, Cambridge, UK). M199 media, trypsin-EDTA, glutamine and penicillin were purchased from Life Technologies (Paisley, UK). Collagenase A (from \textit{Clostridium histolyticum}) was from Roche Diagnostics Ltd (Lewes, UK). Multichamber Tekwells were purchased from Nunc (Naperville, USA). All other culture plastic-ware was from Marathon Laboratory Supplies (London, UK); all flasks were fitted with a vented cap (0.2 \(\mu\)M filter). Loctite 358 adhesive for mounting slides was from Loctite UK (Welwyn Garden City, UK). LPS (from \textit{Escherichia coli} serotype 026:B6), recombinant human TNF-\(\alpha\) and endothelial cell growth supplement (ECGS) along with all other chemicals and tissue culture reagents were from Sigma-Aldrich Company Ltd (Poole, UK).

2.2.2 ISOLATION OF HUVECs.

HUVECs were isolated from fresh umbilical cords using my modifications of a standard technique [316]. Umbilical cords were collected from the Maternity Unit (Royal Free Hospital, London, UK) in pots containing collection M199 media; 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin, 2 mM L-glutamine, 200 \(\mu\)g/ml gentamycin, 250 ng/ml fungizone (amphotericin B) and 20 % (v/v) foetal bovine serum (FBS) in M199 media with Hank's salts. All manipulations of cords were undertaken in a class II laminar-flow cabinet using aseptic technique. Cords were inspected for any damage, e.g. from applying clamps during delivery, and these areas were trimmed away to reduce the chance of contaminating the primary cultures with smooth muscle cells or fibroblasts released after basement membrane disruption. Cords that still remained longer than 20 cm were processed within 12 h of delivery, as described below. The umbilical vein was cannulated with 4 cm lengths of 16 G nasogastric tubing (UnoPlast, Denmark) and secured with ties. The vein was flushed with warm PBS to remove all traces of blood. Thirty ml of 0.2 U/ml (final activity) collagenase A solution in unsupplemented M199 media was freshly prepared to fully distend the cord vein. Whilst clamping off one of the cannulas, the vein was filled with collagenase solution pre-
warmed to 37 °C. Both cannulae were then clamped off securely and the cord incubated at 37 °C for 10 min. After this time, the cord was gently massaged to release loosened endothelial cells and the cell-containing solution drained into 30 ml of 'complete M199 media' (containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20 % (v/v) FBS). The vein was then flushed with PBS to harvest any remaining endothelial cells and the cell suspensions pooled and centrifuged (300 g for 7 min). The cell pellet was resuspended in 5 ml of complete M199 media (until a cell-single suspension was achieved) and, after transferring to a 25 cm² flask, was incubated overnight in an humidified atmosphere at 37 °C with 5 % CO₂ and 95 % air. Next day, the flask was washed with PBS to remove any red blood cells and fresh media was added. The confluence of adherent endothelial cells, designated ‘P₀ cells’, was then assessed by phase-contrast microscopy (Nikon TMS Inverted Microscope; Nikon UK Ltd, Kingston, UK). Depending on the initial yield, cells took 1-2 days to become confluent. Slower growing cultures from poor yields were discarded as they would have to undergo more cell doubling times than the more successful batches. Cells from different cords were not pooled, but kept as separate cultures.

2.2.3 HUVEC SUBCULTURING.

Upon reaching confluency, the P₀ endothelial cells were passaged: by washing with warm PBS, by brief trypsinization (2 min at 37 °C with trypsin/EDTA solution) and then neutralization in complete M199 media, and harvesting by centrifugation (300 g x 5 min) and finally resuspending in fresh media containing endothelial cell growth supplement (25 μg/ml ECGS final concentration). The single cell suspension was reseeded in a 75 cm² flask, pre-coated with 1 % (w/v) gelatin (1:3 split). Few cells were lost during serial passaging; after trypsinization cells were found to be >98 % viable (Section 2.2.4) and >90 % of cells reattached. P¹ cells typically reached 100 % confluency again within 2-5 days, depending upon cell density in the original flask, without the need for ‘cell feeding’ by replacing media. At this stage, harvested P¹ cells were either counted prior to seeding into multichamber Tekwells or 96-well plates for experimentation, or serially passaged 1:3 into 3 flasks to increase the number of cells available for experimentation, or cryopreserved for future use (see following Sections). Note that PBS and complete M199 media were always used pre-warmed to 37 °C in a water bath prior to use with cells.

2.2.4 CELL COUNTING BY TRYPSAN BLUE EXCLUSION ASSAY.

Trypan Blue is used in this dye exclusion procedure to facilitate visualization and counting of both viable cells (which do not take up the dye) and non-viable cells (those with
compromised/ permeable cell membranes which therefore take up the dye). A 30 µl aliquot of single-cell suspension was mixed with an equal volume of 0.4 % (w/v) Trypan Blue solution. Cell viability was determined in a haemocytometer, with a correctly fitted cover slip in place and the chambers precisely filled with the cell/stain mixture, observed under an inverted light microscope (using the x 10 objective lens). The mean cell count of viable cells per 1 mm square with a volume of 10^-3 cm³, from a minimum of 8 squares counted, was used in the calculation below to give the concentration of cells per ml in the original cell suspension:

\[
\text{(mean cell count per square) x dilution factor x 10^4 = viable cell count per ml}
\]

To determine % cell viability in the suspension, both viable and non-viable cells were counted and the viable cell count expressed as a % of the total cell count.

2.2.5 CELL CRYOPRESERVATION.

Batches of confluent P1 endothelial cells were harvested, centrifuged (Section 2.2.3) and the pellet resuspended in 1 ml of pre-chilled complete M199 media containing 10 % (v/v) dimethylsulphoxide (DMSO) as a cryoprotective agent which lowers the freezing point and allows for a slower cooling rate to limit the damage caused by freezing. The whole mixture was immediately transferred to one cryovial and frozen in the vapour phase of a liquid nitrogen tank by slowly lowering the vial nearer to the liquid over a 3 h period. Vials were stored under liquid nitrogen for a maximum of 6 months.

When required, cells were rapidly thawed by immersing the bottom of the vial in a 37 °C water bath and the vial contents immediately added to an excess of complete M199 media, mixed and centrifuged (300 g, 5 min). The pellet was resuspended in fresh media (and supplemented with ECGS) and transferred to a 75 cm² flask. Cells adhered to form a confluent monolayer overnight and were subcultured as described previously (Section 2.2.3).

2.2.6 ECV304 CELL CULTURE.

This spontaneously transformed HUVEC cell line, European Collection of Animal Cell Culture (ECACC) No. 92091712, [312] was donated by Dr D. Hassall (Glaxo Wellcome Research and Development, Stevenage, UK). Cells were maintained as adherent cultures in 175 cm² flasks, pre-coated with 1 % gelatin, in complete M199 media supplemented with 10 % (v/v) FBS but with no ECGS. Cells were subcultured in a similar manner to HUVECs, but more frequently (every 3 d) and reseeded at a lower density (using a ratio of 1:20).
2.2.7 Scanning Electron Microscopy (SEM).

Both HUVECs and ECV304 cells were seeded at $5 \times 10^4$ cells/well in gelatin-coated 8-chamber Tekwells made of permanox plastic and adhered overnight to form confluent monolayers. Wells were then washed with warm PBS, fixed in 3 \% (v/v) glutaraldehyde (pH 7.4) and processed and analysed by the Electron Microscopy Unit (RF&UCMS). Briefly, samples were dehydrated in a graded series of ethanol mixtures and critical point dried before being coated in carbon and gold in a Ion Tech saddle field using an ion source sputter coater (Ion Tech, Teddington, UK). The cells were then viewed in a Philips 501 scanning electron microscope (Pye-Unicam, Cambridge, UK).

2.2.8 Immunocytochemical Analysis of Endothelial Antigens.

Immunostaining for basal and cytokine-induced expression of endothelial markers was performed on confluent cells in gelatin-coated 8-chamber Tekwells (see above). Cells were treated with 50 U/ml (5 \mu g/ml LPS) for 6, 12 or 24 h. After this time, media was removed and the well boundaries carefully removed from the slide. The slide was washed by immersing in a Coplin jar of pre-warmed PBS, followed by a distilled water wash, and then left to air-dry overnight. Individual wells were segregated using a paraffin pen (DAKO Ltd) and cells were fixed with 10 \% neutral buffered formal saline for 10 min. Wells were washed 3 times with PBS and then endogenous cellular peroxidase activity was quenched with 3 \% (v/v) hydrogen peroxide ($H_2O_2$) in PBS for 15 min, before washing again.

Cells were immunophenotyped using a range of commercial mouse anti-human monoclonal antibodies, used on different wells of the same slide, which had previously been titrated and diluted optimally in PBS containing 0.1 \% (w/v) bovine serum albumin (BSA). Antibodies used for immunophenotyping were anti-vWF at 1.2 \mu g/ml, anti-TM at 1 \mu g/ml, anti-CD34 at 1 \mu g/ml, anti-PECAM-1 at 1 \mu g/ml, anti-ICAM-1 at 1 \mu g/ml, anti-VCAM-1 at 5 \mu g/ml and anti-E-selectin at 5 \mu g/ml. Isotype-matched irrelevant antibodies (raised against A. niger glucose oxidase, a protein neither found or induced in mammalian tissues) were used in negative controls on the same slide (at equivalent IgG1 protein concentrations). Primary antibodies were incubated at room temperature for 45 min before washing with PBS. Biotinylated goat anti-mouse antibodies, optimally diluted in 10 \% normal human serum (heat inactivated) in Tris-buffered saline (TBS; 0.05 M Tris and 0.15 M NaCl, pH 7.6) were incubated for 30 min. Note that both reagents in the StreptABC/Complex/HRP Duet kit were titrated and optimally diluted to 1/400. After washing, antibody binding was amplified by an optimally diluted streptavidin/biotin horse radish peroxidase (HRP) complex during a 30 min incubation, and the slide then washed. The slide was then immersed for 5 min in freshly
prepared chromogenic substrate for peroxidase: 0.06% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.001 M imidazole and 0.01% (v/v) \( \text{H}_2\text{O}_2 \) in 0.056 M Tris.HCL (pH 7.6). The substrate for HRP is \( \text{H}_2\text{O}_2 \), the cleavage of which is coupled to the oxidation of the chromogen. The slide was washed thoroughly with distilled water and cell nuclei were counter-stained by a 1 min incubation with Mayer's Haemalum. The slide was washed with distilled water and air-dried in the dark. Coverslips were applied using Loctite 358 adhesive and polymerized under long wave ultraviolet light for 15 mins in the dark room. Slides were stored in the dark to avoid photobleaching prior to microscopic analysis and photography using a Zeiss Photomicroscope III (Carl Zeiss Ltd; Welwyn Garden City, UK) and colour transparency film.

2.2.9 **CELL-BOUND VCAM-1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).**

HUVECs were seeded at 3 x 10^4 cells/well in 96-well plates pre-coated with gelatin and were incubated overnight to allow confluent monolayers to form. Cells were not seeded in the first column (left for application of the reagent blank). Quadruplicate wells were positioned going across the rows, with the maximum of 8 separate treatments going down columns. Cells were treated with either 0.03-100 U/ml TNF-\( \alpha \) for 3-30 h. Plates were washed twice with pre-warmed PBS and then fixed with 10% neutral buffered formal saline for 10 min at room temperature. Wells were washed rigorously (5 x 200 \( \mu \)l PBS per well) after fixing and after subsequent reagent additions. ELISA steps were identical to the immunostaining protocol above, however peroxidase chromogenic substrate was replaced with OPD chromogenic substrate (pH 5.0) containing 0.01% (v/v) \( \text{H}_2\text{O}_2 \). OPD was applied for 1 min (100 \( \mu \)l per well, applied with a multichannel pipette moving across the plate) before stopping the reaction with 1 M sulphuric acid. Absorbances were immediately read at 492 nm \( (A_{492}) \) in a Titertek Multiskan MCC/340 MK II microtitre plate spectrophotometer (Flow Laboratories Ltd; Rickmansworth, UK) against blank substrate plus acid.

2.3 **Results.**

2.3.1 **CHARACTERIZATION OF HUVECS AND COMPARISON WITH THE ECV304 CELL LINE.**

2.3.1.1 **Cell Growth and Morphology.**

In 1973, Jaffe *et al.* first described a method to successfully isolate and culture a pure population of cells using collagenase digestion of the umbilical vein, with subsequent identification of the cells as being endothelial on the basis of both morphological and immunological criteria [316]. I adapted this standard isolation technique to increase the yield of pure endothelial cells, whilst still preventing the introduction of non-endothelial cell types,
smooth muscle cells or fibroblasts; collagenase incubation time was reduced and cords with
the basement membrane disrupted by clamping were discarded (Section 2.2.2). Endothelial
cells were always isolated and cultured without deviating from my optimized protocol, in
order to ensure a standardized procedure. After the first passage, cultures grew well in the
presence of growth factor, but without the need for heparin supplementation which is
thought to potentiate growth factors [317,318].

Every batch of isolated HUVECs consisted of homogeneous, large, polygonal cells
that formed cobblestone monolayers of closely opposed cells when confluent, all
characteristics of endothelial cells [316] (Figure 2.3-1, panel A). There was no visual evidence
of any long, spindle-shaped cells growing in parallel arrays, overlapping or in whorling
patterns, indicating no smooth muscle cell or fibroblast contamination [316]. SEM revealed
that HUVECs were large (30 x 50 μm), flat cells that had distinct cell boundaries with
blebbing of the membrane, and prominent, centrally-located, oval nuclei (Figure 2.3-1, panel
C).

![Figure 2.3-1 Comparison of HUVECs and ECV304 cells by microscopy.](image)

Phase-contrast photography of confluent cultures in 75 cm² flasks reveal that early passage HUVECs
(P¹) have characteristic endothelial cobblestone morphology (panel A) although ECV304 cells were dissimilar
(panel B). Phase-contrast photographs were taken at ×100 magnification using a Nikon TMS inverted
light microscope with a camera attachment. Differences in cell morphology were highlighted by SEM analysis
(Section 2.2.7); ECV304 cells (P³⁰) were smaller and more densely packed at confluence (panel D) than
HUVECs (panel C). White scale bar on electron micrographs represents 50 μm.
In contrast to HUVECs which had a population doubling time of 48-72 h (varying with batch and with time in culture), immortalized ECV304 cells grew at a rate five times that of primary HUVECs, and in the presence of lower concentrations of serum and without need for growth factor supplementation. Indeed, it has been reported that ECV304 cells can even be maintained in serum-free conditions [319]. Cell morphology, as judged by phase-contrast microscopy and SEM, was different to HUVECs (Figure 2.3-1, panels B and D, respectively). ECV304 cells were approximately half the size of HUVECs and were more closely opposed.

2.3.1.2 Immunocytochemical Analysis for Expression of Endothelial Markers.

This immunocytochemical detection of endothelial antigens involved titration of all antibodies (not shown) to achieve the highest specific staining patterns with no background/non-specific staining. An isotype-matched control antibody was always used on one of the 8 wells on the chamber slide and always showed blue counter-stained nuclei but no brown positive staining (Figure 2.3-2, panels A and B). Cells were fixed prior to antibody incubations so that the intracellular pattern of expression of the various antigens could be identified.

HUVECs expressed vWF (alternatively known as factor VIII-related antigen), a classical biochemical marker for endothelial cells, which was distributed in characteristic cytoplasmic inclusions or Weibel-Palade bodies [320] as shown by punctate brown cytoplasmic staining (Figure 2.3-2, panel C). vWF is a large multimeric glycoprotein which mediates platelet adhesion and serves as a carrier for procoagulant factor VIII in the blood [321]. This is a favoured marker for endothelial characterization as vWF is only expressed in endothelial cells, megakaryocytes and platelets. ECV304 cells, tested in parallel in the same immunostaining experiment, were negative for vWF (panel D).

HUVECs were also positive for TM, a thrombin binding protein present on endothelial cell membrane which plays an important role as a natural anticoagulant [322]. TM is not solely an endothelial-specific protein, as it also located in macrophages, keratinocytes and osteoblasts. In HUVECs staining was intense at the membrane but also present at a lower intensity in the cytoplasm (Figure 2.3-2, panel E), whereas ECV304 cells were negative (panel F).
Figure 2.3-2 Immunophenotyping of HUVECs and ECV304 Cells.

Confluent cells on multichamber slides were immunostained (Section 2.2.8) with isotype-matched irrelevant antibodies (panels A and B) or with antibodies against endothelial antigens vWF (C and D) and TM (E and F). Positive staining is brown, while counter-stained nuclei with Mayer's Haemalum are blue. HUVECs (A) and ECV304 cells (B) had no brown positive staining with the negative control antibodies, with only the presence of blue counter-stained nuclei visible. Panel C shows punctate cytoplasmic staining for vWF in HUVECs, while ECV304 are negative (D). Panel E shows predominantly membrane staining for TM, although in contrast, ECV304 cells are negative (F). All light microscope photographs were taken at ×40 magnification.

Cells were stained to detect endothelial CAM expression on resting cells in the ‘basal state’. HUVECs were strongly positive for PECAM-1 (CD31) and ICAM-1 (CD54) expression (Figure 2.3-3, panels A and C, respectively). ICAM-1 is constitutively expressed on a variety of cell types including endothelial cells, while constitutive PECAM-1 is restricted
to endothelial cells and some circulating blood cells including platelets. Staining for both antigens was mostly confined to the membrane (consistent with their roles as adhesive membrane proteins) with some cytoplasmic staining, and was heterogeneously expressed, with some cells staining more markedly than others giving a patchy 'mosaic' distribution of stain. PECAM-1 staining was strongest at intercellular borders between closely opposed cells. ECV304 cells were negative for PECAM-1 (Figure 2.3-3, panel B). In ECV304 cells, staining for ICAM-1 was weak and <50% of the ICAM-1 staining for HUVECs (Figure 2.3-3, panel D). Basal VCAM-1 (CD106) was low with <5% of cells staining positive (Figure 2.3-4, panel A) but was readily induced over a 12 h period by 5 μg/ml bacterial endotoxin, LPS, a component of gram negative bacteria known to cause endothelial activation and induce endothelial CAM expression [82] (Figures 2.3-3, panel E, and 2.3-4, panels B and C). This is consistent with VCAM-1 as a marker for endothelial activation, while being non-constitutively expressed on unactivated endothelium. It should be noted, however, that VCAM-1 is not specific for endothelial cells; it is found in smooth muscle cells (as well as follicular dendritic cells), and so this test alone is not a marker for immunophenotyping primary endothelial cell isolates. VCAM-1 was heterogeneously induced throughout the culture, as previously reported [84], but even after prolonged exposure to LPS, was not as strongly expressed as either ICAM-1 or PECAM-1. In contrast, ECV304 cells were negative for VCAM-1 (Figure 2.3-3, panel F).

In the basal state, my preparations of HUVECs constitutively expressed vWF, TM, PECAM-1 and ICAM-1. Staining for CD34, an adhesion molecule expressed on endothelial cells and haemopoietic progenitor cells [323], was weak in HUVECs with only 10% of cells staining membrane positive (Table 2.3-1). The distribution of CD34 was consistent with its preferential expression on subsets of HUVECs [324]. Although HUVECs were mostly negative for basal VCAM-1 and E-selectin expression (with <5% staining positive), exposure to LPS dramatically upregulated VCAM-1 and E-selectin (Table 2.3-1). E-selectin is a marker which is expressed specifically on cytokine-activated endothelium. Immunostaining of cells treated for 6, 12 and 24 h with LPS, revealed that VCAM-1 was upregulated at 12 h and still elevated at 24 h, while E-selectin was upregulated by 12 h (with strongly positive cells coexisting with negative ones) but had diminished by 24 h. This upregulation possibly reflects the role of E-selectin in early stages of inflammation in vivo, whereas VCAM-1 is thought to characterize the later phases [42]. ICAM-1 was superinduced under the influence of LPS in HUVECs, as demonstrated previously [82,325]. ICAM-1 was hyperinduced by 12 h and remained high at 24 h although ICAM-1 was still not uniformly expressed in the culture, in agreement with earlier observations [325]. In HUVECs,
expression of vWF, TM, CD34 or PECAM-1 was not increased upon endothelial activation with LPS, as previously reported [324]. In sharp contrast, ECV304 cells failed to express endothelial antigens and CAMs; ICAM-1 showed only weak staining which did not increase despite prolonged LPS treatment (Table 2.3-1). In particular, the presence of ICAM-1 does not prove that ECV304 cells have an endothelial origin as ICAM-1 is expressed in a wide variety of cells types.

Figure 2.3-3 Expression of CAMs in HUVECs and ECV304 cells.

Confluent cells on multichamber slides were immunostained (Section 2.2.8) with antibodies against PECAM-1 (panels A and B), ICAM-1 (C and D) and VCAM-1 (E and F). HUVECs constitutively expressed PECAM-1 (A) and ICAM-1 (C), while VCAM-1 expression needed to be induced by LPS treatment (5 μg/ml for 12 h) prior to staining before they were positive for this antigen (E). ECV304 cells were negative for PECAM-1 (B) but expressed ICAM-1 weakly on less than 50% of cells (F). VCAM-1 was not expressed in ECV304, nor was it induced by LPS exposure (F). All light microscope photographs were taken at ×40 magnification.
Figure 2.3-4 LPS treatment induces the expression of VCAM-1 in HUVECs.

When immunostained for VCAM-1 (Section 2.2.8), HUVECs were mostly negative with <5% staining weakly positive (panel A). However, both membrane and cytoplasmic staining of the majority of cells increased with exposure to LPS (5 μg/ml for 12 h) prior to staining (B). LPS-induced VCAM-1 expression was not homogeneous throughout the culture, with some cells responding more to LPS. At higher magnification, it was observed that VCAM-1 was preferentially expressed at the membrane, with some diffuse cytoplasmic staining especially in the perinuclear region where presumably newly synthesized VCAM-1 was undergoing protein maturation before being targeted to the membrane (C). Light microscope photographs A and B were taken at ×40 magnification, C was taken at ×100.
Table 2.3-1 Comparison of endothelial marker expression on HUVEC and ECV304 cells, and response to LPS.

<table>
<thead>
<tr>
<th></th>
<th>HUVEC</th>
<th></th>
<th>ECV304</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No LPS</td>
<td>LPS</td>
<td>No LPS</td>
<td>LPS</td>
</tr>
<tr>
<td>vWF</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>✓</td>
<td>↑</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>E-selectin</td>
<td>✓</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PECAM-1</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>✓</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: expression of constitutive antigens: positive (✓ ✓), weakly positive (✓) or negative (✗). Upregulation by LPS (↑) or no response (−).

I concluded that ECV304 cells were not suitable for endothelial CAM studies; they did not express endothelial antigens or CAMs and were unresponsive to inflammatory stimuli. In contrast, primary HUVEC cultures, isolated by my optimized protocol, were confirmed to be unequivocally endothelial in nature on the basis of my immunophenotyping. Each batch was made up of a purely endothelial population of cells which had retained their functionality. HUVECs remained responsive to activation by LPS although their ability to express VCAM-1 and E-selectin did decline with time in culture (data not shown) and so only early passage cells (i.e. before the 4th passage) were used for future experiments.

2.3.2 Establishment of a VCAM-1 ELISA.

Increasingly, researchers use ELISAs to quantify CAM expression as they combine the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays by using detection antibodies conjugated to an easily assayed enzyme [326]. I established a cell-bound VCAM-1 ELISA, based on my immunohistochemistry protocol, as a quantitative method of measuring both membrane-bound and cytoplasmic VCAM-1 in confluent HUVECs in 96-well plates. Sensitivity was optimized by the streptavidin/biotin/HRP amplification steps, while antibody titration ensured that all VCAM-1 was detected, while, non-specific antibody binding was kept to a minimum. Total cell VCAM-1 i.e. both membrane and cytoplasmic, was detected as cells were fixed prior to antibody incubations. The ELISA steps were essentially the same as the immunostaining protocol, the main exception being that the DAB chromogenic substrate, which yields a precipitate at the site of peroxidase activity, was replaced with a soluble substrate, OPD, which absorbs at 492 nm (A492) with peroxidase.

Basal VCAM-1 was negligible as the anti-VCAM-1 signal was equivalent to that of the
isotype control (Figure 2.3-5, panel A). However, HUVECs responded rapidly to an inflammatory cytokine, TNF-α, giving a 10-fold increased signal for VCAM-1 after 6 h treatment with 100 U/ml TNF-α (Figure 2.3-5, panels A and B). The extent of induction was similar to that reported by others [82-84]. In every ELISA assay, control cells were stained for vWF, a constitutively expressed antigen, to give a positive signal regardless of cytokine-exposure (Figure 2.3-5, panel A).

![Graph A](image)

**Figure 2.3-5 VCAM-1 induction by TNF-α in HUVECs as quantified by ELISA.**

Panel A, confluent HUVECs in 96-well plates were treated with 100 U/ml TNF-α for 6 h; TNF-α was omitted from control wells for unstimulated cells. VCAM-1 ELISA was performed as described in Section 2.2.9. Basal (unstimulated) cells had a negligible level of VCAM-1 (white bar) while after TNF-α (100 U/ml) treatment VCAM-1 was increased 10-fold (black bar). An isotype-matched irrelevant antibody gave a low background signal (light grey bar). The detection of constitutive vWF was the positive control for the ELISA (dark grey bar). Panel B, HUVECs were exposed to a range (0.03–100 U/ml) of TNF-α concentrations over a 6 h period. VCAM-1 was induced in a dose-dependent manner. Results are from representative experiments and each bar shows mean ± SE from quadruplicate wells.
HUVECs readily responded to TNF-α in a dose-dependent manner when stimulated with a range of concentrations (0.03–100 U/ml) for 6 h (Figure 2.3-5, panel B). Although higher amounts were not tested, the curve suggested that the optimal dose was 100 U/ml TNF-α giving the maximal VCAM-1 induction.

Time-course studies indicated that an induction of VCAM-1 by 100 U/ml TNF-α was detectable after 3 h of exposure, with VCAM-1 expression rapidly and significantly increasing over an 8 h period before reaching a plateau. This high level of expression was then sustained for at least 30 h without further addition of cytokine (Figure 2.3-6). This pattern of induction was comparable to another study in HUVECs [81].

![Figure 2.3-6 Time-course for VCAM-1 induction by TNF-α in HUVECs.](image)

Confluent HUVECs in 96-well plates were exposed to 100 U/ml TNF-α for 3–30 h before VCAM-1 quantification by ELISA. Before cytokine addition VCAM-1 expression was undetectable, but was rapidly increased in cells activated by TNF-α. Expression plateaued at 8 h and was sustained for at least 30 h. Results are from one representative experiment and each bar shows mean ±SE from quadruplicate wells.
2.4 Discussion

Umbilical cords are the most readily available source of human endothelial cells and therefore HUVECs remain the most popular endothelial model. Indeed, the majority of our knowledge about endothelial cells comes from the study of these cells. Although macrovascular endothelial cells have been isolated from several human vessels, including the femoral artery, portal vein, pulmonary artery [327] and saphenous vein [82], these vessels are not as easily obtained as umbilical cords. It is even more difficult to obtain vessels susceptible to atherosclerosis, such as the coronary artery. However, investigators have shown that HUVECs do represent a relevant model for studying the regulation of endothelial CAM expression on large vessel endothelium in the adult human [82], and suggest that results can be extrapolated to what may occur in arterial endothelial cells [147]. However, further research is needed to validate this, as endothelial cells from different vascular sites can be heterogeneous in nature, presumably due to their tissue-specific functions (discussed in Chapter 6).

Pure HUVEC cultures were successfully established and, based on both morphological and immunohistochemical criteria, I concluded that early passage HUVECs had retained their endothelial phenotype and functionality, constituting a valid model of human vascular endothelial cells for further experimentation. Every batch displayed a characteristic cobblestone morphology when confluent and displayed typical endothelial antigens and CAMs, some of which were upregulated in response to endotoxin by sensitive immunophenotyping. However, after the 4th passage, LPS responsiveness decreased, an observation that is consistent with other reports in the literature [81] and reflects the tendency for endothelial cells to lose their specialized properties in culture. Although there are concerns that HUVECs are isolated from hypoxic and possibly activated blood vessels [97], my cells were always isolated from freshly obtained umbilical cords and low basal levels VCAM-1 and E-selectin, markers of endothelial activation, suggested that they were isolated in their native state.

Immunohistochemistry was a valuable technique for visualizing both the pattern of cellular expression of endothelial antigens and, in many cases, the heterogeneity of expression, not only within the same culture but also between batches (as commented on previously [328]). HUVEC heterogeneity has been viewed as a disadvantage for endothelial research [329], reinforcing the need to generate a homogenous endothelial cell line to facilitate standardization of results [313]. Alternatively, primary culture heterogeneity may be
viewed as a beneficial quality, reflecting the heterogeneous nature of endothelial cells *in vivo* [97].

Immunohistochemistry, as a visual analysis technique, does have limitations, whereas ELISA offers a sensitive and quantitative method for the immunodetection of VCAM-1. My VCAM-1 ELISA was both sensitive and specific, ensuring all VCAM-1 was detected while maintaining low levels of non-specific/background binding. At this stage, TNF-α, which is found in atherosclerotic lesions [79], was introduced as a better defined and more relevant alternative to LPS for inducing VCAM-1 expression in HUVECs. The use of TNF-α also avoided generating artefacts in subsequent experiments with apoE:phospholipid complexes; phospholipid can bind and neutralize LPS therefore protecting cells from its exposure [330].

It was hoped that ECV304, an immortalized HUVEC cell line [312], might be a suitable and convenient alternative for HUVECs overcoming the disadvantages of using primary cultures, and so these cells were critically evaluated for my subsequent CAM studies. In contrast to primary HUVECs, I found that not only were ECV304 cells morphologically dissimilar to endothelial cells, but that they also failed to express a range of endothelial antigens and CAMs, and were unresponsive to LPS or TNF-α. This cell line could not be classed as endothelial on the basis of my immunocytochemical evidence and was deemed unsuitable for CAM studies with apoE. Although irreversible loss of endothelial-specific functions may have occurred during immortalization, my observations led me to doubt an endothelial origin of ECV304 cells. However, the original author had claimed that although ECV304 cells were vWF negative, they had retained most of the mechanisms of CAM expression and that either cytokines or LPS were able to stimulate expression of these molecules [319]. This discrepancy can now be explained following a statement issued in May 1999 by the ECACC, suppliers of these cells, claiming that 'ECV304 is a false cell line' as 'ECV304 has the same DNA fingerprint as the cell line T-24 (human bladder carcinoma)' and 'does not express characteristics or surface markers typical of an endothelial cell line'. Presumably cross-contamination must have occurred and this regrettable error is now the subject of an investigation by ECACC laboratories.

One of the major problems facing researchers in endothelial cell biology has been the lack of a suitable cell line that could be used as a model for *in vitro* studies. More recently, a number of groups have reported the generation of human endothelial cell lines by several techniques, including spontaneous transformation [331], infection with an amphotropic
recombinant retrovirus [332], transfection with SV40 large T antigen [333], and hybridization of HUVECs with the epithelial cell line A549 [329,334,335]. In many cases, these techniques are now yielding cells with their endothelial morphology and many other characteristics intact. Unfortunately, access to such endothelial cell lines was not available at the time of my studies. Thus, low passage primary HUVECs were deemed the most suitable and accessible endothelial model for CAM studies and were used in all subsequent experiments in this thesis.
Chapter 3
3. CAN PLASMA APOE DOWNREGULATE CAM EXPRESSION IN HUVECS?

3.1 Introduction.

As discussed in Chapter 1, apoE, a surface constituent of circulating plasma lipoproteins, helps protect against atherosclerosis. When the apoE polypeptide is absent or dysfunctional, severe hyperlipidaemia and atherosclerosis ensues [168,234,235], while infusion of apoE in animal models [236] or gene overexpression is protective [237]. In addition to clearing atherogenic lipoprotein particles [168] and participating in local RCT [261], several recent studies infer other anti-atherogenic actions of apoE at lesion sites, where apoE is secreted in abundance by resident cholesterol-loaded macrophages [37,38]. These include the prevention of LDL retention in subendothelial matrix [20], the inhibition of both endothelial [277] and smooth muscle cell proliferation [278], an allele-specific antioxidant activity protecting lipids from peroxidation [269], and a potent anti-platelet action by stimulation of the L-arginine:nitric oxide (NO) signal transduction pathway [216,276].

I have tested the hypothesis that apoE has an additional significant atheroprotective role in the suppression of endothelial CAM upregulation on cytokine-activated endothelium. If tenable, our hypothesis would have consequences for monocyte adhesion and recruitment into focal areas on the arterial subendothelium; these important early events in atherogenesis would be diminished as they are dependent upon early localized endothelial CAM upregulation [23-28,309]. Indeed, decreases in monocyte number or recruitment to the vascular wall have profound effects on atherogenesis by reducing subsequent foam cell formation, an early hallmark of the atherosclerotic process [34,35].

There is some indirect evidence that apoE may play a role in the suppression of endothelial activation and CAM expression. The apoE-deficient (APOE<sup>−/−</sup>) mouse, which develops foam cell and fibroproliferative lesions histologically similar to human atherosclerosis, has markedly elevated VCAM-1 expression in aortic lesions [25,310]. Furthermore, overexpression of apoE in coculture models of aortic wall cells results in a marked reduction in monocyte adhesion and transmigration [311]. Intriguingly, HDL which carries apoE as a minor component, has also been implicated in downregulation of HUVEC CAM expression although this has largely been attributed to its apoAI and apoAII content [83,146-148].
The aim of this study was to investigate whether plasma apoE, either purified and reconstituted in dimyristoylphosphatidylcholine phospholipid vesicles (apoE:DMPC) or as apoE-rich HDL (HDL-E), inhibits the upregulation of CAMs in HUVECs activated by either TNF-α or IL-1β, two inflammatory cytokines found in lesions [79,80]. ApoE:DMPC complexes [336,337] and HDL-E particles [338] have been shown to possess receptor binding and biological activity in other assay systems so were chosen as a model of plasma apoE. Any modulatory effects on CAMs, implicated in the process of atherogenesis and found elevated in human atherosclerotic tissue, including VCAM-1, ICAM-1 and E-selectin, [58-60], were analysed alongside the effects of known CAM suppressors. However, particular attention was paid to VCAM-1 which is increasing described as the most important factor for the selective accumulation of monocytes and T-lymphocytes [23,24,26,27,68]. Although ELISA was a superior analysis technique to immunostaining (described in Chapter 2), I also employed flow cytometry as a powerful tool for monitoring any subtle changes in CAM expression on a cell-by-cell basis throughout the heterogeneous HUVEC populations. Finally, an assay for monocyte adhesion to HUVEC monolayers was established to test for any apoE effect on this functional assay which does not depend on immunodetection of a specific CAM.

3.2 Specialized Materials and Methods.

3.2.1 MATERIALS.

S-Nitroso-L-glutathione (GSNO) was supplied by Alexis Corporation Ltd (Nottingham, UK). Bradford protein assay and Phospholipid B Kits were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK) and from Wako (Neuss, Germany), respectively. Goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibodies were from DAKO Ltd. The fluorescent probe 1',1'-dioctadecyl-3,3,3',3' tetramethylindocarbocyanine perchlorate (DiI/DiIC18(3)) was purchased from Molecular Probes Europe (Leiden, The Netherlands). Recombinant human IL-1β, along with all other chemicals and tissue culture reagents were from Sigma-Aldrich Company Ltd.

3.2.2 PROTEIN MEASUREMENT.

Protein concentrations of purified apoE, apoE:DMPC complexes and HDL samples were measured by the Bradford assay [339]. This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to proteins occurs. The extinction coefficient of a dye-
albumin complex solution is constant over a 10-fold concentration range [340], thus, Beer's Law may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. Over a broader range of protein concentrations, the dye-binding method gives an accurate, but not entirely linear, response.

Dilutions of a BSA protein standard were prepared in distilled water to a final volume of 200 µl. A typical standard curve in the range of 0-20 µg protein/200 µl was freshly prepared each time the assay was performed. The triplicate unknown samples were diluted to 200 µl in distilled water. To each tube, 1 ml of freshly diluted Bradford dye reagent (final concentration 20 %, v/v) was added and the tubes vortexed. After an incubation of 10 min at room temperature, the absorbance at 595 nm (\(A_{595}\)) versus reagent blank was measured using a Kontron Uvikon 930 spectrophotometer (Kontron Instruments Ltd; Watford, UK). The concentration of standards versus their \(A_{595}\) was plotted and the concentration of the test samples determined from the standard curve using Elsevier-Biosoft's Lowry analysis software (Cambridge, UK).

### 3.2.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Fractionation of proteins in polyacrylamide gels is a primary means for their characterization and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used technique to separate both native and denatured proteins. SDS-PAGE separates polypeptides in a complex mixture solely on the basis of molecular size. Firstly, the polypeptide sample is denatured with heat in the presence of SDS and a reducing agent, usually β-mercaptoethanol. In the presence of excess SDS the detergent binds to the polypeptide giving it constant negative charge per mass unit [341,342]. During electrophoresis, SDS-polypeptide complexes will therefore all move towards the anode, and owing to the molecular-sieving properties of the gel, their mobilities are inversely proportional to the \(\log_{10}\) of their molecular weights. If standard proteins of known molecular weight are also run, the molecular weights of the sample proteins can thus be determined.

Mini gels usually 1.5 mm thick were cast in Bio-Rad MiniProtean II electrophoresis cassettes with a 10 or 15 well comb. The acrylamide solution for the resolving gel was prepared using the values given in Table 3.2-1. A discontinuous gel system was used where sharp banding of the loaded polypeptides was first achieved by migration through a stacking gel of high porosity (4 % w/v acrylamide). Thus, the stacking gel was layered above the resolving gel.
Samples (including molecular weight markers) were added 1:1 with 2 x sample buffer; 100 mM Tris.HCl pH 6.8, 4 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, 1 % (v/v) β-mercaptoethanol. Samples were then heated in a boiling water bath for 5 min. The gel cassette was inserted into the electrophoresis chamber, which was then filled with running buffer; 25 mM Tris pH 8.3, 192 mM glycine, 0.1 % (w/v) SDS. The samples were loaded into the bottom of the wells using a micropipetter fitted with a long narrow tip. Electrophoresis was started at a constant current of 20 mA per gel. After the dye front had moved into the resolving gel, the current was increased to 30 mA. When the dye front reached the bottom of the gel, the apparatus was disassembled and the gel carefully removed, cutting the bottom left corner of the gel to aid lane identification. At this stage, the gel was ready for Coomassie staining of the protein bands (as for gels to assess the purity of plasma-isolated apoE, Section 3.2.7). Other follow-on procedures such as electrotransfer and immunoblotting (Sections 4.2.7.3 and 5.2.2) or autoradiography (for radiolabelled polypeptides Sections 5.2.15 and 5.2.16) were carried out as described in different Chapters of this thesis.

<table>
<thead>
<tr>
<th>Solution Components</th>
<th>8% resolving</th>
<th>12% resolving</th>
<th>15% resolving</th>
<th>4% stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>4.6 ml</td>
<td>3.3 ml</td>
<td>2.3 ml</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>29 % (w/v) acrylamide, 1 % (w/v) N, N'-methylenebisacrylamide mix</td>
<td>2.7 ml</td>
<td>4.0 ml</td>
<td>5.0 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10 M Tris HCl buffer</td>
<td>2.5 ml (pH 8.8)</td>
<td>2.5 ml (pH 8.8)</td>
<td>2.5 ml (pH 8.8)</td>
<td>2.5 ml (pH 6.8)</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>*10 % (w/v) ammonium persulphate</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>*N, N, N, N'-tetramethylmethylenediamine</td>
<td>6 μl</td>
<td>4 μl</td>
<td>4 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Table 3.2-1 Solutions for preparing gels for SDS-polyacrylamide gel electrophoresis.

3.2.3.1 Coomassie Blue Staining

After electrophoresis, the gel was transferred to a clean plastic container and about 100 ml of Coomassie stain was added: 0.25 % (w/v) Coomassie brilliant blue R-250 in 50 % (v/v) methanol, 10 % (v/v) acetic acid. After incubation for 1-2 h at room temperature with shaking, the stain was removed and the gel then destained by successive incubations in
destain solution; 30% (v/v) methanol and 10% (v/v) acetic acid. This staining technique has a sensitivity of 0.1-0.5 μg protein per band.

3.2.4 ISOLATION OF PLASMA LIPOPROTEINS FROM HUMAN PLASMA.

3.2.4.1 Blood Sampling.

For all lipoprotein isolations, blood was withdrawn from the donor's antecubital vein. Human apoE3 was purified from the plasma of normal volunteers who were previously phenotyped/genotyped as homozygous apoE3. HDL-E was isolated from liver disease patients with primary biliary cirrhosis (PBC) whose greatly increased plasma apoE levels [338,343] were determined to be >10 mg/dl (normal range 3-7 mg/dl). For apoE and HDL-E isolation, 150 ml of blood per donor was collected into three polyethylene centrifuge tubes (50 ml), each containing a preservative cocktail to inhibit the multiple enzymatic degradations that can occur during and after withdrawal of blood [344] (Table 3.2-2). The tubes were manually agitated during collection, cooled on ice and then centrifuged for 20 min at 2,000 g and 4 °C. The plasma was separated from the sedimented cells and benzamidine and phenylmethylsulphonylfluoride (PMSF) were added to give a final concentration of 1 mM (Table 3.2-3) and used immediately for lipoprotein preparation.

For HDL (HDL₂, HDL₃ and total HDL) isolation, 50 ml of blood was taken from healthy volunteers and added to tubes containing EDTA, sodium azide and NaCl only (Table 3.2-2, see *). No further preservatives were added to the plasma. Both plasma and the corresponding HDL isolated from different donors were kept separate and not pooled through out.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Volume/50 ml Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>*0.2 M EDTA (Na Salt) pH 7.4</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>*0.3 M sodium chloride pH 7.4</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>*sodium azide 2.5 %</td>
<td>200 μl</td>
</tr>
<tr>
<td>chloramphenicol 50 mg/ml in 50% ethanol</td>
<td>80 μl</td>
</tr>
<tr>
<td>gentamicin sulphate 10 mg/ml</td>
<td>400 μl</td>
</tr>
<tr>
<td>kallikrein inactivator 20,000 U/ml</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

Table 3.2-2 Preservative cocktail for blood collection.

Key: * indicates solutions added to blood for HDL₂, HDL₃ or total HDL isolation.
Table 3.2-3 Preservative solutions for lipoproteins.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Volume/50 ml Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzamidine 1 M</td>
<td>50 µl</td>
</tr>
<tr>
<td>PMSF 0.2 M in anhydrous methanol (-20 °C)</td>
<td>250 µl</td>
</tr>
</tbody>
</table>

Table 3.2-3 Preservative solutions for lipoproteins.

3.2.4.2 Sequential, Isopycnic Preparative Ultracentrifugation.
Lipoproteins have lower hydrated densities than the other plasma proteins, permitting their isolation from plasma by floatation ultracentrifugation [345,346]. The density of plasma is increased by the addition of NaCl and/or NaBr and during ultracentrifugation, lipoproteins will float to the surface depending on their density and the prevailing small-solute density of the solution. The density range of plasma lipoproteins is given in Table 3.2-4. Individual lipoprotein classes can be isolated by sequentially increasing the plasma density. Alternatively, HDL can be prepared from plasma after precipitation of the apoB-containing lipoproteins followed by a single ultracentrifugation step (Section 3.2.4.8).

Table 3.2-4 Density classes of plasma lipoproteins.

<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Density Range (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt; 0.940</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.940 - 1.006</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006 - 1.019</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019 - 1.063</td>
</tr>
<tr>
<td>HDL₂</td>
<td>1.063 - 1.125</td>
</tr>
<tr>
<td>HDL₃</td>
<td>1.125 - 1.21</td>
</tr>
</tbody>
</table>

Table 3.2-4 Density classes of plasma lipoproteins.

3.2.4.3 Preparation of Sodium Bromide Density Solutions.

Base density solution (1.006 g/ml).

57.0 g anhydrous NaCl,
0.5 g EDTA (sodium salt),
5 ml 1 M NaOH,
0.5 g sodium azide.

Make up to 5 litres.
Then add 15 ml distilled H₂O.
This solution gives a refractive index value of 1.3345 at 20 °C.
All density solutions were prepared by adding a known weight of solid NaBr to the base density solution. For accurate density determination, the refractive index value for each solution was measured using a refractometer (Bellingham & Stanley Ltd). Fine adjustment of densities were achieved by adding solutions, $d=1.21$ or $1.478 \, g/ml$ (to $\uparrow$ density) and $d=1.006 \, g/ml$ (to $\downarrow$ density).

<table>
<thead>
<tr>
<th>Density (g/ml)</th>
<th>Refractive Index Value</th>
<th>Solid NaBr Added (approx. g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.006</td>
<td>1.3345</td>
<td>-</td>
</tr>
<tr>
<td>1.019</td>
<td>1.3368</td>
<td>-</td>
</tr>
<tr>
<td>1.045</td>
<td>1.3413</td>
<td>0.052</td>
</tr>
<tr>
<td>1.065</td>
<td>1.3448</td>
<td>0.075</td>
</tr>
<tr>
<td>1.10</td>
<td>1.3508</td>
<td>0.150</td>
</tr>
<tr>
<td>1.125</td>
<td>1.3547</td>
<td>0.165</td>
</tr>
<tr>
<td>1.157</td>
<td>1.3620</td>
<td>0.21</td>
</tr>
<tr>
<td>1.182</td>
<td>1.3643</td>
<td>0.25</td>
</tr>
<tr>
<td>1.21</td>
<td>1.3693</td>
<td>0.296</td>
</tr>
<tr>
<td>1.478</td>
<td>1.4160</td>
<td>0.79</td>
</tr>
</tbody>
</table>

NB. Filtered before final adjustment.

Table 3.2-5 Preparation of stock density solutions.

3.2.4.4 Ultracentrifugation.

Bulk lipoprotein fractions were prepared in a 12 x 38.5 ml rotor (Kontron TFT 50.38) either in a Kontron Centrikon T-2060 ultracentrifuge or a Sorvall OTD-65B ultracentrifuge.

3.2.4.5 Adjustment of Plasma Density.

The following equation was used to calculate the volume of density solution required to float lipoproteins:

$$V_1 \cdot d_1 + V_2 \cdot d_2 = (V_1 + V_2) \cdot d_3$$

Where:  
$V_1 =$ Volume of plasma (or ultrafiltrate)  
$V_2 =$ Volume of density solution to be added  
$d_1 =$ Density of plasma (or ultrafiltrate)  
$d_2 =$ Density solution used for adjustment  
$d_3 =$ Density required
Sample Calculation:

Adjustment of density for chylomicron, VLDL, and IDL isolation.

Thus, for a typical isolation in the 29 ml capacity screw-capped polycarbonate centrifuge tubes used: \( V_1 = 19.333 \text{ ml}, V_2 = 9.667 \text{ ml}, d_1 = 1.006 \text{ g/ml}, d_2 = \text{Unknown}, \) and \( d_3 = 1.019 \text{ g/ml} \).

\[
\begin{align*}
(19.333 \times 1.006) + 9.667 \times d_2 &= 29 \times 1.019 \\
19.448998 + 9.667d_2 &= 29.551 \\
9.667d_2 &= 10.102002 \\
d_2 &= 1.045 \text{ g/ml}
\end{align*}
\]

3.2.4.6 Chylomicron, VLDL, and IDL Preparation.

The first step in the purification of apoE is the isolation of the apoE-containing lipoproteins which have \( d<1.019 \text{ g/ml} \) (chylomicrons, VLDL and IDL). Thus, 19.333 ml of plasma was placed in a screw-capped polycarbonate centrifuge tube (Kontron Instruments Ltd) and its density adjusted to 1.019 g/ml by adding 9.667 ml of 1.045 g/ml density solution. The samples were centrifuged for 20 h at 37,000 rpm (105,000 g) and 16 °C. The lipoprotein fraction that floated to the top of the tube was collected in less than 4 ml, using a syringe and needle. The washing step involved refloating the lipoproteins at their density of isolation. Therefore, for this fraction, the lipoproteins were diluted to 29 ml with the stock 1.019 g/ml density solution and recentrifuged. For methods of purifying apoE from these isolated lipoproteins see Section 3.2.7.

3.2.4.7 LDL Preparation.

The solution below the chylomicron, VLDL and IDL fraction was removed and discarded such that 19.333 ml remained in the centrifuge tube. Its density was adjusted to 1.065 g/ml by adding 9.667 ml of 1.157 g/ml density solution and mixed. The samples were again centrifuged for 20 h at 37,000 rpm (105,000 g) and 16 °C. The LDL fraction was not used in my studies so was collected and then discarded.

3.2.4.8 HDL Preparation.

After LDL removal, HDL was prepared either as total HDL (\( d=1.063-1.21 \text{ g/ml} \)) or subfractions: HDL\(_2\) (\( d=1.063-1.125 \text{ g/ml} \)) and HDL\(_3\) (\( d=1.125-1.21 \text{ g/ml} \)). Alternatively, these fractions were prepared from plasma after precipitation of the apoB-containing lipoproteins followed by the appropriate ultracentrifugation steps.
**Total HDL Preparation.**

The solution below the LDL fraction was adjusted to 19.333 ml and its density was adjusted to 1.21 g/ml by adding 9.667 ml of 1.478 g/ml density solution and mixed. The samples were centrifuged for 40 h at 37,000 rpm (105,000 g) and 16 °C, HDL was then removed and washed by recentrifugation at density of isolation (1.21 g/ml).

**HDL\(_{2}\) Preparation.**

The solution below the LDL fraction was adjusted to 19.333 ml and its density was adjusted to 1.125 g/ml by adding 3.29 ml of 1.478 g/ml density solution and 6.38 ml of 1.125 g/ml solution. The isolation and subsequent washing was performed as outlined for total HDL.

**HDL\(_{3}\) Preparation.**

The solution below the HDL\(_{2}\) fraction was removed and discarded such that 19.333 ml remained in the centrifuge tube. Its density was adjusted to 1.21 g/ml by adding 6.13 ml of 1.478 g/ml density solution and 3.54 ml of 1.21 g/ml solution. The isolation and washing was performed as for total HDL.

**Preparation of HDL Following Magnesium/Phosphotungstic Acid Precipitation.**

Due to the specific interaction of apoB with a number of precipitating agents, apoB-containing lipoproteins can be separated from non-apoB-containing lipoproteins. Thus, treating plasma will remove chylomicrons, VLDL, IDL and LDL leaving only HDL, which can be isolated by a single ultracentrifugation step. A number of precipitation methods are available for use, the most popular being magnesium/phosphotungstic acid [345]. One hundred µl of 0.5 M MgCl\(_2\) and 100 µl of 4 % (w/v) phosphotungstic acid (in 0.19 M NaOH) were added to every 1 ml of fresh plasma and mixed well. The plasma was then centrifuged immediately for 20 min at 2,000 g and 20 °C. This step precipitated all apoB-containing lipoproteins, leaving plasma with HDL as the sole lipoprotein. Total HDL (or HDL\(_{2}\) and HDL\(_{3}\)) was isolated by altering the density of the plasma (still at 1.006 g/ml) and performing ultracentrifugation as before.

**3.2.5 HEPARIN-SEPHAROSE PURIFICATION OF HDL-E.**

Total HDL was isolated as described above and then subfractioned by heparin-Sepharose affinity chromatography, a technique that takes advantage of the heparin-binding property of apoE to separate it from contaminating proteins. Total HDL was subfractionated by chromatography at 4 °C in a 10 x 1.5 cm glass column with 10 ml bed volume (Amersham Pharmacia Biotech UK Ltd, St. Albans, UK). The heparin-Sepharose
was equilibrated with buffer 1 (50 mM NaCl, 5 mM Tris.HCl, 25 mM MnCl₂, pH 7.4). Solid MnCl₂ was added to the total HDL to give a final Mn²⁺ concentration of 25 mM and this mixture was then applied to the column via a peristaltic pump at a rate of 0.5 ml/min. The unbound fraction (apoA-rich HDL) was washed away with buffer 1 until the A₂₈₀ returned to the baseline. The bound fraction (apoE-rich HDL) was eluted with buffer 2 (95 mM NaCl, 5 mM Tris.HCl, pH 7.4) and the column was regenerated by sequential washes of buffer 3 (600 mM NaCl, 5 mM Tris.HCl, pH 7.4) to remove additional material (traces of Lp(a) or LDL), and then buffer 1 containing 0.02 % (w/v) sodium azide but excluding MnCl₂. The collected HDL-E fractions were concentrated in Vivaspin 15 ml concentrators (30,000 MWCO; Vivascience Ltd, Binbrook, UK) and then prepared for incubation with cells as described below.

3.2.6 Treatment of Isolated HDL Prior to Cell Incubation.

All HDL preparations (HDL₂, HDL₃, total HDL and HDL-E) were desalted by extensive dialysis against PBS (containing no azide) or, in some cases, using a PD-10 column (Amersham Pharmacia Biotech UK Ltd). All HDL was used on the day of preparation or, if stored at 4 °C, within 2 weeks. Immediately before addition to cells, samples were further dialysed against supplemented M199 media (excluding FBS), sterile filtered using a 0.8/0.2 μM syringe-top dual filter (Supor Acrodisc; Gelman Sciences, Northampton, UK) and assayed for total protein concentration by Bradford assay (Section 3.2.2). Finally, FBS was added back into the media to give a final concentration of 20 % (v/v).

3.2.7 Isolation of Apolipoprotein E: Delipidation and Affinity Chromatography.

The first step in the purification of apoE from the plasma of apoE3/3 individuals was the isolation of triglyceride-rich apoE-containing lipoproteins chylomicrons, VLDL and IDL (d<1.019 g/ml) by ultracentrifugation floatation (Section 3.2.4.6). Next, the lipoproteins were lyophilized, delipidated with organic solvents [347] and then the apolipoproteins (except for insoluble apoB) were resolubilized in a guanidium-containing buffer. ApoE was then purified from this apolipoprotein solution by heparin-Sepharose affinity chromatography [348] and its purity verified by SDS-PAGE (Section 3.2.3).

3.2.7.1 Lipoprotein Lyophilization.

The d<1.019 g/ml lipoprotein preparation was separated into 4 x 200 ml conical glass tubes, snap frozen in liquid nitrogen and freeze-dried until only 1-2 ml remained. At this stage the frozen lipoproteins were either stored at -70 °C or immediately delipidated.
3.2.7.2 Delipidation.

The lipoprotein preparations were defrosted and vigorously stirred magnetically. To each tube of stirring lipoprotein, 60 ml of ice cold methanol was added, followed by 140 ml of diethyl ether. The tubes containing precipitated apolipoprotein were allowed to settle on ice. The solvent was removed by aspiration and the precipitate washed in 120 ml diethyl ether and allowed to settle again. This wash was repeated twice. The final pellet was allowed to dry until moist. Each pellet was then dissolved in about 25 ml of 0.2 M Tris.HCl, pH 8, containing 2 M guanidium HCl. The solubilized apolipoproteins were pooled and transferred into two 50 ml centrifuge tubes and centrifuged at 2,000 g for 15 min to remove the insoluble apoB. The supernatant was filtered and transferred to dialysis tubing and dialysed versus 4 x 21 changes of 25 mM ammonium bicarbonate.

3.2.7.3 Heparin-Sepharose Affinity Chromatography.

The dialysed apolipoprotein solution in 25 mM ammonium bicarbonate (supplemented with 0.1 % v/v mercaptoethanol) was bound to a 1 ml Hi-trap heparin-Sepharose column (Amersham Pharmacia Biotech UK Ltd) pre-equilibrated in the same buffer. The column was washed with 25 ml equilibration buffer, and then the apoE was eluted with 0.75 M ammonium bicarbonate. The apoE-containing fractions were pooled and assayed for protein content using the Bradford assay (Section 3.2.2). Purity was assessed by performing SDS-PAGE on a 15 % gel (Section 3.2.3). This procedure typically produced apoE of >95 % purity with albumin being the major contaminant. The pure apoE was separated into 0.5 mg aliquots and stored at -70 °C for up to 1 year.

3.2.8 Preparation of ApoE:DMPC Complexes.

Although apoE-containing lipoproteins bind to apoE receptors, purified apoE devoid of lipids does not [337]. The apoE must be recombined with phospholipid to confer an appropriate structural conformation to restore its receptor binding and biological activity [336]. The procedure below describes recombining the apoE with DMPC phospholipid, to produce apoE:DMPC complexes that have been shown to bind with high affinity to the apoE receptors [337].

3.2.8.1 Production of DMPC Vesicles.

DMPC (40 mg) was weighed into a 50 ml glass beaker, to which 10 ml of PBS was added. The DMPC was allowed to hydrate for 10 min at 20 °C and then sonicated (Sanyo Soniprep 150, small probe, setting an amplitude of 8 microns) for 30 min or until the solution was slightly translucent. The DMPC liposomes were then micro-centrifuged at
13,000 g for 5 min to remove the titanium released from the sonication probe. This solution of DMPC vesicles was stored at room temperature.

3.2.8.2 Production of ApoE:DMPC.

An aliquot of apoE, isolated as described in Section 3.2.7, was reduced by the addition of β-mercaptoethanol (0.5 μl/100 μg of apoE) for 30 min at room temperature. This is essential as both apoE3 and apoE2 form intramolecular disulphide bonds, which hamper apoE-lipid interactions. The DMPC vesicles were added to the protein (3.75 mg of vesicles per 1 mg of apoE), and the mixture was incubated on a rocking platform for 3 h at room temperature to allow apoE to incorporate into the vesicles. ApoE:DMPC complexes were then separated from any uncomplexed, free apoE by flotation ultracentrifugation [337]. Thus, the apoE:DMPC complexes were adjusted to a density of 1.21 g/ml, centrifuged at 105,000 g for 20 h, and then the translucent apoE:DMPC collected carefully from the top of the tube. The apoE:DMPC complexes were homogeneous discs, 18.8±1.5 nm in diameter and 3.5±0.4 nm in width, which had a tendency to stack in multiples of 5 or more (Figures 3.3-1 and 4.3-10) as assessed by negative staining transmission electron microscopy (TEM, Section 3.2.8.3). After extensive dialysis against PBS, the apoE:DMPC preparations, stored at 4 °C, were stable and retained full biological activity for up to 1 month. Several different batches of apoE:DMPC were used and each preparation was checked for apoE activity as judged by its ability to inhibit agonist-induced platelet aggregation (methods and data not shown; assays performed by Dr D. Riddell in our group [276]). For experiments with HUVECs, apoE:DMPC and control samples of phospholipid carrier (DMPC), were further dialysed against supplemented M199 media (excluding FBS) and sterile filtered immediately prior to use. Protein and DMPC concentrations were measured using commercially available methods (Sections 3.2.2 and 3.2.8.4, respectively). Finally, FBS was added back into the media to give a final concentration of 20 % (v/v).

3.2.8.3 Characterization of DMPC Complexes by Electron Microscopy.

The morphology of the DMPC liposomes and apoE:DMPC complexes was examined using negative staining TEM [349]. Briefly, 1 μg of apoE:DMPC protein was placed on a Formvar-coated grid for 5 min at 23 °C. The excess fluid was dried with filter paper, after which 10 μl of 1 % uranyl acetate (pH 2.5) was placed on the grid and immediately dried. The grids were subsequently viewed with a Philips 501 electron microscope and each sample photographed (Electron Microscopy Unit, RF&UCMS).
3.2.8.4 **Phospholipid Measurement.**

Phospholipid concentration of both DMPC vesicles and apoE:DMPC complexes were measured after dialysis against supplemented M199 media (excluding FBS) and sterile filtering. Phospholipid content was quantitatively determined by a one-step enzymatic colourimetric method using the Phospholipid B Kit following the manufacturer's instructions.

Briefly, a phospholipid standard curve (0-60 µg) was freshly prepared from a stock phospholipid solution each time the assay was performed. To standards and triplicate samples (20 µl in M199 media), 1 ml of colour reagent was added. Tubes were then incubated at 37 °C for 10 min before the A505 versus reagent blank was measured. Test samples were determined from a standard curve using Biosoft's Lowry analysis software. The presence of M199 media in samples was found not to interfere with the assay, nor was it found to contain any phospholipid.

3.2.9 **Cell-Bound VCAM-1 ELISA.**

This cell-bound ELISA for the measurement of VCAM-1 was performed essentially as described in Section 2.2.9. Briefly, HUVECs were seeded at 3 x 10^4 cells/well in 96-well plates pre-coated with gelatin and were incubated overnight to allow confluent monolayers to form. Cells were preincubated with varying concentrations of either apoE:DMPC (10-200 µg/ml) or HDL (0.2-1.5 mg/ml) for 24 h, washed with media and then treated with either 100 U/ml TNF-α (or suboptimal concentrations as indicated) for the last 6 h. Variations to this protocol included the use of serum-free M199 media supplemented with 1 % (w/v) BSA and the replacement of TNF-α with IL-1β (1 ng/ml or 100 U/ml) or LPS (5 µg/ml or 50 U/ml). For positive controls of VCAM-1 downregulation, parallel wells were either preincubated for 40 h with 17β-estradiol (10 µM) prior to TNF-α addition, or GSNO (200 µM) was added in a coincubation with cytokine. Plates were washed twice with pre-warmed PBS and fixed for VCAM-1 ELISA (Section 2.2.9). Absorbances were read at 492 nm (A492) against blank substrate. Finally, all traces of the chromogenic substrate were removed by washing with PBS and the relative amount of cellular protein per well was measured using the Bradford reagent. Briefly, monolayers were solublized with 100 µl 0.1 M NaOH (per well) during a 2 h incubation at 37 °C, 100 µl Bradford reagent added and optical density measured at 620 nm (A620) against blank substrate (with NaOH).
3.2.10 **Flow Cytometry Analysis of Cell Surface CAMs.**

Flow cytometry is a rapid and sensitive technique for making simultaneous measurement of the multiple physical or biological characteristics of a large number of suspended individual cells as they interact with a focused laser beam of defined wavelength. Cells are hydrodynamically focused to a serial cell flow, and at the time of laser-intercept, each cell generates scattered incident light; forward scatter (FS) light dependent on cell size and side scatter (SS or right-angle) light dependent on cellular internal granularity/complexity. Prestaining the cells with fluorochrome dyes or by fluorescent immunolabelling of antigenic epitopes results in a fluorescence light emission impulse (proportional to the extent of dye binding) after laser light excitation. All optical signals generated by a single cell are simultaneously recorded by photomultiplier tubes, processed into digital format and saved in Listmode data files to be recalled for further analysis.

These analyses were carried out after seeding HUVECs at $1.5 \times 10^5$ cells/well in 12-well plates and incubating overnight; apoE:DMPC/HDL experiments were then performed as outlined in Section 3.2.9. Plates were subsequently cooled on ice, the cells gently removed using a cell lifter (Corning Costar; High Wycombe, UK) and incubated in suspension for 45 min at 4 °C with either anti-VCAM-1 (5 μg/ml), ICAM-1 (1 μg/ml), E-selectin (5 μg/ml) or IgG1 negative control antibodies (at equivalent IgG1 protein concentrations) diluted in 0.1 % (w/v) BSA in PBS. After washing twice in cold PBS, primary antibody binding or negative control antibody binding was detected using goat anti-mouse FITC-conjugated antibodies (50 μg/ml in 0.1 % BSA in PBS) incubated at 4 °C for an additional 45 min. The cells were washed and then fixed in 1 % (w/v) paraformaldehyde in PBS as a single-cell suspension (confirmed by phase-contrast microscopy) for analysis by a fluorescence-activated cell sorter (FACS, Coulter Epics Elite; Coulter, Hialeah, USA) equipped with an argon ion laser providing an excitation wavelength of 488 nm (Cyontics model 2201, San Jose, USA), and standard computer and electronics. The cell population (excluding debris) was gated, using a ‘bit-map gate’, on a dual parameter display of side (SS) and forward scattered (FS) light using linear amplifications. FITC fluorescent emissions between 520-530 nm were collected using a 550 nm dichroic long pass filter, a 525 nm band pass filter and standard instrument optics. Analysis was performed on 5,000 gated events per sample. Log fluorescence signals were stored as Listmode data files. Figures were created using WinMDI Version 2.8 software (internet address: http://facs.scripps.edu).
3.2.11 MONOCYTE ADHESION ASSAY.

3.2.11.1 Fluorescent Dil-Labeling of THP-1 Monocytes.

THP-1 cells, a human cell line established from acute monocytic leukemia cells resembling monocytes [350], were cultured as a continuous suspension in 25 mM HEPES buffered RPMI-1640 media (ICN Pharmaceuticals Ltd; Basingstoke, UK), supplemented with 10 % (v/v) FBS, 100 µg/ml streptomycin, 100 IU/ml penicillin and 2 mM glutamine as described previously [351]. A stock solution of Dil fluorescent probe (15 mg/ml in DMSO, stored at 4 °C and protected from light) was pre-warmed to 37 °C and 50 µg Dil was added per 0.5 x 10^6 monocytes in every 1 ml of complete RPMI-1640 media (total DMSO concentration <0.5 %). Monocytes were incubated for 30 min at 37 °C to allow incorporation of the lipophilic probe, harvested by brief centrifugation (400 g, 5 min) and then washed twice in warm media to remove traces of unincorporated probe. Monocytes were labelled uniformly, as judged by fluorescence microscopy using a Nikon Diaphot (fitted with a 100 W mercury lamp and using the filter set up to provide an excitation wavelength of 488 nm), and were >95 % viable after the labelling procedure as assessed by Trypan Blue Exclusion (Section 2.2.4).

3.2.11.2 Adhesion Assay.

Confluent HUVECs in 96-well plates (3 x 10^4 cells per well) were exposed to either media containing 100 U/ml TNF-α (5 ng/ml) or to TNF-α-free media. In controls to suppress monocyte adhesion, HUVECs were pre-incubated for 24 h with media containing a final concentration of 1 mM L-arginine (adjusted for an L-arginine content of 333 µM in the media formulation) before adding TNF-α (100 U/ml) into the media for the last 6 h. Dil-labelled THP-1 monocytes in complete RPMI-1640 media were added to HUVEC monolayers (1 x 10^5 per well in 200 µl) and incubated for 30 min at 37 °C. Wells were washed twice with warm media and then once with warm PBS to remove any non-adherent cells before fixation with 1 % (w/v) paraformaldehyde in PBS. Plates were stored at 4 °C and wrapped in foil to exclude light prior to analysis using a Perkin Elmer Luminescence Spectrometer LS 50B (Perkin Elmer Applied Biosystems, Warrington, UK). Total fluorescence was measured for each well using excitation and emission wavelengths of 514 and 578 nm, respectively, subtracting any autofluorescence from HUVEC controls (without Dil-monocytes added).
3.2.11.3 SEM.

HUVECs were seeded at $1 \times 10^5$ cells per well in 8-well chamber permanox Teckwells and incubated overnight for confluent monolayers to form. Wells were treated with either 100 U/ml TNF-α (5 ng/ml) or with TNF-α-free media for 6 h. THP-1 monocytes were then added (3.3 $\times 10^5$ per well) at a ratio of 1:3.3 (HUVECs:Dil-monocytes as above) and incubated for 30 min at 37 °C. Wells were washed with pre-warmed media and then PBS, fixed in 3 % (v/v) glutaraldehyde (pH 7.4) and processed and analysed by the Electron Microscopy Unit (RF&UCMS), see Section 2.2.7.

3.2.12 CI1STH CELL CULTURE.

Cells of this spontaneously transformed HUVEC cell line were a gift from Dr G. Cockerill (BHF Cardiovascular Medicine Unit, Hammersmith Hospital, London, UK). These cells were cultured as described elsewhere [331]. Essentially, CI1STH cells were maintained in the same way as HUVECs (Section 2.2.3) with the exceptions that the amount of growth supplement in the media was increased to a final concentration of 50 μg/ml and that, due to a slower rate of proliferation, cells were passaged every 7 days and reseeded at high density (usually 1:2 split). Many experiments with HDL incubations were performed with both HUVECs and CI1STH cells in parallel, having been seeded at 3 $\times 10^4$ cells/well in 96-well plates pre-coated with gelatin.

3.2.13 STATISTICAL ANALYSIS.

Statistical analysis for n=3 independent experiments was performed using Student's t-test; results are shown as mean ±SE and P<0.05 was considered significant.

3.3 Results.

3.3.1 CHARACTERIZATION OF PURIFIED APOE AND APOE:DMPC COMPLEXES.

Every preparation of apoE, isolated from plasma of individuals homozygous for apoE3, was >95 % pure with only traces of albumin, as assessed by 15 % SDS-PAGE (Figure 3.3-1, panel A). Before incubation with cells, apoE needed to be recombined with phospholipid to restore its receptor binding and biological activity [336]. ApoE:DMPC complexes were produced, which bind with high affinity to apoE receptors [337] and have anti-platelet action [276]. Prior to complexing with apoE, DMPC liposomes were unilamellar spherical vesicles with a diameter of 35.9±3.6 nm and formed aggregates in solution, as assessed by negatively staining the liposomes and performing TEM analysis (Figure 3.3-1,
Panel B). ApoE:DMPC complexes appeared as homogeneous disc-like structures of 18.8±1.5 nm in diameter and 3.5±0.4 nm in width, which had a tendency to stack in multiples of 5 or more (Figure 3.3-1, panel C).

![Figure 3.3-1](image)

**Figure 3.3-1** Analysis of purified apoE by SDS-PAGE, and apoE:DMPC complexes by electron microscopy.

Panel A, purified apoE (1 µg sample) was analysed by 15% SDS-PAGE and Coomassie Blue staining (described in Section 3.2.3). Note, the apoE doublet, which is due to differences in glycosylation of the mature polypeptide. Suspensions of DMPC liposomes alone (panel B) and complexes of apoE with DMPC (apoE:DMPC; panel C) were subjected to negative staining TEM (details in Section 3.2.8.3). Electron micrographs show that DMPC liposomes were unilamellar vesicles with a diameter of 35.9±3.6 nm (panel B) whereas, apoE:DMPC complexes were homogeneous discs 18.8±1.5 nm in diameter and 3.5±0.4 nm in width, which had a tendency to stack (panel C). The white scale bar represents 100 nm.

**3.3.2 Physiological Concentrations of ApoE:DMPC Complexes Fail to Downregulate Cytokine-Induced VCAM-1 in HUVECs as Assessed by ELISA.**

A cell-bound VCAM-1 ELISA, described in Chapter 2, was used as a quantitative method of measuring VCAM-1 in confluent HUVECs. Total cell VCAM-1 i.e. both membrane and cytoplasmic, was measured as cells were fixed prior to antibody incubations. Although basal VCAM-1 was negligible, HUVECs responded rapidly to TNF-α, giving a 10-
fold increased signal for VCAM-1 after 6 h treatment with 100 U/ml (5 ng/ml) TNF-α (Figure 3.3-2). A range of apoE:DMPC concentrations ranging from low to supra-physiological levels (10-200 µg protein/ml) were preincubated with HUVECs overnight before cytokine stimulation for 6 h, but failed to decrease VCAM-1 induction beyond the phospholipid carrier control (DMPC) level, even at high concentration (Figure 3.3-2). Neither DMPC nor apoE:DMPC preincubation altered basal VCAM-1 expression (data not shown) confirming preparations were free of endotoxin contamination which might have activated the cells in a similar way to TNF-α or IL-1β (Section 2.3.1.2). Coincubation experiments were also performed in which cells were simultaneously exposed to apoE:DMPC and TNF-α, but no change in VCAM-1 induction was noted. Similarly, use of a sub-optimal dose of TNF-α, 1 U/ml, which limited VCAM-1 induction to 30 % of the standard 100 U/ml amount (Section 2.3.2), did not reveal a suppressive effect of apoE:DMPC (Figure D in Appendix II).

One explanation for these negative findings is that our HUVEC preparations may lack the ability to restrict upregulation of VCAM-1; alternatively, TNF-α may be an inappropriate cytokine to evaluate apoE modulation of CAM expression. These possibilities were examined by use of a NO donor and 17β-estradiol, both of which limit VCAM-1 induction by cytokines [101,105,107-109,112-114], and by testing another inflammatory cytokine, IL-1β. We found that a 6 h coincubation with 200 µM GSNO inhibited TNF-α-induced stimulation of VCAM-1 by 20.9±3.3 % (P<0.001; Figure 3.3-3, panel A). Similarly, a long 40 h preincubation with one high dose of 17β-estradiol (10 µM), which is reported to maximally inhibit VCAM-1 upregulation [113], caused 60.4±3.0 % inhibition (P<0.001; Figure 3.3-3, panel A). Neither GSNO nor 17β-estradiol was cytotoxic; no change in morphology or loss of cells was evident by phase-contrast microscopy and protein assays confirmed that cellular protein was not lost. Thus, the reduction of VCAM-1 by these agents appeared to be specific. Use of IL-1β supported these observations. VCAM-1 was readily stimulated by this cytokine (at 100 U/ml or 1 ng/ml) to an equivalent extent as with TNF-α, but the induction was not suppressed by the addition of apoE:DMPC to parallel wells (Figure 3.3-3, panel B). By contrast, GSNO and 17β-estradiol were effective inhibitors reducing expression of VCAM-1 by 23.0±2.8 % (P<0.001) and 43.6±3.2 % (P<0.001), respectively.
Figure 3.3-2 ApoE:DMPC has no effect on TNF-α-induced expression of VCAM-1 - analysis by ELISA.

HUVECs were incubated overnight with a range of apoE:DMPC concentrations (10-200 µg protein/ml) and then stimulated with 100 U/ml TNF-α for 6 h to induce VCAM-1 expression. Basal (unstimulated) cells had a negligible level of VCAM-1 (white bar) while after TNF-α treatment for 6 h VCAM-1 was increased 10-fold (black bar). Preincubation of cells with apoE:DMPC (diagonal bars) had no effect on the subsequent VCAM-1 induction as compared to the DMPC control (grey bar). An isotype-matched irrelevant antibody gave a low background signal (light grey bar). The results are from one representative experiment and each bar shows mean ± SE of the ELISA from quadruplicate wells. Three other experiments using different preparations of HUVECs and apoE:DMPC gave similar results.
Figure 3.3-3 Expression of cytokine-induced VCAM-1 is partially inhibited by GSNO, a nitric oxide donor, or by 17β-estradiol.

HUVECs were incubated for 6 h with 100 U/ml of either TNF-α (A) or IL-1β (B) to stimulate VCAM-1. Incubating HUVECs with 200 μM GSNO decreased VCAM-1 expression induced by TNF-α or IL-1β (both 100 U/ml). Preincubation with 10 μM 17β-estradiol for 40 h before cytokine stimulation caused a more pronounced inhibition, while addition of 0.02 % ethanol, the carrier, had no effect. In marked contrast, parallel wells containing apoE:DMPC (50 μg protein/ml) failed to suppress VCAM-1 expression and, indeed, was moderately stimulatory (10.5±1.8 % and 11.3±3.9 % with TNF-α and IL-1β, respectively; both P<0.05). However, this effect appeared due to the DMPC carrier and was not investigated further. Results show normalized data from three independent experiments performed in quadruplicate (mean ±SE).
3.3.3 **Flow Cytometric Analysis Confirms that ApoE:DMPC Has No Effect on HUVEC VCAM-1.**

Sensitive flow cytometry or FACS analysis for immunodetection of a large number of individual cells was used to verify our ELISA data. HUVECs were removed by gentle scraping and binding of primary antibody at the cell surface was detected using a FITC-conjugated secondary antibody (Section 3.2.10). The FITC fluorescence profiles are shown in Figure 3.3-4. Basal VCAM-1 was negligible with the majority of cell fluorescence values under 10 units (panel A), giving a similar profile to that of isotype-treated control cells (not shown). After 4 h exposure to TNF-α there was a clear right shift in the fluorescence peak, indicating upregulation of cell surface VCAM-1 (panel B). Preincubation with 50 μg protein/ml apoE:DMPC did not alter the fluorescence profile or mean fluorescence intensity (panel C), although 17β-estradiol was effective at suppressing VCAM-1 expression, as shown by the left shift in the fluorescence profile and by the corresponding fall in mean fluorescence (panel D). Although some HUVECs did appear more sensitive to 17β-estradiol, we found no evidence for a subpopulation of apoE-responsive cells which the ELISA would not distinguish; the FACS fluorescence profiles provided clear evidence that VCAM-1 expression on the cell surface was not suppressed by apoE:DMPC.

3.3.4 **Neither ICAM-1 Nor E-Selectin Are Suppressed by ApoE:DMPC.**

The ability of apoE:DMPC to regulate expression of two other endothelial CAMs was also examined: ICAM-1, another member of the IgSF, and E-selectin, which belongs to the selectin family of CAMs. Flow cytometry was used to establish that ICAM-1 was constitutively expressed on basal HUVECs giving a wide distribution of fluorescence from 0-100 units on the FACS histogram (Figure 3.3-5, panel A). Indeed, flow cytometry was particularly valuable for immunodetection of heterogeneous levels of ICAM-1 as, even in the basal state, the high levels of expression were above the limits of ELISA detection. After 4 h exposure to TNF-α, ICAM-1 was super-induced and most of the population expressed a higher level of cell surface ICAM-1 (panel B). However, neither apoE:DMPC (panel C) nor GSN0 or 17β-estradiol (data not shown) were able to inhibit this induction by TNF-α.

Although basal levels of E-selectin were negligible (<10 fluorescence units) apart from a separate subpopulation that were weakly positive (Figure 3.3-6, panel A), a 4 h incubation with TNF-α resulted in most cells expressing a high level of E-selectin (panel B). This induction was unaffected by preincubation with apoE:DMPC (panel C) but partially prevented by 17β-estradiol (panel D).
Figure 3.3-4 Flow cytometric analysis of TNF-α-induced expression of cell surface VCAM-1.

For FACS experiments, anti-VCAM-1 binding to HUVEC suspensions was detected using a secondary FITC-conjugated antibody before fixation in 1% (w/v) paraformaldehyde in PBS. Intact cells were gated on forward scatter versus side scatter amplifications and 5,000 gated cells were analysed for FITC fluorescence. Histograms show a 4 decade log^10 fluorescence scale against the number of gated cells or 'events' with corresponding fluorescence values. Basal VCAM-1 expression was negligible (A), but after 4 h exposure to TNF-α there was upregulation of cell-surface VCAM-1 (B). Preincubation with 50 µg protein/ml apoE:DMPC failed to alter VCAM-1 expression (C), although 17β-estradiol was an effective downregulator (D). Samples were analysed in duplicate but only single measurements are shown for a representative experiment. The results were confirmed in two independent assays.
Figure 3.3-5 Flow cytometric analysis of TNF-α-induced ICAM-1 expression.

Binding of anti-ICAM-1 was detected using FITC-labelled secondary antibodies and analysed by flow cytometry as described in Figure 3.3-4. The level of constitutive ICAM-1 was wide ranging in the basal state (A), but, following incubation with TNF-α (100 U/ml), most of the population had a higher level of cell surface ICAM-1 (B). ApoE:DMPC (50 μg protein/ml) did not affect this cytokine-induced expression of ICAM-1 (C). The histograms shown are from one representative experiment but were reproduced in other independent experiments.
Although basal expression of E-selectin was negligible with only a sub population being positive (A), cell-surface E-selectin accumulated after addition of TNF-α (100 U/ml) for 4 h (B). This induction of E-selectin was unaffected by incubation with apoE:DMPC (50 μg protein/ml) (C), whereas 17β-estradiol could prevent much of the upregulation (D). The histograms shown are from one representative experiment but were reproduced in other independent experiments.

3.3.5 MONOCYTE ADHESION ASSAY.

The process of monocytes adhering to endothelial cells is complex and requires a cascade of cell-cell interactions mediated by many different CAMs (as discussed in Chapter 1). Therefore, a sensitive fluorescent assay of monocyte adhesion to HUVEC monolayers under non-flow conditions was established to test whether apoE:DMPC could affect this functional process. THP-1 cells, a human cell line established from acute monocytic leukemia cells [350], were used as a model for human blood monocytes in these assays. These cells have been widely used to study monocyte/macrophage involvement in atherogenesis having retained numerous monocyte characteristics and representing a homogeneous population, avoiding the limitations of isolating human blood monocytes [352]. THP-1 monocytes were labelled with Dil (DiIC₃₄(3)), a highly fluorescent membrane probe commonly used to label either cell membranes (e.g. to study leukocyte adhesion to endothelium [353]) or lipoprotein particles (e.g. to study cellular interactions [351,354]). Dil is amphiphilic and associates with the cell membrane in the same way as phospholipids; its two hydrocarbon tails of 18 carbons in length imbed parallel to the phospholipids, while the fluorescent part lays parallel with the surface of the cell [355]. Importantly, once
incorporated, Dil does not exchange or transfer between membranes [353,356,357] nor does it significantly affect cellular function including cell-cell adhesion [358].

When a suspension of Dil-monocytes was incubated for 30 min with HUVECs and then non-adherent cells removed by washing, TNF-α-stimulated HUVEC monolayers supported approximately a 2-fold increase in adhesion above unstimulated cells as assessed by cell counting. Adherent Dil-monocytes per field increased from 117±4 to 269±2 with 6 h TNF-α treatment (100 U/ml) counting a minimum of 10 fields by fluorescence microscopy (using the x 10 objective lens). This increased monocyte adhesion was also demonstrated by SEM using non-labelled monocytes (Figure 3.3-7).

![Figure 3.3-7 Scanning electron micrographs of THP-1 monocytes adhering to HUVEC monolayers.](image)

**Figure 3.3-7** Scanning electron micrographs of THP-1 monocytes adhering to HUVEC monolayers.

*THP-1 monocytes were incubated with HUVEC monolayers for 30 min before washing away non-adherent cells and fixing for SEM analysis. Basal monocyte adhesion (A) was increased when HUVECs were stimulated with TNF-α for 6 h prior to monocyte incubation (B). White scale bar represents 50 μm.*
However, cell counting can be subject to bias and so further analyses used a fluorescence plate reader for detection. The increase in Dil-monoocyte adhesion after HUVEC TNF-α treatment was verified by this technique; the fluorescence signal at 578 nm increased from 5±2 to 10±3 fluorescent units upon TNF-α-exposure. However, preincubation with apoE:DMPC had no effect in this functional assay (Figure 3.3-8). By contrast, a 24 h preincubation with 1 mM L-arginine, the physiological substrate for NOS and a known suppressor of monocyte adhesion [359], significantly inhibited this 2-fold increase by 64±16 % (Figure 3.3-8). This suppression was comparable to a report in which L-arginine supplementation (1 mM) decreased human monocyte adhesion to HUVECs to a similar degree and corresponded with the downregulation of both VCAM-1 and ICAM-1 [359].

![Figure 3.3-8 Monocyte adhesion to TNF-α-treated HUVEC monolayers is inhibited by L-arginine, a known suppressor of CAM expression.](image)

HUVECs were activated with TNF-α (100 U/ml) for 6 h and then incubated for 30 min with Dil-monoocytes. Adhesion was assessed by detection of fluorescent emissions at 578 nm (after excitation with a 514 nm laser) with a fluorescence plate reader. When HUVECs were pretreated with 1 mM L-arginine prior to TNF-α-exposure, Dil-monoocyte adhesion was reduced to 36±16 % (grey bar, *P<0.05) from the level of the TNF-α-stimulated control (100±7.2 %, black bar). L-arginine supplementation had no effect on the level of adhesion to non-stimulated monolayers (data not shown). Preincubation of HUVECs with apoE:DMPC (50 µg protein/ml) did not reduce Dil-monoocyte adhesion (diagonal bar). Graph shows one experiment performed in quadruplicate. Data were calculated as the fluorescent emission corresponding to TNF-α-induced monocyte adhesion (minus the basal level) and expressed as a % of the TNF-α control (=100 %).
3.3.6 **HDL-E Fails to Downregulate VCAM-1 Expression.**

Although my synthetic apoE:DMPC preparations were biologically active, as assessed by their ability to inhibit platelet aggregation (data not shown) [276], they appeared not to have an effect on the cytokine-mediated upregulation of CAMs or on the process of monocyte adhesion to HUVEC monolayers. However, as purification protocols may attenuate apoE biological activity [360], it was prudent to test the potential CAM-modulatory effect of HDL-E, the minor subclass of HDL rich in apoE, that is considered more representative of native circulating apoE particles. HDL-E was isolated from the plasma of cirrhotic patients with greatly increased apoE levels [343] and, importantly, was potent in platelet assays [338].

Confluent HUVEC monolayers were preincubated for 16 h with HDL-E at a high physiological level (1.5 mg HDL-E protein/ml) before treatment with TNF-α (100 U/ml) for 6 h and analysis of cell-surface VCAM-1 by flow cytometry. Pretreatment with HDL-E did not alter the fluorescence profile or mean fluorescence intensity of TNF-α-stimulated HUVECs (Figure 3.3-9). This analysis technique was also used in IL-1β stimulations, and still showed that HDL-E failed to modulate VCAM-1 (data not shown). Furthermore, HDL-E was benign in ELISA assays (97.1±7 % VCAM-1 induction vs. 100±1.6 % for control, n=3, Table 3.3-1). Each HDL-E batch was biologically active, as judged by inhibition of platelet aggregation [338] (data not shown) and, as HDL-E incubation did not alter basal VCAM-1 expression (data not shown), was considered free from endotoxin contamination that might have activated the cells in a similar way to cytokines. In all experiments, the findings were not influenced by any cytotoxicity as judged by microscopic evaluation.

The result that HDL-E was benign in VCAM-1 experiments was puzzling as total HDL has been described as a potent inhibitor of CAM upregulation by cytokines [83]. Indeed, it is thought that the apoAI and apoAII components of HDL are essential for such an affect [83,146-148] and although our HDL-E was enriched in apoE, apoAI was still the major apolipoprotein and so an inhibitory effect was expected. These discrepant observations clearly needed to be pursued further.
Figure 3.3.9 Flow cytometric analysis reveals the inability of HDL-E to suppress TNF-α-induced VCAM-1 expression.

Confluent HUVEC monolayers were pre-incubated for 16 h with plasma-purified HDL-E (1.5 mg protein/ml) in serum-containing media before exposure to TNF-α (100 U/ml) for 6 h. FACS histograms show a 3-decade log fluorescence scale against the number of gated cells or 'events' with corresponding fluorescence values. Low basal cell surface VCAM-1 expression (A), was increased after 6 h exposure to TNF-α (B). A 16 h preincubation with 1.5 mg/ml HDL-E failed to reduce VCAM-1 expression (C). Samples were analysed in duplicate but only single measurements are shown for a representative experiment. The results were confirmed in other independent assays using different batches of HUVECs and HDL-E preparations.
Treâhfltc&t VCAJVM liidtiedon (%  control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VCAM-1 Induction (% of control)</th>
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<tbody>
<tr>
<td>Total HDL</td>
<td>106.6±1.9*</td>
</tr>
<tr>
<td>HDL$_3$</td>
<td>106.9±7.7</td>
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<tr>
<td>HDL$_2$</td>
<td>111.5±4.7</td>
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<tr>
<td>HDL-E</td>
<td>97.1±7.0</td>
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Table 3.3-1 Inability of HDL subclasses to inhibit VCAM-1 induction.

Preincubation of HUVECs with either HDL$_3$, HDL$_2$, or HDL-E for 16 h failed to suppress VCAM-1 upregulation by 100 U/ml TNF-α (P>0.05). Data is from three independent experiments performed in quadruplicate (mean □ SE). Moreover, total HDL was moderately stimulatory causing 7±2% induction above the TNF-α control (*P<0.01, hatched bar, n=10), although no stimulatory effect on the level of basal VCAM-1 was detected (data not shown). No cytotoxicity was caused by any of the HDL treatments. Data are expressed as VCAM-1 induction (above the basal level) normalized to a % of the TNF-α control (100±1.6 %).

3.3.7 Lack of VCAM-1 inhibition by HDL - Flow Cytometric and ELISA Analyses.

Total HDL from normal, healthy individuals was substituted for HDL-E in the previous group of experiments with analyses performed by both FACS and ELISA. Preincubation of HUVECs with 1.5 mg HDL protein/ml for 16 h before TNF-α treatment, did not alter the fluorescence profile or mean fluorescence intensity (Figure 3.3-10).

ELISA verified the FACS data. Preincubating cells for 16 h with total HDL (1.5 mg protein/ml) before cytokine stimulation failed to restrict VCAM-1 induction, even at suboptimal TNF-α concentrations with limited VCAM-1 response (Figure 3.3-11). Indeed, when 9 individual donors of HDL and 3 batches of HUVECs were tested, total HDL proved to be mildly, but significantly, stimulatory (7±2% greater induction than with TNF-α alone, P<0.01, Table 3.3-1). HDL$_2$ and HDL$_3$ subfractions were also ineffectual at downregulating VCAM-1 expression (Table 3.3-1) in contrast to a report that both subfractions are inhibitory [146].
Figure 3.3.10 Inability of HDL to downregulate TNF-α-induced VCAM-1 in HUVECs.

FACS histograms show a 3-decade log fluorescence scale against the number of gated cells or 'events' with corresponding fluorescence values. Basal cell surface VCAM-1 expression (A) was increased after 4 h exposure to 100 U/ml TNF-α (B). A 16 h preincubation with total HDL (1.5 mg protein/ml) failed to reduce VCAM-1 expression (C), although in parallel wells, preincubation with 10 μM 17β-estradiol was an effective downregulator (D). Samples were analysed in duplicate but only single measurements are shown for a representative experiment. The results were confirmed in three independent assays using different batches of HUVECs and HDL preparations.
H.U.V.E:C monolayers were preincubated for 16 h with HDL (1.5 mg protein/ml) and then stimulated with TNF-α for 6 h to induce VCAM-1 expression. Decreasing concentrations of TNF-α (100-0.1 U/ml) were used to examine whether an inhibitory effect of total HDL could be revealed. ELISA gave a low background signal, as shown by isotype-matched irrelevant antibodies (grey bar), while unstimulated cells had insignificant basal VCAM-1 expression (white bar). A 10-fold induction above basal was seen after treatment with 100 U/ml TNF-α and this was reduced in a stepwise manner with decreasing additions of cytokine (black bars). Preincubation with total HDL (hatched bars) had no effect on either basal VCAM-1 or on subsequent VCAM-1 induction as compared to the corresponding TNF-α controls. Cellular protein was unaltered by any of the treatments described. Results are from a representative experiment performed in quadruplicate (mean ± SE) but were reproduced in two other independent assays.
Several additional studies were carried out in which experimental conditions were varied. In each case, and using 10 different preparations of low-passage HUVECs, HDL consistently failed to suppress TNF-α-induced induction of VCAM-1. Thus, use of a range of HDL concentrations (0.1-1.5 mg/mL) and pre-incubation times (1, 6 and 16 h), or adding TNF-α without prior removal of the HDL [83], all failed to reduce VCAM-1 induction (data not shown). Neither was a potential HDL suppressive activity on VCAM-1 upregulation counteracted by lipoproteins or other constituents of serum-containing media; parallel experiments on HUVECs cultured in serum-free media containing BSA (1%, w/v) showed no inhibitory effect of HDL (115±0.4 % vs. 100±7 % for control cells). Similarly, the inability of HDL to downregulate VCAM-1 was not due to the initial stimulus or to the source and mode of preparation of HDL. Two other agents, IL-1β (1 ng/ml or 100 U/ml) and LPS (5 μg/ml or 50 U/ml) readily achieved upregulation of VCAM-1 but these inductions were not suppressed by HDL pre-treatment (104±5 % vs. 100±3 % for IL-1β control; 113±9 % vs. 100±8 % for LPS control). A range of low to high physiological concentrations (0.1-1.5 mg protein/ml) were used in some experiments, but none of the HDL doses had an modulatory effect on VCAM-1. Many of these experiments were performed using a transformed HUVEC cell line (C11STH cells from Dr G. Cockerill) in parallel to my low passage HUVECs using Dr G. Cockerill’s protocols [83,331]. Although C11STH cells were found to have poor expression of certain endothelial markers, grossly abnormal morphology and a low rate of proliferation, they were activated by TNF-α or IL-1β to induce an equivalent level of VCAM-1 expression to HUVECs. However, in every instance, HDL was unable to inhibit this induction (data not shown). Inter-donor variation of the HDL was also eliminated by using blood from a total of 14 healthy individuals, while reducing the time to isolate HDL, by precipitating apoB-containing lipoproteins before ultracentrifugation and by use of desalting columns, made no difference to our ELISA or FACS measurements.

3.3.8 HDL IS UNABLE TO INHIBIT VCAM-1 AND E-SELECTIN EXPRESSION IN HUMAN CORONARY ARTERY ENDOTHELIAL CELLS (HCAECs).

After completing my HDL/HUVEC studies, similar experiments with HDL using primary cultures of HCAECs, a model directly relevant to blood vessels affected by atherosclerosis [117,118], were performed in collaboration with Dr S. Allen (Department of Cardiothoracic Surgery, Harefield Hospital, Middlesex, UK). In brief, confluent HCAEC monolayers were preincubated for 1 h with total HDL concentrations ranging from low to high physiological levels (0.25–2 mg protein/ml) before cytokine stimulation with TNF-α (10
ng/ml) for 4 h and analysis by flow cytometry. In control wells, without HDL pretreatment, 54±10 % of cells treated with TNF-α were positive for VCAM-1 expression after 4 h as compared to 6±1 % before stimulation (Figure 3.3-12). Increasing amounts of HDL had no effect on either basal VCAM-1 (data not shown) or on the subsequent upregulation of VCAM-1 by TNF-α (Figure 3.3-12). Similar findings were noted for E-selectin expression; cells positive for cell surface E-selectin increased from 16±9 % to 64±18 % after TNF-α stimulation, but HDL was ineffective at suppressing this upregulation, even at high concentrations (Figure 3.3.13).

Although insufficient for maximum downregulation, a 1 h preincubation time with HDL is reported to effectively inhibit CAM expression in HUVECs (~50 % maximal inhibition) [19]. Nevertheless, to exclude the possibility that HDL effects on HCAECs were dependent on exposure time, we extended the preincubation period to 16 h before addition of cytokine; no significant inhibition of either VCAM-1 or E-selectin was observed (data not shown). Other experiments tested the major HDL subfraction, HDL₃, as this is considered more inhibitory than HDL₂ [146], but this also failed to downregulate TNF-α-induced VCAM-1 or E-selectin. In all experiments, the findings were not influenced by cytotoxic effects of HDL, as judged by microscopic evaluation and Trypan Blue exclusion tests for cell viability.
Figure 3.3.12 HDL does not suppress TNF-α-induced VCAM-1 expression in HCAECs.

These experiments were performed by S. Khan (Department of Cardiothoracic Surgery, Harefield Hospital, Middlesex, UK). HCAECs were isolated from normal, non-atherosclerotic coronary arteries and cultured as described previously [117,118]. Confluent HCAEC monolayers were pre-incubated for 1 h with total HDL (0.25-2 mg protein/ml) in serum-containing media before washing with warm HDL-free media and exposure to TNF-α (10 ng/ml) for 4 h. For FACS analysis of CAM expression, cell suspensions were prepared by trypsin treatment before primary antibody binding and negative control antibody binding (at equivalent IgG protein concentrations) were detected using a secondary R-phycocerythrin (RPE)-conjugated antibody (DAKO Ltd). For each well, 5,000 gated intact cells were analysed for the percentage of cells staining positive for RPE fluorescence, indicating levels of cell surface VCAM-1, using a Coulter Epics XL-MCL FACS. Basal VCAM-1 expression (white bar) was low (6±1 % of cells positive) but was markedly increased by TNF-α stimulation (black bar) with expression of VCAM-1 reaching 54±10 %. A pre-incubation of 1 h with total HDL, before treatment with cytokine, did not significantly alter VCAM-1 expression (hatched bars). Bar graphs show mean ±SE of 4 independent experiments using different donors of total HDL and batches of HCAECs.
Figure 3.3.13 HDL does not suppress TNF-α-induced E-selectin expression in HCAECs.

These experiments were performed by S. Khan. Confluent HCAEC monolayers were pre-incubated for 1 h with total HDL (0.25-2 mg protein/ml) in serum-containing media before washing with warm HDL-free media and exposure to TNF-α (10 ng/ml) for 4 h. FACS analysis of E-selectin expression was essentially as described in Figure 3.3.12. For basal E-selectin expression (white bar) 16±9 % cells were positive but were markedly increased by TNF-α stimulation (black bar) with expression of E-selectin reaching 64±18 %. Pre-incubation of total HDL before treatment with cytokine, did not significantly alter TNF-α-induced E-selectin expression (hatched bars). Bar graphs show mean ±SE of 4 independent experiments using different donors of total HDL and batches of HCAECs.
3.4 Discussion

3.4.1 Plasma-Purified ApoE is Ineffective at Inhibiting CAM Upregulation in HUVECs.

In addition to clearing remnant lipoprotein particles and participating in RCT, several recent studies infer anti-atherogenic actions of apoE at lesion sites (discussed in Chapter 1). If tenable, our hypothesis that apoE could limit endothelial CAM upregulation by pro-inflammatory cytokines would be a significant additional anti-atherogenic role; monocyte adhesion and recruitment to focal areas of the arterial subendothelium, important early events in atherogenesis which are dependent upon localized endothelial CAM upregulation, would be diminished.

Using my VCAM-1 ELISA (described in Chapter 2), I demonstrated that each batch of early passage HUVEC cultures was responsive to TNF-α and that induced VCAM-1 expression was susceptible to downregulation by two different inhibitory agents. ELISA data showing that GSNO reduces VCAM-1 induction by 20-25% is consistent with other reports that NO donors down-regulate cytokine-stimulated VCAM-1 [101,105,107-109,114]. VCAM-1 gene expression is redox-sensitive and NO represses gene transcription, in part, by inhibition of NF-κB, a redox-sensitive transcription factor [85,107,109]. In addition, NO prevents formation of lipid hydroperoxides which may act as intracellular messenger signals in the activation of NF-κB [105]. We also confirmed [113] that our HUVEC cultures were responsive to 17β-estradiol, inasmuch as both IL-1β and TNF-α-induced VCAM-1 were strongly suppressed (40-60%) by this sterol. However, the mechanism underlying this effect is complex as 17β-estradiol not only upregulates endothelial NOS [111], but may also affect VCAM-1 transcription, possibly by estradiol receptor complexes interacting with components of the NF-κB signalling pathway, negatively affecting transcription [113].

Nevertheless, despite careful characterization of our HUVEC cultures and demonstration of VCAM-1 downregulation by known suppressors, we found that physiological or supraphysiological amounts of plasma-purified apoE did not affect cytokine-induced expression of VCAM-1. Thus, although we verified that our apoE:DMPC vesicles were biologically active as a platelet antagonist, they had no effect on VCAM-1 expression. Furthermore, they were ineffective in both pre- or coincubation studies with either TNF-α or IL-1β, and they failed to limit VCAM-1 induction by sub-optimal doses of TNF-α. These findings by ELISA were verified using FACS analysis for immunodetection of cell surface VCAM-1. This was important as, during immunocytochemical phenotyping of HUVECs,
we noted that CAM expression varied both in the basal state and in response to cytokines within the same culture, although the population was purely endothelial. However, although some HUVECs did appear more sensitive to 17β-estradiol, we found no evidence for a subpopulation of responsive cells which the ELISA would not distinguish; the FACS fluorescence profiles provided clear evidence that VCAM-1 expression on the cell surface was not suppressed by apoE:DMPC. Similarly, apoE:DMPC vesicles were also unable to suppress TNF-α-mediated hyper-induction of ICAM-1 and upregulation of E-selectin, two other CAMs implicated in inflammation and activation of vascular endothelium.

Plasma-purified apoE had no influence on the expression of endothelial VCAM-1, ICAM-1 or E-selectin, nor did it affect monocyte adherence to HUVEC monolayers, either before or after cytokine induction. L-arginine supplementation was inhibitory in this functional assay, consistent with another report [359]. ApoE:DMPC data was strengthened by studies using HDL-E, a native apoE-containing lipoprotein subclass of bulk plasma HDL, in place of apoE:DMPC complexes. Thus, HDL-E also failed to downregulate VCAM-1, implying that plasma apoE is unlikely to be important in limiting endothelial activation. However, whether apoE can regulate CAM expression in another model of vascular endothelium, such as in vitro co-cultures of human aortic endothelial and smooth muscle cells [361], or in vivo where apoE secreted by resident macrophages may be specifically targeted, remains to be investigated.

3.4.2 HDL IS INEFFECTIVE AT INHIBITING CAM UPREGULATION IN HUVECS.

We then tried to reproduce the original observation of VCAM-1 suppression by total HDL in HUVECs [83]. However, although induction of CAM expression by IL-1β or TNF-α was consistent with other reports [82,83], neither ELISA or FACS analysis could reveal an inhibition of VCAM-1 expression after prolonged preincubation of cells with HDL. Indeed, a modest induction (7 %, P<0.01) above the cytokine-stimulated level of VCAM-1 was seen after pretreatment with HDL (1.5 mg protein/ml), whereas a marked inhibition of about 90 % had been anticipated [83]. This inability of HDL to downregulate VCAM-1 was also seen with sub-optimal doses of TNF-α when only limited induction occurred.

Our discrepant data were not due to a general poor responsiveness of the HUVECs, as positive controls established that CAM expression was subject to downregulation. It is also unlikely that our HDL lacked biological potency; previous preparations, using identical methodology, have shown characteristic functional properties towards several cell types, including those cultured in vitro [136,362,363] or used ex vivo [137,338,364,365]. Donor
variation, which is reported to influence inhibitory activity [146], was excluded since we sampled HDL from 14 healthy individuals, both male and female with ages ranging from 20–50 y, while procedural changes to reduce isolation time also failed to yield HDL capable of inhibiting CAM expression. In addition, all HDL preparations had a minimal effect on basal VCAM-1 expression (see Figure 3.3-11); an important observation, as contamination of HDL with endotoxin or oxidation products might have stimulated VCAM-1 expression and hence counteracted any downregulatory effect of the HDL. Finally, none of our experimental conditions caused cytotoxic effects, as judged by microscopic examination of cells, and by assays for cell viability and loss of cellular protein.

Although primary cultures of HUVECs are a well-established system to study human vascular endothelial cell biology in vitro and have been used extensively in CAM research [82], primary cultures of HCAECs are a model directly relevant to blood vessels affected by atherosclerosis [117,118] and therefore a preferred model to test the anti-atherogenic actions of HDL. However, we failed to demonstrate a reduced induction of VCAM-1 or E-selectin by TNF-α when cells were preincubated with HDL, or with its two major subfractions, HDL₂ and HDL₃, even though different plasma donors and HCAEC batches were used.

Although there are few reports of native HDL effects on CAM expression in HUVECs [83,145,146], several groups have studied reconstituted HDL particles (rHDL), mainly apoAI:phospholipid complexes. However, these studies have been contentious, possibly because purified apolipoproteins have variable activity [360]. Thus, while the original authors suggested that downregulatory effects of rHDL on CAM were comparable to native HDL [83], other reports found that rHDL only partially reduced (~30 %) VCAM-1 in HUVECs [147,148], while suppression of ICAM-1 and E-selectin was marginal (<10 %) [366].

We attempted to exchange materials and reagents with the author of the original report [83], but, for reasons outside our control, the discrepancy in our findings were not resolved. The only major difference in culture conditions and experimental set up was their routine use of heparin supplementation (0.05-20 mg/ml) in all culture media [83,146,147], equating to ~10-4,000 times the physiological dose in human plasma of ~5 μg/ml [367]. As discussed in Chapter 2, it was deemed unnecessary to supplement my media with heparin. The use of high doses of heparin in culture may cause artefacts [368], as heparin alters endothelial function by modulating ICAM-1 transcription in HUVECs [369], by affecting endothelial
interactions with leukocytes [370], and by decreasing NO production [371]. Heparin can also bind TNF-α, limiting its availability to interact with target cells [372].

In summary, although the hypothesis that HDL diminishes expression of CAMs on arterial endothelium, a prerequisite for capture of circulating leukocytes, is an attractive explanation for HDL anti-atherogenicity, we found no evidence for such a regulatory effect in human arterial endothelial cells in vitro. This finding is consistent with the failure of apoAII to prevent monocyte binding to human aortic endothelial cell monolayers [373] and, importantly, with a recent study in vivo which showed that apoAI inhibited atheroma formation in apoE-deficient mice by mechanisms independent of VCAM-1 suppression [310]. Thus, although further studies are needed to understand HDL interactions with vascular endothelial cells, we conclude that attenuation of CAM induction on arterial endothelium is unlikely to contribute to protective actions of HDL in vivo.
Chapter 4
4. DOES LOCALLY-SECRETED APOE INHIBIT VCAM-1 EXPRESSION IN HUMAN ENDOTHELIAL CELLS?

4.1 Introduction.

In the previous Chapter, I outlined that apoE, either plasma-purified and reconstituted into phospholipid vesicles (apoE:DMPC) or as apoE-rich HDL (HDL-E), failed to inhibit endothelial activation in response to cytokines. I concluded that bulk plasma apoE was unlikely to modulate CAM expression in vivo. However, the apoE purification process could have caused artefactual results. Subtle differences in the conformation of apoE can alter its biological activity [167], while procedures to isolate apoE from plasma lipoproteins, including ultracentrifugation, delipidation and denaturation may disrupt endogenous conformation and change its oxidation state [360]. Indeed, purified apoE can exhibit a different biological activity from native protein [360]. Even the apoE-containing HDL used in Chapter 3 was isolated from plasma by prolonged ultracentrifugation and Mn^{2+}-heparin chromatography. Thus, this differs from native HDL-E particles. ApoE forms labile associations with lipoproteins and ultracentrifugation causes apoE to dissociate from the particle surface [374] or even redistribute to other lipoprotein classes [375]. It is therefore reasonable to assume that native preparations of unpurified apoE secreted from cultured cells could potentially be more physiologically relevant to study its effects on endothelial activation.

In vivo, endothelial cells are exposed to an abundance of locally-derived apoE synthesized by the cholesterol-loaded macrophages found within atherosclerotic plaques [37,38]. Therefore, although HDL-E and apoE:DMPC were inactive, we could not exclude the possibility that locally-secreted, cell-derived apoE may suppress endothelial CAM expression. Establishing an in vitro model of endothelial exposure to locally-synthesized apoE to test this hypothesis needed careful consideration. An obvious model would be the co-culture of monocytes/macrophages (either the human THP-1 cell line or preferably human blood monocytes/macrophages) with endothelial cells. However, as well as secreting apoE in culture [183], macrophages also produce another apolipoprotein, serum amyloid A3, a component of certain HDL subfractions [376]. Additionally, macrophages release numerous other biologically relevant secretory factors relating to their role in lipid metabolism, in immune and inflammatory responses, in growth control, in matrix accumulation and remodelling, and in thrombosis during atherogenesis [32]. These include enzymes (e.g. LPL, lipoxygenases, matrix-degrading metalloproteinases), chemoattractants or chemokines (e.g.
MCP-1), growth regulatory molecules (e.g. platelet-derived growth factor or PDGF, fibroblast growth factor), prostaglandins (e.g. prostaglandin E2), factors controlling blood clotting (e.g. tissue factor and plasminogen activator inhibitor-1 or PAI-1) and superoxide [1,31]. It is presently unknown whether these substances can influence the activation state of the endothelial cells and modulate endothelial CAM expression. However, in addition to these factors, macrophages secrete substances known to have direct effects on endothelial cells [1,31]: endothelial mitogens (e.g. vascular endothelial growth factor, VEGF and transforming growth factor-α, TGF-α), antagonists of endothelial proliferation (e.g. TGF-β), cytokines that both modulate growth and stimulate endothelial CAMs (e.g. IL-1β, TNF-α) [377], and NO that affects redox sensitive NF-κB and therefore CAM expression [101,105,107-109,114].

In addition, co-cultures lead to cell-cell cross-talk that actually induces monocytes to secrete many of the above substances, making the system even more complex [378-382]. Furthermore, co-culturing monocytes with endothelial cells directly stimulates endothelial CAM expression to augment such cell-cell interactions in a positive feedback mechanism, partly mediated by the production of cytokines [149-151]. Co-cultures also enhance endothelial response to exogenous cytokines [152] or to LPS [153] and attenuate release of biologically active NO [383], which can regulate CAM expression. Moreover, adding TNF-α to stimulate endothelial VCAM-1 expression would also activate macrophages, altering the pattern of their secretory products, including apoE secretion [252]. Clearly, studying cell-derived apoE effects on endothelial cells in a macrophage-endothelial co-culture system would not allow unequivocal interpretation of the results.

To avoid such complicated cell-cell interactions, two alternative in vitro models were devised to mimic effects of macrophage-released apoE on endothelium in vivo. The first approach involved transiently transfecting HUVECs with an apoE expression plasmid so that the HUVECs produced their own source of native apoE without the need to introduce another cell type into the system. The second model was a co-culture of HUVECs with recombinant Chinese hamster ovary (CHO) cells overexpressing human apoE.

4.2 Specialized Materials and Methods.

4.2.1 MATERIALS.

Iscove's modified DMEM media, dialysed FBS, non-essential amino acids (MEM), CHO-SFMII media and LipofectAMINE Reagent were purchased from Life Technologies. Apo-Tek ApoE Kits and Endofree Plasmid Maxi Kits were purchased from PerImmune Inc.
(Rockville, USA) and Qiagen Ltd (Crawley, UK), respectively. Hybond ECL nitrocellulose membrane, secondary antibody-HRP conjugates, enhanced chemiluminescence (ECL) substrate and Hyperfilm ECL were all supplied by Amersham Pharmacia Biotech UK Ltd. HYDRAGEL LIPO + Lp(a) kit was purchased from Sebia, Issy-les-Moulineaux, France.

Plasmids pCMV.0, pCMV.apoE2, pCMV.apoE3, and pCMV.apoE4 were all supplied by T. Athanasopoulos (Royal Holloway, University of London, UK). The cDNA for each apoE isoform had been excised from the corresponding pPUC19 (Dr J. Breslow, Rockefeller University, New York, USA) and ligated into pCMV.0 (originally purchased as pCMV.β from Clontech Laboratories Ltd, Basingstoke, UK). Recombinant CHO cells stably-expressing human apoE isoforms were generated by A. Tagalakis in our group. Monoclonal apoE antibodies (clone F5M3/A10) were a kind gift from Dr R. James (Hôpital Cantonal, Geneva, Switzerland). All other reagents, unless otherwise stated in the text, were purchased from Sigma-Aldrich Company Ltd.

4.2.2 Preparation and Purification of Plasmid DNA to Transfect HUVECs.

4.2.2.1 Transformation of Plasmid DNA in Competent E. Coli.

Expression plasmids encoding the cDNA for apoE isoforms (pCMV.apoE2, -3 or -4, for expression of apoE2, apoE3 and apoE4, respectively) and control plasmid without apoE cDNA insert (pCMV.0) were amplified in E. coli. For each plasmid, a 50 μl aliquot of competent E. coli bacteria (strain DH5α) was thawed on ice and mixed with 10 ng (~2 μl) of plasmid DNA. The bacteria were incubated on ice for 30 min and then heated at 42 °C for exactly 90 s before being returned to ice for 2 min, to induce a transient state of ‘competence’ during which time the bacteria took up the plasmid DNA. Next, 125 μl of L-broth (LB) medium was added and the tubes incubated for 45 min at 37 °C with shaking at ~150 rpm. To select the transformants, the contents of each tube were spread onto LB agar plates containing 100 μg/ml ampicillin, incubated at room temperature for 1 h and then incubated at 37 °C overnight. Since the plasmids contained an ampicillin resistance gene, only transformed cells grew. Positive colonies were selected and grown for 4 h in a sterile 20 ml tube containing 3 ml of LB media containing 100 μg/ml ampicillin with constant shaking (200 rpm) at 37 °C. This starter culture was then added to 100 ml of LB media (with 100 μg/ml ampicillin) in a conical flask and incubated overnight with shaking at 37 °C. Glycerol stocks of recombinant bacterial cultures were made for future use by mixing glycerol 1:1 with the bacteria and then storing 1 ml aliquots at -70 °C. The majority of the bacteria were harvested by centrifugation ready for isolation of plasmid DNA (see below).
4.2.2.2 Isolation and Purification of Endotoxin-Free Plasmid DNA.

The Endofree Plasmid Maxi Kit is designed to purify plasmid DNA by anion-exchange technology, whilst eliminating endotoxins/LPS which are membrane components of Gram-negative bacteria \textit{E. coli} released during the lysis step of plasmid purification. Endotoxin-free plasmid DNA is essential for transfection of primary human cells, such as HUVECs, which are difficult to transfect and are sensitive to endotoxin [384]. Removal of endotoxin contaminants improves transfection efficiencies, whilst ensuring that cells are not ‘activated’ (Section 4.3.1).

Following the manufacturer’s instructions for this kit, expression plasmids encoding the cDNA for apoE isoforms and the control plasmid were isolated from recombinant bacterial cultures, culminating in isopropanol precipitation of the plasmid DNA and reconstitution of the DNA pellet in endotoxin-free buffer. Prior to transfection experiments, plasmid preparations were further purified and sterilized by ethanol precipitation and resuspension in sterile endotoxin-free PBS. The purity and concentration of plasmid preparations was measured by UV spectrophotometry of diluted samples (typically 1/100 dilution) and determined using the equation below. At 260 nm, an optical density ($A_{260}$) of 1 corresponds to approximately 50 µg/ml of doubled stranded DNA.

$$\text{Plasmid DNA concentration (µg/µl)} = (A_{260} \times \text{dilution factor} \times 50 \, \text{µg/ml}) \div 1000$$

The ratio between readings at 260 and 280 nm ($A_{260}:A_{280}$) provides an estimate of purity of the DNA, since protein and phenol contaminants have an absorbance at 280 nm ($A_{280}$). Only plasmid preparations with a ratio of 1.8:1 to 2:1 ($A_{260}:A_{280}$) were considered pure enough for transfection; they consistently showed negligible cytotoxicity towards HUVECs, as assessed by phase-contrast microscopy and assays of cellular protein. Finally, apoE expression plasmid preparations were confirmed as having the correct apoE cDNA insert by genotyping [385,386]. This procedure, involving restriction digestion and agarose gel analysis, was performed by A. Tagalakis.

4.2.3 Transient Transfection of HUVECs with ApoE Expression Plasmids and Subsequent ApoE and VCAM-1 Measurements.

4.2.3.1 Cationic Liposome-Mediated Transfection.

LipofectAMINE Reagent is a stable liposome formulation. It is composed of the polycationic lipid 2,3-dioleloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (or DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (or DOPE) in a ratio of 3:1, DOSPA:DOPE (w/w). The positive
and neutral lipids form liposomes that can complex with nucleic acids such as plasmid DNA. The DNA-liposome complex then interacts with cell membranes (with a net negative charge) and facilitates the uptake of DNA into the cells. LipofectAMINE was chosen due to its success with 'hard-to-transfect' cells. The manufacturer's protocol was optimized for efficient HUVEC transfection and limited cytotoxicity. Adjustments included altering cell density at transfection, LipofectAMINE:DNA ratios and time periods for transfection (see below).

4.2.3.2 Optimized HUVEC Transfection Protocol.

HUVECs were seeded at a density of 2 x 10^4 cells per well in 96-well plates and allowed to adhere overnight to form an 80% confluent monolayer. Prior to transfection, plasmid preparations were incubated with LipofectAMINE in 'basic M199 media' excluding anti-bacterial agents and FBS, in a concentrated stock solution; 40 µg/ml DNA and 300 µg/ml LipofectAMINE (1:7.5 ratio of DNA to LipofectAMINE) in 10% of the final volume required for transfection. DNA-liposome complexes were allowed to form during a 15 min incubation at room temperature before being diluted in basic M199 media to give a final concentration of 4 µg/ml DNA and 30 µg/ml LipofectAMINE. HUVECs were washed with basic M199 media at 37 °C before incubation with 100 µl of transfection mixture per well (0.4 µg DNA and 3 µg LipofectAMINE per well of 2 x 10^4 cells). Cells were transfected for 1 h at 37 °C before the media was replaced with 200 µl of 'complete M199 media' supplemented with 5% (v/v) FBS and incubated for a further 48 h to allow apoE expression. Note, I had previously shown that reducing the concentration of FBS to 5% (v/v) still gave comparable expression of VCAM-1 (either basal or TNF-α-stimulated) to those cells in 20% FBS (data not shown).

4.2.3.3 VCAM-1 Measurements on Transfected HUVECs.

For the last 6 h of the 48 h post-transfection incubation period, TNF-α was added to the wells from a 100 U/ml stock in media to give a final concentration of 10 U/ml (0.5 ng/ml), a sub-optimal dose for VCAM-1 induction (Section 2.3.2), mixing thoroughly by gentle pipetting to ensure cells were equally exposed to the cytokine. TNF-α-free media was added to controls for basal VCAM-1 expression. At the end of this incubation, 200 µl of media from each well was immediately frozen at -20 °C until assayed for apoE content by ELISA (see below). HUVEC monolayers were washed with warm PBS, fixed and analysed for VCAM-1 by ELISA (Section 2.2.9). Finally, cellular protein was determined by solublizing
the monolayers, adding Bradford reagent and measuring the optical density at 620 nm ($A_{620}$), as described in Section 3.2.9.

4.2.3.4 Calculation of VCAM-1 ELISA Results.

VCAM-1 expression was calculated for each well as a ratio of VCAM-1:protein values ($A_{492}$ VCAM-1 assay:$A_{620}$ protein assay) for TNF-α stimulated cells; non-stimulated wells were then subtracted to give ‘VCAM-1 induction above basal expression’. Data were then normalized to % of the TNF-α control VCAM-1 induction, i.e. the pCMV.0 mock transfection (control=100%), due to variable VCAM-1 expression in different HUVEC batches. Subsequent data in this Chapter are expressed in this format.

4.2.3.5 Commercial ELISA for ApoE Measurement.

Cell culture samples for apoE determination were measured using the Apo-Tek ApoE Kit, following the manufacturer's protocol. Media from transient transfections was analysed undiluted. The detection limit of the ELISA was found to be 10-15 ng/ml apoE.

4.2.4 CHO CELL CULTURE.

The mutant CHO cell line, deficient in dihydrofolate reductase (CHO<sup>dhfr</sup>), was purchased from the ECACC (No. 94060607). These cells, with epithelial morphology, lack the DHFR gene so that they are defective in endogenous purine and pyrimidine base synthesis; as such, they have an absolute requirement for hypoxanthine and thymidine. Cells were maintained as adherent cultures in ‘CHO cell media'; Iscove’s modified DMEM media supplemented with 10 % (v/v) dialysed FBS, 2 mM glutamax-I (L-alanyl-L-glutamine), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.1 mM hypoxanthine and 16 μM thymidine. Recombinant CHO<sup>dhfr</sup> cells secreting human apoE2 (designated as CHO<sup>apoE2</sup> cells), apoE3 (CHO<sup>apoE3</sup>) and apoE4 (CHO<sup>apoE4</sup>) were supplied by A. Tagalakis. Briefly, CHO<sup>dhfr</sup> cells had been stably transfected with the expression plasmids p7055.E2, p7055.E3 and p7055.E4 encoding the cDNA for human apoE isoforms apoE2, E3 and E4, respectively, using Transfectam Reagent (Promega, Southampton, UK) and the manufacturer's protocol. Successfully transfected cells were easily selected by virtue of the vector's selectable DHFR gene, allowing the cells to simultaneously produce apoE and dhfr, thus enabling growth in CHO cell media without supplementation with hypoxanthine or thymidine [387]. CHO<sup>apoE2</sup> cells were also subcloned to achieve a clone capable of secreting 2-fold higher levels of apoE as detected by apoE ELISA (Section 4.2.3.5). Media was removed from a 75 cm<sup>2</sup> flask of subconfluent CHO<sup>apoE2</sup> cells after a 24 h collection period and was analysed for secreted apoE (diluting the media 1/10 - 1/100 prior to the assay). Cellular protein was determined
by solubilizing the cells directly in the flask with 0.1 M NaOH during a 2 h incubation at 37 °C and taking an aliquot for Bradford protein assay (Section 3.2.2). In CHO cell media this subclone of CHOapoE2 cells typically secreted 75-80 μg apoE/mg cell protein/24 h.

### 4.2.5 Co-Culture of HUVECs with CHO Cells.

For co-culture experiments, HUVECs were seeded in 96-well plates at a density of 7 x 10^3 cells per well with 100 μl of complete M199 media containing 5 % (v/v) FBS and left to adhere for 2 h. To obtain a ratio of 4:1 HUVEC:CHO cells, 1.75 x 10^5 CHOapoE2 cells (or CHOdhfr cells in controls) were added to each well of HUVECs in 100 μl of complete M199 media giving a total well volume of 200 μl. CHO cells adhered to the plastic in any spaces between HUVECs as judged by phase-contrast microscopy. Co-cultures were incubated for 48 h, with the addition of TNF-α straight into the existing media for the last 6 h (final TNF-α concentration of 10 U/ml or 0.5 μg/ml). Media from each well was immediately frozen at -20 °C until assayed by apoE ELISA. Monolayers were washed, fixed and analysed for VCAM-1 by ELISA (Section 2.2.9) and cellular protein by Bradford assay (Section 3.2.9).

### 4.2.6 Production of CHO Cell-Conditioned Media.

To produce CHO cell-conditioned media for HUVEC experiments CHOapoE2, CHOapoE3 or CHOapoE4 cells were grown to 80 % confluency in CHO media and then changed to 7 ml complete M199 media containing 5 % (v/v) FBS for 24 h conditioning. As controls, CHOdhfr cells were treated identically in parallel flasks. Conditioned media was sterile filtered using a 0.8/0.2 μM filter (Gelman Sciences) and assayed for apoE content by ELISA (Section 4.2.3.5) using 1/10 - 1/100 dilutions of media. Conditioned media was then used immediately for a 24 h incubation with confluent HUVECs in 96-well plates (3 x 10^4 cells per well). For the last 6 h, TNF-α was added into the media (final well concentration of 10 U/ml or 0.5 ng/ml) before the monolayers were washed and fixed for VCAM-1 measurement by ELISA (Section 2.2.9) and cellular protein determination (Section 3.2.9).

For experiments requiring more concentrated CHO cell apoE, media was filtered and then concentrated in Vivaspin 15 ml concentrators (10,000 MWCO; Vivasience Ltd) at 2,500 g for 15 min. After an apoE ELISA, the media was adjusted accurately to the desired apoE concentration by dilution with the concentrator flow through (devoid of apoE as checked by ELISA) and then was sterile filtered before addition to cells. Control CHOdhfr media was treated similarly.
4.2.6.1 ELISA for VCAM-1, E-Selectin and vWF.

In addition to VCAM-1 measurement, E-selectin and vWF expression were also measured in parallel wells. As before, HUVECs were incubated for 24 h with CHO cell-conditioned media (from CHO<sup>apoE2</sup> cells or CHO<sup>dhfr</sup> cells as controls), with addition of TNF-α for the last 6 h (final well concentration of 10 U/ml or 0.5 ng/ml). Monoclonal antibodies to E-selectin and vWF were used at concentrations of 5 and 1.2 μg/ml respectively, diluted in 0.1 % BSA in PBS. VCAM-1, E-selectin and vWF were then all assayed by ELISA (Section 2.2.9).

4.2.7 Characterization of CHO Cell-Secreted ApoE.

4.2.7.1 Ultracentrifugation Isolation of ApoE:Lipid Particles in Serum-Free Media.

Any CHO cell-secreted apoE particles that contained lipid were isolated from serum-free media, CHO-SFM II, by a single ultracentrifugation floatation step. A subconfluent 75 cm<sup>2</sup> flask of CHO<sup>apoE2</sup> cells (3 x 10<sup>7</sup> cells total) was switched to 8 ml CHO-SFM II media for a 48 h collection of secreted apoE. Conditioned media was filtered to remove cell debris and its density adjusted to 1.21 g/ml as outlined in Section 3.2.4. The sample was then centrifuged at 105,000 g for 20 h at 16 °C. Eighteen fractions of 0.5 ml were carefully removed from the top of the tube with a circular pipetting motion and stored for a maximum of 1 week at 4 °C. Samples were analysed by apoE ELISA (Section 4.2.3.5), phospholipid assay, immunoblotting of SDS-PAGE and agarose gels, and negative-staining TEM.

4.2.7.2 Phospholipid Assay.

Phospholipid concentration of the 18 fractions was determined using the Phospholipid B Kit (Section 3.2.8.4). To increase sensitivity of the enzymatic phospholipase colourimetric method, 200 μl of sample was analysed and standards of phospholipid were in the lower range of 0-15 μg.

4.2.7.3 SDS-PAGE and Immunoblotting Using ECL Substrate.

The 18 fractions were subjected to 15 % SDS-PAGE under reducing conditions (Section 3.2.3) before electrophoretical transfer to Hybond ECL nitrocellulose membrane using a Trans-Blot Semi-Dry transfer cell (Bio-Rad Laboratories). Following transfer, the membrane was blocked overnight at 4 °C in ‘blocking buffer’ (5 % w/v non-fat dry milk, 0.1 % v/v Tween-20, 0.2 % w/v 2-chloracetamide in PBS) to block any non-specific binding sites and then immunoblotted for apoE. Briefly, primary anti-apoE monoclonal antibody (clone F5M3/A10) was diluted 1/3,000 in ‘wash buffer’ (0.1 % v/v Tween-20, 0.2 % w/v 2-chloracetamide in PBS) and incubated with the blot for 1 h at room temperature. The blot
was then washed 6 x 5 min in wash buffer and incubated for 1 h with 1/10,000 dilution of anti-mouse secondary antibody-HRP conjugate. After washing as before, Blots were visualized by using an ECL substrate. Hyperfilm ECL X-ray films were exposed to the membrane for 5 s only.

4.2.7.4 Agarose Gel Electrophoresis and Immunoblotting.

Aliquots (10 μl) of fraction 1 from ultracentrifugation, and in some cases fresh samples of media containing CHO cell-secreted apoE, were analysed by electrophoresis on alkaline buffered (pH 8.8) 0.8 % agarose gels using the HYDRAGEL LIPO + Lp(a) Kit according to the manufacturer's protocol. Samples of fresh plasma (2 μl per lane) were used as controls to monitor migration in the gel. Electrophoresis was carried out using a Sebia K20 apparatus for 2 h at 50 V and 30 mA to allow separation of lipoprotein particles. After this procedure, the gel was cut into two. One half of the gel was dried and stained (for 15 min) with the lipid stain, Sudan black (0.044 % w/v, in 53 % ethanol). The gel was then rapidly destained with 45 % ethanol to reveal lipoprotein bands. The particles on the other half of gel were transferred to Hyperfilm ECL nitrocellulose for immunoblotting. Briefly, the nitrocellulose was cut to size, soaked in PBS and placed on top of the gel taking care not to trap air bubbles between gel and membrane. This was followed by 2 sheets of PBS-soaked filter paper (Whatman International Ltd, Maidstone, UK) and 4 sheets of dry filter paper. A glass plate was placed on top to distribute a 100 g weight. After transfer for 1 h, the gel was dried and stained with Sudan black to check efficiency of transfer, while the membrane was blocked and immunoblotted (Section 4.2.7.3) using 1/10,000 primary monoclonal anti-apoE and 1/10,000 anti-mouse-HRP conjugate. Hyperfilm ECL X-ray films were exposed to the membrane for 2 s only.

4.2.7.5 Negative Staining TEM.

ApoE particles which had been isolated by ultracentrifugation (fraction 1) were negatively stained and examined using TEM (Section 3.2.8.3). A sample of apoE:DMPC was also analysed for comparison.

4.2.8 Statistical Analysis.

Values in text, tables and figures were expressed as the mean ±SE. Statistical differences were determined by Student's t-test, ANOVA test or Pearson correlation as appropriate and considered significant if \( P<0.05 \). Analyses were performed using InStat version 3.01 (GraphPad Software, San Diego, USA) and SigmaPlot for Windows (Jandel Scientific, Erkrath, Germany).
4.3 Results.

4.3.1 VCAM-1 is Downregulated in HUVECs Expressing ApoE - I - A Model for Locally-Secreted ApoE.

The first in vitro model mimicking exposure of endothelium to locally-secreted apoE involved transfecting HUVECs with an expression plasmid encoding human apoE cDNA. This enabled HUVECs to secrete apoE and therefore exposes the monolayer to a local and cell-derived source. The plasmids provided were designated pCMV.apoE2, pCMV.apoE3 and pCMV.apoE4 encoding the three common polymorphic forms: apoE2 (Arg158Cys), apoE3 (wild-type) and apoE4 (Cys112Arg), respectively. This vector includes all the segments in an array necessary to permit transcription, splicing and polyadenylation of the cloned cDNA [388] (Figure 4.3-1). ApoE expression and secretion using pCMV.apoE had been previously confirmed by transiently transfecting HEK 293 or C2C12 cells in culture, with subsequent detection by immunoblotting [389] (T. Athanasopoulos).

Primary cultures of endothelial cells are notoriously difficult to transfect, however, some groups have successfully transiently transfected HUVECs to express recombinant proteins [390,391]. In my study, LipofectAMINE transfection reagent was successfully used to transfect HUVECs using a modification of the manufacturer's protocol (Section 4.2.3.2). LipofectAMINE was used in conjunction with sterile, plasmid DNA that had been prepared using the Endo-Free Maxi Prep Kit in order to remove all traces of this Gram negative wall component derived from amplification of the plasmids in E. coli (Section 4.2.2.2). It was important that neither LipofectAMINE nor plasmid preparations had endotoxin contaminants that could induce VCAM-1 in a similar manner to TNF-α (described in Chapter 2), and therefore confound results. Furthermore, endotoxin has been shown to reduce the efficacy of transfection reagents [384]. Basal expression of VCAM-1 after transfection was compared with non-transfected controls in every ELISA experiment to check for absence of endotoxin. The transfection procedure caused negligible cytotoxicity as judged by the absence of changes in cell morphology by phase-contrast microscopy, in VCAM-1 expression by ELISA and in cellular protein content per well.

The empty plasmid without insert pCMV.0 (3,693 bp) was used as a transfection control and although apoE was undetectable in these control wells, it was readily detected in media from pCMV.apoE transfectants. Interestingly, the VCAM-1 induction was significantly reduced in pCMV.apoE3 transfectants by 49.1±1.45 % as compared with the pCMV.0 controls (100±6.9 % induction) with this effect also apparent when transfecting with pCMV.apoE2 (62.3±8.8 % reduction, Figure 4.3-2). The accumulated apoE in both
pCMV.apoE2 and pCMV.apoE3 wells was equivalent (0.10±0.02 vs. 0.12±0.02 μg/ml, respectively, n=4) and at these concentrations there was no significant difference between isoforms in their ability to reduce VCAM-1 (P>0.05, ANOVA test). We ruled out the effect being purely a reflection of cell death during pCMV.apoE transfection (possibly resulting in less cells remaining to respond to cytokine). The transfection procedure had been optimized to reduce cytotoxicity, moreover, each well was subjected to protein assay after VCAM-1 ELISA and then the data calculated to take into account any slight changes in cellular protein over the lengthy experiment (Section 4.2.3.4).

Figure 4.3-1 Simplified plasmid diagram showing the main features of pCMV.apoE.

The expression plasmid pCMV.apoE (4731 bp) was supplied by T. Athanasopoulos, the apoE cDNA being excised from pPUCl9.apoE (Dr J. Breslow) and ligated into pCMV.0 (originally purchased as pCMV.β from Clontech Laboratories Ltd). The plasmid includes an human cytomegalovirus immediate early gene promoter (CMV IE), a simian virus polyadenylation signal (SV40 poly A) so that mRNA transcripts will have a polyadenylated 3' end, a selectable marker conferring resistance to ampicillin to E. coli hosts (Amp') and an E. coli origin of replication (pUC ori) [388]. The plasmids provided were designated pCMV.apoE2, pCMV.apoE3 and pCMV.apoE4 encoding the three common polymorphic forms: apoE2 (Arg1158Cys), apoE3 (wild-type) and apoE4 (Cys112Arg), respectively.
Figure 4.3-2 Transfecting HUVECs with apoE expression plasmids suppresses VCAM-1 expression.

Subconfluent HUVECs were transfected with pCMV::apoE2 or pCMV::apoE3 plasmids, incubated for 48 h with TNF-α (10 U/ml) added for the last 6 h. In parallel wells, pCMV.0 was used for transfectant controls. Results were calculated for each well as ratio of VCAM-1 ELISA:cellular protein values (\(A_{492}\) VCAM-1 ELISA: \(A_{630}\) protein assay) for TNF-α-stimulated values minus non-stimulated controls to give VCAM-1 induction (above basal expression). Results here are expressed as data normalized to a % of the VCAM-1 induction in pCMV.0 transfectant controls representing 100 % VCAM-1 induction (100±6.9 %, black bar). VCAM-1 induction by TNF-α was significantly reduced to 37.7±8.8 % and 50.9±4.45 % of the control VCAM-1 induction when cells were transfected with pCMV::apoE2 and pCMV::apoE3, respectively, and compared to pCMV.0 controls (both \(*P<0.01\), ANOVA test). The concentration of apoE in the media was 0.12±0.02 μg/ml (apoE2) and 0.10±0.02 μg/ml (apoE3) as measured by apoE ELISA. At these equivalent doses there was no difference in the ability of apoE isoforms to suppress VCAM-1 induction. Data shown is for one experiment performed in triplicate.
In order to confirm this initial observation, experiments with pCMV::apoE2 were reproduced in a further 5 independent experiments using 5 different batches of HUVECs. By correlating the VCAM-1 induction and apoE content of individual wells over a range of apoE concentrations, a strong dose-dependent inverse relationship between apoE produced and VCAM-1 expression was revealed ($r=-0.757$, $P<0.001$, Pearson correlation, $n=20$; Figure 4.3-3). Thus, the regression line showed that 50% VCAM-1 inhibition was achieved at $\sim 0.16 \mu g/ml$ apoE.

In similar transfection experiments substituting pCMV::apoE3 for pCMV::apoE2, the level of apoE3 also significantly correlated with VCAM-1 induction ($r=-0.815$, $P<0.001$, Pearson correlation, $n=8$; Figure 4.3-4, regression line not shown). Intriguingly, when HUVECs were also transfected with pCMV::apoE4, VCAM-1 was downregulated (at most by 79.5%) but without any apoE4 being detected in the media (Figure 4.3-4). If apoE had been secreted into the media it should have been detected since the apoE ELISA kit does not discriminate between apoE isoforms and assays were undertaken with appropriate controls, however, the concentration of apoE4 could be below the detection limit of the assay (10-15 ng/ml apoE). The reason for this finding remains unclear without further experimentation, but possible explanations are discussed later on in this Chapter.

These data show that by exposing HUVECs to a locally-secreted source of apoE (either apoE2 or apoE3), VCAM-1 induction by TNF-α is downregulated indicative of a suppression of endothelial activation. The levels of apoE found to give an effect (ranging from $0.013-0.189 \mu g/ml$ HUVEC apoE) were much lower ($\sim 1000$-fold lower) than the level of apoE:DMPC used in Chapter 3 ($10-200 \mu g/ml$ apoE protein in apoE:DMPC). However, apoE measurements from transfectants only represent the concentration in the bulk media and the amount of apoE at the cell surface at the source of secretion may be far higher (as discussed in Section 4.4.1.2); it may be these doses (unmeasurable in the present assay) that are crucial for the effect.
Figure 4.3-3 Secretion of apoE2 by transfected HUVECs correlates with a decrease in VCAM-1 expression.

HUVECs were transfected with pCMV.apoE2, incubated for 48 h with TNF-α (10 U/ml) added for the last 6 h. In parallel wells, pCMV.0 was used for transfectant controls. VCAM-1 results were calculated as in Figure 4.3-2 and data expressed as % VCAM-1 induction of the pCMV.0 transfectant controls (22 controls with mean 100.3±3.4 % VCAM-1 induction). ApoE was undetectable in control wells when HUVECs were transfected with pCMV.0 (○) but readily detected in media from cells transfected with pCMV.apoE2 (●) as measured by apoE ELISA. The level of secreted apoE2 was varied in some experiments by transfecting with differing amounts of pCMV.apoE2. Values for both VCAM-1 induction and apoE content were determined for individual wells. There was a strong inverse relationship between the apoE produced and the VCAM-1 induction by TNF-α (correlation coefficient r=-0.757, P<0.001, Pearson correlation, n=20). Graph shows a total of 5 independent experiments using 5 different batches of HUVECs.
Figure 4.3-4 Transfection of HUVECs with expression plasmids encoding apoE isoforms decreases VCAM-1 expression.

HUVECs were transfected with pCMV apoE2 (●), pCMV apoE3 (▲) or pCMV apoE4 (△), incubated for 48 h for apoE with TNF-α (10 U/ml) added for the last 6 h. In parallel wells, pCMV 0 was used for transfectant controls (□). Results were calculated as in Figure 4.3-2 and expressed as % VCAM-1 induction of the pCMV 0 transfectant controls (22 controls with mean 100.3±3.4 % VCAM-1 induction, shown as ±SE bars). Values for both VCAM-1 induction and apoE content were determined for individual wells. There was a strong inverse relationship between the apoE3 produced and the VCAM-1 induction (r=-0.815, P<0.01, Pearson correlation, n=8, 2 independent experiments performed in quadruplicate with 2 batches of HUVECs, correlation line not shown). In pCMV apoE4 transfected wells no apoE was detected, however, VCAM-1 was still reduced in pCMV apoE4 transfectants (n=5, 2 independent experiments performed in quadruplicate with 2 batches of HUVECs).
4.3.2 **CO-CULTURING HUVECs WITH RECOMBINANT CELLS RELEASING APOE SUPPRESSES VCAM-1 INDUCTION - II - A MODEL FOR LOCALLY-SECRETED APOE.**

The HUVEC transfection model was successful in showing that local cell-derived apoE, in contrast with purified apoE, was a potent inhibitor of VCAM-1 expression. However, there were concerns that by transfecting HUVECs and hijacking their protein production machinery to produce apoE, the same HUVECs could have less capability to produce other proteins, such as VCAM-1, in response to cytokine-stimulation. Thus, suppression of VCAM-1 might not be a direct effect of the apoE but a transfection artefact. Alternatively, the suppression of VCAM-1 may have simply reflected an intracellular action of apoE. In response to these concerns another *in vitro* model of endothelial exposure to locally-secreted apoE was established. However, this time the endothelial cells themselves were not the site of apoE production.

This system involved co-culturing HUVECs with recombinant CHO cells secreting apoE2 (CHO^apoE2^ cells) and thus ruling out involvement of both LDL-R which is expressed HUVECs, and LRP1, although this is very poorly expressed in these cells [226-229]; apoE2 binds poorly to both the LDL-R (only 1% of apoE3 binding activity [167]) and LRP1 (only 40% of apoE3 binding [392]). The CHO cell line has emerged as one of the most favoured for the commercial production of recombinant proteins that most closely resemble the naturally occurring human proteins [393,394] and has been used by us and other groups to stably express human apoE [176,395-398]. Our CHO^dhfr^ cells had been transformed with a high level apoE expression plasmid, p7055.E2.

Firstly, in preliminary experiments, it was established that CHO cells themselves did not express either basal or TNF-α-stimulated VCAM-1, or antigens capable of cross-reacting with the monoclonal anti-VCAM-1 (as measured in CHO cell-bound VCAM-1 ELISA, data not shown). Next, the HUVEC seeding density was optimized: 1) to allow CHO cells enough space between HUVECs in which to adhere, 2) to ensure that HUVECs were confluent enough for VCAM-1 to be expressed, and 3) to avoid overcrowding and ultimately cell death by the end of the 48 h co-culture period. Finally, the CHO^apoE2^ cell seeding density was also optimized so that the accumulated apoE dose (as determined by apoE ELISA at the end of the incubation period) was within the range of 0.01-0.2 μg/ml apoE which we knew had a downregulatory effect on VCAM-1 (*Section 4.3.1*). CHO^dhfr^ cells were used in parallel control wells at the same cell density as recombinant cells. The final seeding density ratio of HUVECs to CHO^apoE2/dhfr^ cells was 4:1. This allowed the CHO cells to be evenly distributed throughout the well as observed by phase-contrast microscopy. Importantly, CHO cells did
not activate the HUVECs in the co-culture, as determined by assaying basal VCAM-1 expression (data not shown).

As before, apoE was allowed to accumulate in the media for 48 h before addition of TNF-α straight into the media (final TNF-α concentration 10 U/ml). In this experiment VCAM-1 was significantly downregulated by 16.1±2.4 % (P<0.05, Student’s t-test) in HUVECs co-cultured with CHOapoE2 cells, where the concentration of apoE in the media was 0.136±0.002 μg/ml, when compared to the co-culture controls (Figure 4.3-5).

Thus, by incubating HUVECs with a local source of apoE, cytokine-stimulated VCAM-1 had been significantly reduced. This model confirms the transfection data and reinforces the observation that locally-secreted apoE is active in down-regulating VCAM-1. However, if we compare this to the transfection data (Figure 4.3-3) we may have expected a reduction in VCAM-1 of as much as 60 % with an apoE2 concentration of 0.136±0.002 μg/ml, instead of the 16.1±2.4 % reduction observed here. Again, we do not know the apoE concentration at the cell surface, possibly accumulated by endothelial HSPG, and may explain the difference (Section 4.4.1.2). Moreover, CHO cell-secreted apoE could be less active at down-regulating VCAM-1; a potential difference in efficacy of HUVEC versus CHOapoE2 cell apoE could lie in the nature of the secreted apoE particles dependent on cell type, but difficult to measure in these models due to low concentrations of apoE.

4.3.3 HUVEC-CONDITIONED MEDIA CONTAINING APOE DOWNREGULATES VCAM-1.

As cell-derived apoE can display different biological activities to plasma purified apoE [360], it was considered that the apoE, secreted either from transfected HUVECs or from recombinant CHO cells, may itself be active at downregulating VCAM-1 without the need for local production. To investigate this possibility, apoE-containing media from HUVEC transfectants was incubated with untransfected HUVECs. Thus, half of the media from transfected wells was steriley collected after a 48 h incubation (but without cytokine addition) and was immediately incubated for 24 h with untransfected HUVECs. Media from pCMV.O transfected wells was used in controls. The original transfection experiment continued (with half of the usual well volume) by adding TNF-α (10 U/ml) and then assaying for VCAM-1 induction and apoE concentration. In the ‘transfer experiment’, TNF-α was also added for the last 6 h and VCAM-1 induction measured +/- apoE exposure.
Figure 4.3-5 VCAM-1 induction by TNF-α is suppressed when HUVECs are cocultured with recombinant CHO cells (CHOapoE2) expressing apoE2.

HUVECs and CHOapoE2 cells were seeded into 96-well plates at a ratio of 4:1 (HUVECs:CHOapoE2 cells) and co-cultured for 48 h, adding TNF-α (10 U/ml) for the last 6 h. CHOwt cells were used in parallel co-culture controls. Results were calculated as described in Figure 4.3-2 and bars show % VCAM-1 induction (±SE) for a single experiment performed with quadruplicate wells. VCAM-1 was reduced to 83.9±2.4 % (grey bar) of the control VCAM-1 induction (100±4.6 %, black bar) in wells where CHOapoE2 cells had secreted 0.136±0.062 μg/ml apoE (*P<0.05, Student’s t-test). This result was confirmed in another independent experiment (data not shown).

As expected, expression of VCAM-1 in transfected HUVEC was reduced by 32.6±7.8 % by locally-secreted apoE (at 0.12±0.03 μg/ml), with VCAM-1 induction correlating closely with apoE content (r=−0.89, P<0.01, Pearson correlation, n=8; graph not shown). Moreover, when the same media was transferred to other (untransfected) HUVECs, VCAM-1 was reduced in the apoE-containing wells (12.9±2.2 % reduction in VCAM-1, P<0.05, Student’s t-test; Figure 4.3-6) albeit to a lesser extent than transfected HUVECs. It seems that an apoE concentration as low as 0.12±0.03 μg/ml is active at downregulating VCAM-1 independent of a local source and that this is inherent in the unpurified, cell-synthesized nature of the apoE.
Figure 4.3-6 When media from pCMV.apoE2 HUVEC transfectants is transferred to non-transfected HUVECs, VCAM-1 is suppressed.

HUVEC-conditioned media from transfection experiments was collected and incubated with non-transfected HUVECs for 24 h (with TNF-α added for the last 6 h). Results were calculated as described in Figure 4.3-2. This bar graph shows VCAM-1 induction (±SE) as a % of the cell-conditioned media control induction (100±3.1%, black bar) for a single experiment performed with quadruplicate wells for controls and a total of 8 wells containing apoE2 at 0.12±0.03 μg/ml. VCAM-1 was found to be reduced to 87.1±2.2% (*P<0.01, Student's t-test) in the cells incubated with conditioned media containing apoE2.

4.3.4 CHO Cell-Conditioned Media Containing ApoE Downregulates VCAM-1.

If HUVEC-conditioned media containing apoE suppresses VCAM-1 then does CHO apoE2 cell-conditioned media have the same effect? To test this notion, cultures of CHO apoE2 cells were switched to complete M199 media (with 5% FBS) to be conditioned for 24 h. CHO apoE2 cells were capable of secreting 78±9 μg apoE/mg cell protein/24 h (or approximately 3.25 μg apoE/mg cell protein/per h) in CHO cell media (Iscove's modified DMEM media supplemented with 10% FBS). This compares favourably to reports from other groups, with secretion even ~3-fold higher than reported previously in recombinant CHO cells (1.25 μg apoE/mg cell protein/per h [398]). ApoE production in M199 media
under the same collection conditions slightly dropped to 55±9 µg apoE/mg cell protein/24 h (or 2.29 µg apoE/mg cell protein/per h) and varied with each collection, thus giving a range of apoE concentrations to incubate with HUVECs. Control cell-conditioned media was collected for each experiment; control CHO\textsuperscript{trans} cells at approximately the same confluency were exposed to the same volume of M199 media for exactly the same incubation period. All media was sterile filtered before incubation with confluent HUVECs for 24 h (with the last 6 h in the presence of TNF-α, 10 U/ml). VCAM-1 ELISA revealed that there was a strong inverse relationship between the level of apoE in the conditioned media and VCAM-1 induction (r=-0.697, P<0.001, Pearson correlation, n=10; Figure 4.3-7).

![Graph showing the relationship between VCAM-1 induction and concentration of apoE2.](image)

**Figure 4.3-7 CHO cell-conditioned media containing apoE downregulates HUVEC VCAM-1.**

M199 media was conditioned by incubation with control CHO\textsuperscript{trans} cells (△) or recombinant CHO\textsuperscript{apoE2} cells secreting human apoE2(●). Media was then incubated for 24 h with confluent HUVECs, adding TNF-α for the last 6 h. Results were calculated as described in Figure 4.3-2 and expressed as % VCAM-1 induction of the CHO\textsuperscript{trans} cell media controls (30 controls with mean VCAM-1 induction of 100.2±1.3 %, shown as □ ±SE bars). There was a strong inverse relationship between the level of apoE in the media and VCAM-1 induction (r=-0.697, P<0.001, Pearson correlation). Data is from 10 independent experiments performed in quadruplicate using 6 different batches of HUVECs.
Therefore, at these concentrations of apoE, unpurified cell-derived apoE added endogenously to HUVECs is active at suppressing VCAM-1. At 5.5±0.2 µg/ml apoE, VCAM-1 was suppressed by 23.4±2.2 % (P<0.001, ANOVA test, n=3; Figure 4.3-8). This concentration may be too low to represent the concentration of apoE in plasma (normal physiological range for healthy subjects is 30-70 µg/ml [168]) but is within the physiological range for macrophage-produced apoE whose contribution to the total circulating apoE is considered to be ~10 % [235,257]. This concentration may have physiological relevance. At 5 µg/ml, apoE has potent antioxidant ability \textit{in vitro} [269] and can protect against atherosclerosis in apoE-deficient mice that have undergone adenovirus-mediated gene transfer [242].

As with the HUVEC transfection experiments, the suppression of VCAM-1 was not just a phenomenon associated with apoE2. VCAM-1 was successfully downregulated by apoE3 and apoE4, in CHO\textsuperscript{apoE3} and CHO\textsuperscript{apoE4} cell-conditioned media respectively, with the concentration of apoE being inversely related to VCAM-1 induction (r=-0.639 for apoE3 and r=-0.5295 for apoE4, both P<0.001, Pearson correlation, both n=4; graphs not shown). However, fewer of these experiments were conducted than for apoE2 and at present it is impossible to deduce whether there is a true difference in the abilities of the different apoE isoforms to downregulate VCAM-1.

\textbf{4.3.5 APOE SPECIFICALLY DOWNREGULATES VCAM-1.}

It was determined whether CHO\textsuperscript{apoE2} cell-conditioned media containing apoE could affect the expression of other endothelial markers, namely vWF and E-selectin. At 5.5±0.2 µg/ml apoE2 there was no effect on levels of constitutive vWF expression (which is not dependent on TNF-α stimulation), indicating that endothelial function was maintained (Figure 4.3-8). TNF-α-induced expression of E-selectin was also unchanged by preincubating with apoE2, showing that apoE was not interfering with the ability of the cells to respond to cytokine (for example by non-specifically masking TNFRs) and that the effect may be specific for VCAM-1. The intracellular mechanisms controlling the regulation of CAM expression, although incompletely understood, are thought to consist of divergent regulatory pathways. A selective effect on CAM expression by apoE may have physiological relevance. Recently it was demonstrated that E-selectin does not contribute significantly to monocyte recruitment in a novel \textit{in vivo} model of monocyte homing to atherosclerotic plaques, whereas VCAM-1 played a major role [67]. Additionally, it is endothelial VCAM-1 and not E-selectin that is upregulated at atherosclerosis-prone sites in apoE-deficient mice [25]. Therefore, it is desirable that anti-atherogenic apoE primarily reduces VCAM-1 rather
than E-selectin as an anti-inflammatory mechanism to limit progression of the disease. However, apoE effects have yet to be tested on other members of the IgSF and selectin families of CAMs, such as ICAM-1, PECAM-1 and P-selectin.

Figure 4.3-8 CHO cell-derived apoE specifically downregulates VCAM-1 without altering endothelial function or E-selectin response to TNF-α.

Confluent HUVECs were incubated for 24 h with CHOapoE cell-conditioned media containing 5.5±0.2 μg/ml apoE2, adding TNF-α (10 U/ml) for the last 6 h. CHOapoE cell-conditioned media was used in controls. Induction of VCAM-1, E-selectin and expression of vWF were measured in parallel wells by ELISA (Section 4.2.6.1) and compared to controls (VCAM-1, vWF and E-selectin levels in presence of control media = 100±1.2 %, black bar). Results were calculated as described in Figure 4.3-2 and bars show % endothelial antigen expression (mean ±SE). VCAM-1 was reduced to 77.6±2.2 % of the control induction (*P<0.001, Student's t-test), whereas in the same experiments vWF and E-selectin were unaffected (99.8±4.5 % and 100±4.2 %, respectively) as compared to their appropriate controls. Data is from 3 independent experiments performed in quadruplicate using 2 batches of HUVECs.
4.3.6 CHARACTERIZATION OF RECOMBINANT apoE SECRETED FROM CHO^apoE2 CELLS.

Unpurified CHO cell-derived apoE downregulates VCAM-1 expression in HUVECs independent of it being produced in the vicinity of the cells. Therefore, what was different between the active cell-secreted apoE and the apoE:DMPC particles that had proved to be benign? In order to answer this question apoE particles secreted into serum-free CHO-SFMII media were characterized by ultracentrifugation, by apoE, phospholipid and cholesterol assays, by electron microscopy and by immunoblotting of SDS-PAGE and agarose gels.

CHO^apoE2 cells secreted 92±10 µg apoE/mg cell protein/h (or ~3.8 µg apoE/mg cell protein/h) in CHO-SFMII media. Conditioned media was collected and ultracentrifugated at density 1.21 g/ml to float apoE:phospholipid particles. Eighteen 0.5 ml fractions were taken from the top of the tube, separated by SDS-PAGE and analysed by anti-apoE immunoblotting and densitometry (using a Bio-Rad Model GS-670 Imaging densitometer) to give the amount of apoE in each fraction as a % of the total. The first 6 fractions had 35 % of the total apoE, with the 7th fraction having no apoE present; the last 11 fractions contained 65 % of the apoE (Figure 4.3-9).

![Figure 4.3-9 Immunoblotting of apoE in fractions separated by ultracentrifugation.](image)

CHO-SFMII media was conditioned by CHO^apoE2 cells for 24 h before the density of the media was adjusted to 1.21 g/ml and subjected to ultracentrifugation at 105,000 g, 16 °C for 48 h. Eighteen 0.5 ml fractions were collected and, after 15 % SDS-PAGE, immunoblotted for apoE with ECL detection (Section 4.2.7.3). Fractions are labelled 1 to 18, with 1 being the 1st fraction collected from the top of the tube.
Only the 1st fraction contained detectable levels of phospholipid (12.7 µg/ml phospholipid). The apoE content of this fraction was 20.5 µg/ml (13% of the total apoE), thus giving a ratio of apoE:phospholipid as 1:0.63 (w/w). These apoE particles were phospholipid-poor compared with apoE:DMPC preparations (ratio of 1:3.75, w/w). A detailed characterization of the phospholipid composition of the cell-secreted apoE particles is necessary since the assay used does not differentiate between sphingomyelin, lecithin or lysolecithin.

Cell-secreted apoE:phospholipid particles were spherical in morphology with diameter of 14±3 nm (mean of 40 particles measured) as judged by negative staining TEM (Figure 4.3-10, panel A). These particles were directly compared with samples of apoE:DMPC which were discs of 18.8±1.5 nm in diameter that had a tendency to stack in multiples of 5 or more (Figure 4.3-10, panel B).

**Figure 4.3-10 Analysis of CHO cell-secreted apoE:phospholipid particles and comparison with apoE:DMPC complexes by electron microscopy.**

Samples of CHO cell-derived apoE:phospholipid particles isolated by ultracentrifugation (1st fraction, panel A) as well as apoE:DMPC for comparison (panel B) were subjected to negative staining TEM (Section 4.2.7.5). Electron micrographs show isolated apoE:phospholipid particles as individual spheres of 14±3 nm diameter which do not appear to aggregate (panel A) and apoE:DMPC particles as discs of 18.8±1.5 nm diameter with a tendency to stack and aggregate (panel B). Both micrographs were taken at ×125,000 magnification. The white bar represents 100 nm.
The majority of apoE:phospholipid spheres isolated by density ultracentrifugation migrated with γ mobility in agarose gels (with a minority of particles with α mobility) as detected by immunoblotting (Figure 4.3-11). In the control lane of human plasma, HDL particles with α mobility were found to contain apoE. The larger VLDL particles, with pre-β mobility, were the only particles not to transfer to nitrocellulose efficiently (as assessed by Sudan black staining of the blotted gel, not shown).

Figure 4.3-11 CHO cell-secreted apoE:phospholipid particles have γ mobility in agarose gels as detected by immunoblotting.

A 300 ng sample of CHO cell-secreted apoE isolated by ultracentrifugation (1st fraction) was subjected to agarose gel electrophoresis and immunoblotted for apoE using ECL detection (Section 4.2.7.4). In the control lane, human plasma apoE-containing particles with α mobility (HDL-E particles) were detected. The majority of the CHO cell-secreted apoE:phospholipid particles had γ mobility with a minor species having α mobility.

However, experiments on HUVECs were not carried out using apoE secreted under serum-free conditions and the particles responsible for the effect on VCAM-1 may be of an entirely different nature. CHO<sup>apoE2</sup> cell-conditioned M199 media containing 5% (v/v) FBS was concentrated and subjected to agarose gel electrophoresis and immunoblotted for apoE (Figure 4.3-12). The lane for media conditioned by control CHO<sup>apoE2</sup> cells showed no traces of apoE, while apoE secreted from the CHO<sup>apoE2</sup> cells was located in 2 separate bands within the range for pre-β and α mobility. There was also an intense, smeared signal representing particles of unknown nature, migrating with pre-α mobility. No γ-migrating particles were detected in the CHO<sup>apoE2</sup> cell-conditioned HUVEC media although traces were detectable in
the human plasma control. This indicates that although the apoE secreted by \( \text{CHO}^{\text{apoE2}} \) cells has the potential to form particles with \( \gamma \) mobility under serum-free conditions, these may represent only a very minor fraction (perhaps beyond the detection limit of this technique) in the serum-containing media. Since FBS appears to consist of mostly HDL particles (demonstrated on Sudan black stained gels, Figure 4.3-12, left hand side), it is possible that the apoE secreted could be interacting with the endogenous lipoproteins in the media to create apoE-rich lipoproteins and that it is these particles that are active at downregulating VCAM-1. At present, we do not know which of these multiple-mobility apoE-containing lipoproteins represent the active constituents of the \( \text{CHO}^{\text{apoE2}} \) cell-conditioned media.

**Figure 4.3-12 Distribution of apoE in serum-containing media as detected by immunoblotting of agarose gels.**

\( M199 \) media containing 5 % (v/v) FBS was conditioned for 24 h by incubation with \( \text{CHO}^{\text{apoE2}} \) cells. Media was concentrated 10-fold and 300 ng of apoE (determined by apoE ELISA) was subjected to agarose gel electrophoresis and immunoblotting using ECL detection (Section 4.2.7.4). Media conditioned by incubation with control \( \text{CHO}^{\text{apoE2}} \) cells was also concentrated 10-fold and used as a control. Panel A, shows Sudan black staining of the samples and an arrow marks the origin where samples were applied. Lane 1 shows human plasma lipoproteins separated by their characteristic mobility. Lane 2 is the control cell-conditioned media and lane 3 is apoE-containing conditioned media, both showing faint smears corresponding to particles with \( \alpha \) mobility (HDL) in the bovine serum. From the same gel, panel B shows the same samples immunoblotted for apoE and bands visualized by ECL. Human plasma has apoE-containing particles with \( \gamma \), pre-\( \beta \) and \( \alpha \) mobilities (corresponding to \( \gamma \)-LpE at the origin then VLDL and HDL, respectively). Although there were no bands present in the control conditioned media lane, multiple bands were detected in the media containing apoE sample, with pre-\( \beta \), \( \alpha \) mobility and pre-\( \alpha \) mobility, as compared to the human plasma control.
4.4 Discussion.

4.4.1 Local Production of ApoE.

The experiments performed in this Chapter demonstrate that when HUVECs are subjected to an endogenous source of apoE, in two in vitro models to mimic vascular endothelium exposure to macrophage apoE, cytokine-induced VCAM-1 expression is suppressed. However, it subsequently appeared that the VCAM-1-modulatory ability of all apoE isoforms was not wholly dependent upon its local production. Thus, when cell-conditioned media containing apoE was transferred to other non-apoE-secreting cells, VCAM-1 was still suppressed, albeit to a lesser extent. This indicated that a proportion of apoE’s activity was inherent in its newly synthesized, cell-derived origin when secreted from either transformed HUVECs or recombinant CHO cells.

Recombinant cell-secreted apoE has been shown to have biological activities similar to native apoE [269,396,399] and by using unpurified apoE, to minimise conformational alteration [360] (discussed in Section 4.1), undoubtedly ensured a more physiologically relevant way of studying the effects of native apoE on HUVECs than using purified plasma apoE in apoE:DMPC complexes. Therefore, it was interesting that this cell-secreted apoE was active in terms of VCAM-1 regulation, especially as only low concentrations (~5 μg/ml) of apoE were needed for the effect. Indeed, these doses resembled the estimated level of circulating apoE that is contributed by macrophages (~10 % of plasma apoE [235,257]) and raises the possibility that the downregulation of endothelial VCAM-1 by macrophage apoE in vivo is a realistic mechanism for apoE to exert an anti-atherogenic effect. Indeed, this proposal is strengthened by the observations that transfected aortic endothelial cells overexpressing apoE support less monocyte recruitment in an in vitro co-culture system representative of the human aortic wall [311], and that VCAM-1 is upregulated in vivo in apoE-deficient mice [25].

It was interesting that the level of locally-secreted apoE in the bulk media from transfected HUVECs was ~100-fold lower than in the CHOapoE cell-conditioned media used directly on HUVECs, but still gave the most dramatic downregulatory effect on VCAM-1. This potency at extremely low levels of locally-secreted apoE may have been due to a number of reasons, including i) intracellular effects of apoE in the transfected cells, and ii) entrapment at the cell surface of secreted apoE by endothelial HSPG.

4.4.1.1 Intracellular effects of apoE.

In primary macrophages, over 50 % of newly synthesized apoE is directly targeted from the trans-Golgi network to the lysosomes for degradation, without reaching the plasma
membrane, possibly involving an intracellular receptor for apoE [400]. Importantly, intracellular pools of apoE have been described in hepatocytes and it is speculated that these might participate in intracellular lipid metabolism and transport [401]. Intracellular apoE has also been implicated in the facilitation of cholesterol efflux from cholesteryl ester-loaded macrophages to extracellular acceptors [402], but other roles for intracellular apoE are poorly understood in these cells. Interestingly, the interaction of apoE with cytoplasmic proteins in cells of the brain is thought to influence the development of Alzheimer's disease [403]. Although apoE is known to be an extracellular antioxidant not dependent on lipid association [269], it is unknown whether it can have a similar function when in an intracellular pool; in transfected HUVECs the presence of an intracellular antioxidant may alter the redox balance and therefore alter redox-sensitive VCAM-1 transcription in response to TNF-κ.

These intracellular effects may not be relevant in vivo where the native endothelium does not express apoE, but may have implications for gene therapy, whereby transfecting endothelial cells to overproduce apoE may reduce endothelial activation in vivo and have anti-atherogenic potential. Such intervention has already been shown to ameliorate atherosclerosis and modulate cholesterol levels [404].

4.4.1.2 ApoE entrapment by endothelial HSPG.

In addition to intracellular effects, VCAM-1 downregulation could also have been enhanced by apoE entrapment on endothelial HSPG creating a higher concentration of apoE in the immediate extracellular milieu.

It is known that macrophages and Hepatoma G2 (HepG2) cells, rapidly rebind much of the apoE they secrete by virtue of pericellular HSPGs [405-407]. Such sequestration in macrophages significantly modulates the uptake and degradation of apoE and could influence lipoprotein interactions with various receptors for which apoE serves as a ligand [407]. Indeed, 40% of newly synthesized apoE by HepG2 cells is held in a cell-associated pool in dynamic equilibrium with the secreted apoE [408] and it has been postulated that HSPGs on the surface of hepatocytes in vivo are responsible for the initial sequestration and uptake of chylomicron remnants in the space of Disse [409]. Such a 'secretion-recapture' model for apoE may also exist in my transfected HUVECs. Vascular endothelial cells also have cell-surface HSPGs that can anchor LPL and together enhance interactions with apoE [20]. Several classes of HSPGs have been identified on HUVECs [410,411]. It is therefore possible that much of the apoE secreted by transfected HUVECs is concentrated at the cell surface by a HSPG-mediated mechanism, and is available to somehow elicit a limiting effect.
on endothelial activation. Furthermore, apoE has been shown to stimulate HSPG production specifically in endothelial cells [275], thus increasing the likelihood of an apoE retention/accumulation mechanism in my transfection model. Further experiments using heparin or heparinase to displace HSPG (and LPL) from HUVEC cell surface are needed to verify this hypothesis.

Interestingly, there is variable apoE handling and retention, dependent on apoE isoform, by HSPG on cultured neurons, fibroblasts and HepG2 cells [412,413]. ApoE4 accumulates by HSPG retention less readily than apoE3 in these cells. However, apoE4 does have increased surface binding to macrophages and enhanced re-uptake of apoE4 [414] and, if true for HUVECs, may explain its absence in the media of my pCMV.apoE4-transfected HUVECs. However, differential apoE isoform retention by endothelial HSPGs is yet to be reported. Alternatively, apoE4 might be more avidly cleared from the media by an unknown mechanism. It is known that in vivo apoE4 is kinetically different from apoE3, being catabolized twice as fast as apoE3 [415].

If sequestering of locally-secreted macrophage apoE by endothelial cells occurs in vivo, then this extracellular pool could be important for apoE to elicit its effects on the endothelium such as inhibition of endothelial proliferation [277], reduction of monocyte recruitment [311] and suppression of VCAM-1 expression.

4.4.2 CELL-DERIVEDAPOE HAS BIOLOGICAL ACTIVITY.

All the experiments on VCAM-1 in this Chapter used unpurified cell-derived apoE and this absence of purification may have account for its effectiveness versus exogenous apoE:DMPC. It would have been advantageous, time permitting, to purify the CHO cell apoE to compare with unpurified cell-conditioned media. Alternatively, the inherent characteristics of newly synthesized apoE particles may contribute most to their biological activity. Interestingly, newly secreted and functionally active apoE, from in vitro cultures of both human monocyte macrophages [261] and HepG2 cells [416], contains excess sialic acid residues on its carbohydrate chains when compared to the circulating form of plasma apoE. In the brain, locally-produced astrocyte-derived apoE also has a higher degree of sialylation [182]. Excessive sialylation leads to extensive charge differences [417] and may affect the apoE conformation with subsequent altered cellular interactions. Recombinant CHO cells are not only capable of glycosylating the proteins they produce [393,394] but, in the case of apoE, can synthesize multiple sialylated forms [176,395]. This raises the possibility that my CHO cell apoE is of a heterogeneous nature with a range of corresponding biological activities. In future experiments, the activity of CHO cell apoE could be compared with
apoE secreted from CHO ldlD cells reversibly defective in glycosylation [395], in order to
test whether the degree of sialylation is crucial for the activity of recombinant apoE in
downregulating VCAM-1.

It may be of great importance that CHOapoE2 cells were capable of secreting particles,
distinct from apoE:DMPC and with similarity to macrophage-secreted apoE. ApoE was
found in discrete spherical particles with a diameter of 14±3 nm that floated at a density of
<1.21 g/ml and had γ mobility in agarose gels. These particles were poorly-lipidated,
containing only traces of cholesterol and phospholipid. This initial characterization indicates
that cell-derived apoE particles were different from apoE:DMPC complexes in terms of
particle morphology and in the extent of lipidation, both factors that can influence its
conformation and therefore determine biological activity of the apoE [167]. Indeed, from
these preliminary analyses, it appears that the cell-secreted apoE particles in serum-free media
resembled biologically active γ-LpE. This lipid-poor plasma lipoprotein exhibits γ mobility in
agarose gels [127] and is found in vivo; it has apoE as its sole protein, and sphingomyelin as its
major phospholipid component, and is also spherical in morphology with diameters in the
range of 12-16 nm. Importantly, macrophages secrete apoE-containing nascent HDL
particles in this form [127]. These particles are active in the early steps of RCT by removing
accumulated cell cholesterol in an autocrine/paracrine pathway in vitro that is independent of
exogenously added cholesterol acceptors [264,414]. Indeed, expression of γ-LpE in vivo by
apoE transgene macrophages in APOE+: mice, restores the cholesterol efflux capacity of
apoE-deficient plasma [262], although this does not exclude other functions of γ-LpE that
might limit atherosclerosis in this model. Our γ-LpE-like particles have not yet been tested
for cholesterol efflux ability from cholesterol-loaded macrophages, but have proved to be
potent inhibitors of platelet aggregation (Dr D. Riddell, unpublished data). It remains to be
seen whether these particles are a good recombinant model for studying the effects of
macrophage apoE in vivo. Furthermore, these particles may be applicable for the study of
spherical astrocyte-derived apoE particles in the CSF that transport cholesterol and
phospholipid. Minimally-lipidated recombinant apoE from different cell types has been
successfully used for the study of apoE on neurite outgrowth [418] and allele-specific
antioxidant activity [269]. In order to clarify the suitability of these recombinant apoE
particles to represent newly secreted particles found in vivo, a more comprehensive
characterization of the recombinant CHO cell-secreted apoE is currently being undertaken
by Dr A. von Eckardstein (Institute of Arteriosclerosis Research, Münster, Germany).
Although the recombinant CHO cells had the potential to produce \( \gamma \)-LpE-like particles under serum-free conditions, the apoE for HUVEC experiments was secreted into media suitable for HUVECs, which was supplemented with FBS (5%, v/v). Thus, an important question was raised. Was the biological activity towards VCAM-1 regulation inherent in these poorly-lipidated, cell-derived apoE particles or was it dependent on their interaction with other lipoproteins found in FBS? ApoE secreted in the presence of FBS over a 24 h period was no longer associated with particles with \( \gamma \) mobility in agarose gels, but was detectable in lipoprotein bands with pre\( \beta \), \( \alpha \) or pre-\( \alpha \) mobility. ApoE has a high affinity for lipid and binds to the phospholipid monolayer on lipoprotein surfaces. It therefore seems likely that apoE2, initially secreted as \( \gamma \)-migrating particles, had redistributed over time onto the HDL particles present in FBS. This was especially probable because, out of all the plasma lipoprotein classes, apoE2 associates preferentially with HDL [419] and because HDL is the predominant lipoprotein in FBS. As the conformation of apoE is sensitive to its lipid and particle environment, the recombined apoE-HDL particles, containing a range of lipids, may influence the helical content and ordering of the apoE [167] and may confer its activity in modulating VCAM-1. Interestingly, macrophage apoE can also combine with HDL to form apoE-containing HDL or HDL-E [168]. This HDL can interact with lipoprotein receptors by virtue of the presence of apoE and can efflux cholesterol from lipid-loaded macrophages [265] and participate in its redistribution and/or its reverse transport. Indeed, the possibility of HDL particles rich in cell-derived apoE being responsible for the effect on VCAM-1 is intriguing, especially in the light of my earlier work showing that plasma purified HDL-E was benign (Section 3.3.6). Using conditioned media without purification, the apoE-containing HDL may have retained its native biological activity.

Unfortunately, I haven’t yet ruled out the possibility that the difference in apoE activity, when comparing cell-derived to apoE:DMPC, is a reflection of the dosage. The concentrations of CHO cell-synthesized apoE were ~10-fold lower than those used with apoE:DMPC. Ideally, apoE:DMPC should also have been also tested at these levels (~5 \( \mu \)g/ml apoE) and, by the same token, CHO cell apoE should have been tested within the range of apoE:DMPC (10-200 \( \mu \)g/ml). It remains to be seen whether apoE:DMPC at lower concentrations is active in downregulating VCAM-1 or whether higher concentrations of CHO cell apoE lose their ability to modulate VCAM-1. ApoE can exhibit biphasic behaviour, dependent on its concentration [269] and this may also be a factor in the regulation of VCAM-1 by apoE.
Chapter 5
5. EXPLORATION OF A POTENTIAL MECHANISM FOR THE
DOWNREGULATION OF VCAM-1 BY CELL-DERIVED APOE.

5.1 Introduction.

In the previous chapter, it was discovered that cell-derived apoE downregulated
HUVECs VCAM-1 expression by an unresolved mechanism. Importantly, NOS, the enzyme
responsible for NO production, can be regulated by apoE in cells of the vasculature. ApoE
has been reported to induce activation of eNOS in platelets [276] with the production of NO
inhibiting platelet aggregation, via an apoE receptor-dependent mechanism [214-217].
Furthermore, in cells of the vascular wall such as macrophages [304] and vascular smooth
muscle cells [278], apoE causes similar activation of the inducible enzyme, iNOS. The
corresponding release of NO in smooth muscle cells is associated with apoE’s cytostatic
functions of inhibiting cell proliferation and migration which may protect against vascular
diseases including atherosclerosis [307]. In macrophages, NO synthesized by iNOS is
thought to be an important autoregulatory inhibitor of vascular inflammation, in part by
inhibiting monocyte-endothelial adhesion [308], and therefore modulation of this enzyme by
apoE is likely to be yet another function of this anti-atherogenic molecule.

NO is a potent intracellular messenger and endogenous inhibitor of vascular lesion
formation in vivo [420]. Significantly, this is partly due to its ability to reduce monocyte
adhesion to the endothelium [107,293,359], by suppressing VCAM-1 induction by cytokines
[101,105,107-109,114]. It has been known for several years that NO regulates transcription
events for NF-κB-dependent pro-inflammatory genes, including VCAM-1, and limits
endothelial activation in response to cytokines in human vascular endothelial cells [105,107].
In vitro, not only have exogenous NO donors been shown to downregulate cytokine-induced
VCAM-1 [101,105,107-109,114], but endogenously cell-produced NO has the same effect
[107,109,359].

The inhibitory effect of NO on VCAM-1 transcription is common in many different
models of vascular endothelium including human saphenous vein endothelial cells (HSVCEs)
[101,107,109], aortic endothelial cells [107,114] and HUVECs [105,108]. Moreover, using my
preparations of early passage HUVECs, I have also been able to show that these cells are
capable of responding to a NO donor by downregulating VCAM-1 (Section 3.3.2). This
strengthens the novel and attractive hypothesis that in my HUVECs, apoE modulates
VCAM-1 expression by NO release, possibly by apoE binding to a signal-transducing apoE
receptor capable of triggering a signalling cascade to ultimately activate NOS. It was logical, therefore, to probe for the involvement of NOS and NO release in the downregulation of VCAM-1 by cell-derived apoE. The preliminary experiments in this Chapter explore the plausibility of the proposed apoE-NO link in the endothelium as the mechanism responsible for the effect of apoE on VCAM-1.

5.2 Specialized Materials and Methods.

5.2.1 Materials.

2-ethyl-isothiopseudourea (ethyl-ITU) was obtained from Calbiochem-Novabiochem Ltd (Nottingham, UK). QuickPrep Micro mRNA Purification Kit, cGMP Radioimmunoassay Kit, PRO-MIX L-[³⁵S] In Vitro Cell Labelling Mix, Amplify reagent, Hyperfilm ML high performance autoradiography film and NHS-Sepharose HiTrap columns were all supplied by Amersham Pharmacia Biotech UK Ltd. Moloney murine leukaemia virus (MuLV) reverse transcriptase, GeneAmp RNA PCR Kit and AmpliTaq Gold PCR DNA Polymerase System were from Perkin Elmer Applied Biosystems. Endothelial NOS (eNOS) antibody was supplied by Transduction Laboratories (Kentucky, USA). Complete Mini protease inhibitor cocktail tablets were purchased from Roche Diagnostics Ltd.

Extracts of recombinant CHO cells expressing LRP8 (CHOLRP8) were a gift from Dr X Sun (MRC Lipid Team, Hammersmith Hospital, London, UK). The anti-peptide anti-sera 'αLRP8Ins' and the corresponding peptide were commissioned from Genosys Biotechnologies Europe Ltd (Cambridge, UK). All other reagents, unless otherwise stated in the text, were purchased from Sigma-Aldrich Company Ltd.

5.2.2 Western Blotting of HUVEC eNOS.

A 75 cm² flask of confluent HUVECs (P⁻) was washed with warm PBS then cooled on ice. Cells were gently scraped in 1.5 ml ice cold PBS using a cell scraper and a 30 µl aliquot kept for determining the cell count before the rest of the cells were pelleted by centrifugation (300 g, 5 min). The cell pellet was drained of excess PBS and taken up in 20 µl boiling 1 x sample buffer (125 mM Tris.HCl pH 6.8, 2 % w/v SDS, 5 % v/v glycerol, 0.003 % w/v bromophenol blue and 1 % v/v β-mercaptoethanol), resuspended and boiled at 95 °C for 5 min. A confluent flask of CI1STH cells (P³⁰) was treated similarly. The 20 µl samples (1 x 10⁶ HUVECs/CI1STH) along with 5 µl of positive control lysate (human aortic endothelial cell or HAEC lysate, supplied with the antibody), were subjected to 8 % SDS-PAGE under
reducing conditions and then immunoblotting (Section 4.2.7.3) using a primary eNOS antibody at a 1/1,000 dilution and then a 1/1,000 dilution of anti-rabbit secondary antibody-alkaline phosphatase conjugate. After the final wash, the membrane was stained using freshly prepared alkaline phosphatase substrate: 66 µl nitro blue tetrazolium or NBT and 33 µl bromochloroindolyl phosphate or BCIP (both 5 %, w/v in 70 % dimethylformamide, v/v) in 10 ml alkaline phosphatase buffer consisting of 100 mM Tris.HCl (pH 9.5), 100 mM NaCl and 5 mM MgCl₂. Purple bands appeared after 20 min. Washing the blot in copious amounts of distilled water stopped the development reaction. The blot was then dried and stored in the dark.

5.2.3 INHIBITING NOS ACTIVITY WITH ETHYL-ITU.

HUVECs were preincubated in 96-well plates for 2 h with the NOS inhibitor, ethyl-ITU, (100 µM) prior to adding CHO cell-conditioned media from CHO°apoE2 cells or CHO°hfe-cells as controls (Section 4.2.6) which also contained 100 µM ethyl-ITU. Ethyl-ITU was used from a freshly made 2 mM stock in media which was diluted 1/20 to give a final concentration of 100 µM in cell-conditioned media. Control wells were without ethyl-ITU added. HUVECs were incubated for 24 h with CHO cell-conditioned media, with addition of TNF-α for the last 6 h (final TNF-α concentration of 10 U/ml or 0.5 µg/ml). VCAM-1 was measured by ELISA (Section 2.2.9) and cellular protein also determined (Section 3.2.9).

5.2.4 CYCLIC GMP ASSAY.

HUVECs were plated in 12-well plates (0.7 x 10⁵ cells/well) and allowed to adhere overnight. Wells were then incubated for 24 h with 400 µl of 5-7 µg/ml apoE2 in CHO°apoE2 cell-conditioned media (Section 4.2.6), adding TNF-α for the last 6 h (final TNF-α concentration of 10 U/ml or 0.5 µg/ml). After this time, cells were washed with warm PBS and lysed with 133 µl of 20 % (v/v) HClO₄ for 30 s before neutralization with 266 µl 1.08 M K₃PO₄. Samples were centrifuged (2,000 g for 15 min at 4 °C) and, after acetylation, the supernatant assayed for intracellular cyclic guanosine 3',5'-monophosphate (cGMP) content by Dr D. Riddell using a commercial radioimmunoassay kit.

5.2.5 RT-PCR TO IDENTIFY HUVEC LRP8.

PCR (polymerase chain reaction) is a relatively simple but sensitive technique by which a DNA or cDNA template is amplified many thousand- or million-fold quickly and reliably [421]. The PCR process amplifies short (usually 100-500 bp) segments of a longer DNA molecule. A typical amplification reaction includes the sample of target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates
(dNTPs), reaction buffer and magnesium. The reaction is placed in a thermal cycler and exposed to a series of optimized temperature and time adjustments, referred to as one cycle of amplification. Each cycle of PCR amplification consists of three steps: 1) production of single-stranded DNA templates by denaturation of double stranded DNA, 2) annealing the two oligonucleotide primers and 3) synthesis of a copy from each strand of template by DNA polymerase. Theoretically, each PCR cycle doubles the amount of targeted template sequence in the reaction with ten cycles theoretically multiplying the sequence a thousand-fold and 20 cycles by a million-fold.

In order to apply PCR methodology to the study of RNA, the RNA sample must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called 'reverse transcription' (RT). I used MuLV reverse transcriptase to produce a DNA copy of the HUVEC mRNA template using an oligo(dT) primer. After this initial RT step, the procedure follows the temperature cycling steps of the basic PCR reaction, amplifying the target sequence as a DNA molecule.

5.2.6 MESSENGER RNA EXTRACTION.

Messenger RNA (mRNA) was extracted from HUVEC, megakaryocytic HEL cells and CHO cells (2 x 10^6 cells for each cell type) using the QuickPrep Micro mRNA Purification Kit adhering to the manufacturer's instructions. Briefly, the cells are extracted in a buffered solution containing guanidinium thiocyanate, ensuring the rapid inactivation of endogenous ribonucleases (RNases). The mRNA was purified by oligo(dT)-cellulose chromatography in a spun-column format, by virtue of the poly(A) tracts on mRNA molecules which hydrogen-bond with oligo(dT). As mRNA is extremely sensitive to RNases, care was taken to prevent contamination during mRNA isolation and subsequent handling. Gloves were worn for all manipulations and disposable RNase-free plastic-ware was used. Solutions were treated with diethylpyrocarbonate (DEPC) and autoclaved.

The isolated mRNA was subjected to ethanol precipitation and washed twice with 75 % ethanol. Finally, the mRNA pellet was air-dried and reconstituted in 20 μl DEPC-treated water and the mRNA content determined by UV spectrometry (see equation below). An optical density of 1 at 260 nm (A_{260}) corresponds to approximately 40 μg/ml for single stranded RNA. All mRNA samples were stored at -70 °C prior to use.

\[ \text{[RNA]} \, \text{ng/μl} = A_{260} \times 40 \, \mu g/ml \]
5.2.7 **RT Protocol.**

One μg or 1/4 of the total amount of RNA isolated from each cell type was converted to cDNA with MuLV reverse transcriptase using the GeneAmp RNA PCR Kit.

A master mix for RT was prepared by adding the reagents in the order and proportions shown below to 0.2 ml thin-walled PCR tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM MgCl₂ Solution</td>
<td>4 μl</td>
<td>5 mM</td>
</tr>
<tr>
<td>10 x PCR Buffer II</td>
<td>2 μl</td>
<td>1x</td>
</tr>
<tr>
<td>DEPC Water</td>
<td>5.2 μl</td>
<td>--</td>
</tr>
<tr>
<td>dATP (100 mM stock)</td>
<td>0.2 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>dGTP (100 mM stock)</td>
<td>0.2 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>dTTP (100 mM stock)</td>
<td>0.2 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>dCTP (100 mM stock)</td>
<td>0.2 μl</td>
<td>1 mM</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1 μl</td>
<td>1 U/μl</td>
</tr>
<tr>
<td>MuLV Reverse Transcriptase</td>
<td>1 μl</td>
<td>2.5 U/μl</td>
</tr>
<tr>
<td>Oligo d(T)16</td>
<td>1 μl</td>
<td>2.5 pM</td>
</tr>
<tr>
<td>mRNA extracted from cells or DEPC water control</td>
<td>5 μl</td>
<td>≤ 1 μg total RNA</td>
</tr>
<tr>
<td><strong>Total volume, including sample.</strong></td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

Tubes were incubated in a Stratagene PCR Robocycler (Stratagene Ltd; Cambridge, UK) as follows:

<table>
<thead>
<tr>
<th>Annealing</th>
<th>Reverse Transcription</th>
<th>Denaturation of Enzyme</th>
<th>Cooling</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C for 10 min</td>
<td>42 °C for 20 min</td>
<td>95 °C for 5 min</td>
<td>4 °C for 5 min</td>
</tr>
</tbody>
</table>

5.2.8 **General Protocol for PCR Amplification.**

One-quarter of the total amount of cDNA (5 μl) from each cell type was amplified with the GeneAmp AmpliTaq Gold PCR DNA polymerase system. A master mix for amplification was prepared by adding the reagents in the order and proportions shown below to 0.2 ml thin-walled PCR tubes. The incubation steps were optimized for each template and primer pair combination using the PCR Robocycler.
### 5.2.9 Assessment of RNA Integrity.

The quality and purity of the starting mRNA template is crucial to the success of RT-PCR. As the efficiency of the first strand synthesis reaction can be related to the quality of the RNA template and significantly influences the success of the subsequent amplification, the template must be intact and free of contaminating genomic DNA. Therefore, the quality of mRNA was assessed by RT-PCR for U1A RNA [422]. U1A is a 'housekeeping' RNA species which forms part of the U1A spliceosome complex [423] and is required by all living cells. Briefly, 500 ng aliquots of the RT reaction mixture (containing cDNA) were subjected to PCR with 5 μM U1A1 primer (GGC CCG GCA TGT GGT GCA TAA) and 5 μM U1A2 primer (CAG TAT GCC AAG ACC GAC TCA GA) in a total volume of 50 μl. After heating at 95 °C for 5 min, amplification proceeded for 35 cycles, with denaturation for 30 s at 95 °C, annealing of primers for 30 s at 56 °C and extension for 30 s at 72 °C. Finally, the reaction was completed by an extension step at 72 °C for 10 min. Reaction products were visualized and photographed under UV light after electrophoresis of 10 μl of the product in a 2 % agarose gel containing 0.3 μg/ml ethidium bromide (see below). A successful mRNA preparation gave a clean PCR product of ~230 bp in length.

### 5.2.10 Agarose Gel Electrophoresis of DNA.

The results of a PCR reaction were conveniently analysed using agarose gel electrophoresis, followed by staining the DNA with ethidium bromide and visualization by UV irradiation of the gel [421]. The minigel apparatus (Horizon minigel apparatus, Life
Technologies) was set up as recommended by the manufacturer. Table 5.2-1 outlines the separation ranges for typical gel concentrations. The required weight of agarose (AquaPor LE GTAC agarose; National Diagnostics, Hull, UK) was added to the appropriate amount of 1 x Tris borate EDTA (TBE) buffer (from a 10 x stock; National Diagnostics). The mixture was heated in a microwave oven until the agarose just dissolved (usually 2 min) with mixing at regular intervals. The solution was cooled to 50-60 °C and, after addition of ethidium bromide (0.5 μg/ml), was poured into the cast. The gel was allowed to set for ~30 min at room temperature. The comb and blocks were removed and a sufficient volume of 1 x TBE buffer containing ethidium bromide (0.3 μg/ml) was added. The PCR products were mixed 9:1 with 10 x loading buffer (10 mM Tris.HCl, pH 7.5 containing 50 mM EDTA, 10 % v/v Ficoll 400, 0.25 % w/v bromophenol blue and 0.25 % v/v xylene cyanol FF) and loaded (10-20 μl) into the wells. The gel was run at a constant voltage of 125 V for ~30 min or until the dye front had migrated 2 cm from the bottom of the gel. After electrophoresis, the gel was visualized and photographed under UV light.

<table>
<thead>
<tr>
<th>DNA Size (bp)</th>
<th>Gel Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 3,000</td>
<td>2.00 %</td>
</tr>
<tr>
<td>150 - 4,000</td>
<td>1.75 %</td>
</tr>
<tr>
<td>200 - 5,000</td>
<td>1.50 %</td>
</tr>
<tr>
<td>300 - 8,000</td>
<td>1.25 %</td>
</tr>
<tr>
<td>400 - 12,000</td>
<td>1.00 %</td>
</tr>
<tr>
<td>1,000 - 23,000</td>
<td>0.75 %</td>
</tr>
</tbody>
</table>

Table 5.2-1 Separation ranges for typical agarose gel concentrations.

5.2.11 SPECIFIC RT-PCR AMPLIFICATION OF HUVEC LRP8 mRNA.

To confirm the presence of LRP8 mRNA in HUVECs, a specific sequence encompassing LRP8 cytoplasmic tail, was amplified from HUVEC and CHO<sup>LRP8</sup> cell cDNA, essentially as described previously [212]. Briefly, 2 μl of the RT reaction mixtures were subjected to 'hot-start PCR' using the GeneAmp AmpliTaq Gold PCR DNA polymerase system with 1 μM sense primer: oligonucleotide 2546 5'-GAA ACT GGA ACG GGA AGA AC-3' and 1 μM of antisense primer: oligonucleotide 2918 5'-GAG GCA CGA AGG GGG TGA T-3' in a total volume of 50 μl. After heating at 95 °C for 10 min, amplification proceeded for 40 cycles, with denaturation for 30 s at 95 °C, annealing of primers for 1 min
at 63 °C, and extension for 1 min at 72 °C. Additional Taq was added after 20 cycles and the reaction was completed by an extension step at 72 °C for 10 min. Products (10 µl aliquot) were visualized and photographed under UV light after electrophoresis in a 2% agarose gel containing 0.3 µg/ml ethidium bromide.

5.2.12 **LONG-RANGE RT-PCR of HUVEC LRP8.**

To amplify the full-length open reading frame of LRP8, HUVEC, megakaryocytic HEL cell and CHO<sup>LRP8</sup> cell cDNA were subjected to GC-rich RT-PCR using the Advantage-GC PCR kit (Clontech Laboratories UK Ltd), in the presence of 0.5 M GC melt, according to the manufacturers instructions. Briefly, 2 µl of each cDNA preparation were subjected to 35 cycles with primer annealing at 64 °C for 30 s and elongation at 68 °C for 3 min in a total volume of 50 µl (sense primer, oligonucleotide 24: 5'-TCT CCG GCT TCT GGC GCT-3', and antisense primer, oligonucleotide 2918: 5'-GAG GCA CGA AGG GGG TGA T-3' both at 300 nM). Reaction products (10 µl aliquot) were analysed by electrophoresis in a 1% agarose gel containing 0.3 µg/ml ethidium bromide, purified and cloned into pGEM-T (Promega) for automated fluorescent sequencing (Oswel Services, Southampton, UK).

5.2.13 **METABOLIC LABELLING OF HUVECs with [<sup>35</sup>S]METHIONINE.**

HUVECs were metabolically labelled with PRO-MIX L-[<sup>35</sup>S] In Vitro Cell Labelling Mix. This mix contains approximately 70% L-[<sup>35</sup>S]methionine and 30% L-[<sup>35</sup>S]cysteine allowing both amino acids to be optimally incorporated into proteins being synthesized when used in conjunction with methionine/cysteine-deficient media.

5.2.13.1 **Radioactive Labelling Procedure.**

One 75 cm<sup>2</sup> flask of HUVECs at ~50% confluency (1 x 10<sup>6</sup> cells total) was washed twice with warm PBS (to remove traces of methionine/cysteine from media/FBS). Cells were then incubated for 4 h at 37 °C with 5 ml of 'labelling media': methionine/cysteine-deficient RPMI 1640 media (ICN Pharmaceuticals Ltd) with 10% (v/v) dialysed FBS, 3.6 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µCi/ml L-[<sup>35</sup>S]methionine/L-[<sup>35</sup>S]cysteine (total of 250 µCi of radioactive label added to the flask). After this period, the labelling media was removed and replaced by complete M199 media (20% FBS), to 'chase' incorporation of the label, and incubated for a further 2 h. The cells were then washed twice with warm PBS and after brief cooling on ice, were gently scraped in 1.5 ml ice cold 'sonication buffer' (Complete Mini protease inhibitor cocktail in PBS). The cells were transferred to a 20 ml tube and the flask rinsed in more cold sonication
buffer giving a final volume of 8 ml cell suspension.

5.2.13.2 Preparation of Radioactively Labelled Cytosolic and Membrane Fractions.

The [³⁵S]-labelled HUVECs in sonication buffer were disrupted by ultrasonication using a Sanyo Soniprep 150. The suspension, kept on ice throughout, was subjected to 7 x 20 s bursts at 8 μm amplitude with 20 s cooling intervals. Lysates were freed of any intact cells, membranes, organelles and mitochondria and any contaminating titanium from the probe by ultracentrifugation at 19,000 g for 30 min at 4 °C and re-centrifugation at 100,000 g for 1 h (both steps using pre-chilled rotors). The pellet and supernatant were then both used for two different procedures:

1. The membrane pellet was dissolved in 'cell lysis buffer': 10 mM EDTA, 10 mM EGTA, 10 mM N-ethylmaleimide, 2.2 % v/v DMSO, 1 % v/v Triton X-100, Complete Mini protease inhibitor cocktail, 50 mM HEPES, 100 mM NaCl. This was then centrifuged to remove insoluble material and the soluble membrane fraction used for immunoprecipitation of LRP8 (Section 5.2.15).

2. The supernatant containing cytosolic proteins was subjected to affinity chromatography to isolate proteins capable of binding to the cytoplasmic tail of LRP8 (Section 5.2.16).

5.2.13.3 Scintillation Counting.

Five µl aliquots of radioactive material were kept at each stage of the labelling procedure for counting in 5 ml Cocktail T scintillation fluid to measure emissions of β-radiation. Scintillation cocktail contains a mixture of organic solvents and 'fluors'. When bombarded with radiation these compounds are excited, emit photons of light and this fluorescence can be detected by phototubes and quantified.

5.2.14 Preparation of Anti-LRP8 Antibodies (αLRP8Ins) – DR D. RIDDELL.

An anti-peptide antiserum designated 'αLRP8Ins' was supplied by Dr D. Riddell. The antiserum was directed against a peptide of 17 amino acids (residues 865-881: CLGETREPEDPAPALKE) corresponding to a region within the unique cytoplasmic insert of human LRP8, as deduced from the published cDNA [211], and was prepared by immunizing rabbits with the KLH-conjugated peptide (Genosys Biotechnologies Europe Ltd). The sensitivity, specificity and titre of αLRP8Ins had been previously assessed by Western blotting using recombinant CHO cell extracts from cells overexpressing LRP8 with
and without cytoplasmic insert. It should be noted that because αLRP8Ins was commissioned before alternative splicing of LRP8 was recognised, it is only useful for the detection of LRP8 variants containing the cytoplasmic insert.

5.2.15 IMMUNOPRECIPITATION OF HUVEC LRP8.

The [35S]-labelled HUVEC membranes were dissolved in 'cell lysis buffer' (Section 5.2.13.2) and incubated overnight with 10 μl pre-immune serum at 4 °C, continually mixing on a rotating wheel. Protein A-Sepharose beads (50 μl) were added for 3 h and the mixture centrifuged for 1 min at 13,000 g. The supernatant was divided into 2 x 450 μl aliquots. One half was incubated overnight with 10 μl pre-immune sera, the other with 10 μl αLRP8Ins, and then each for a further 3 h with 50 μl protein A-Sepharose. The beads were isolated by centrifugation as before, washed 10 x with cell lysis buffer, and then boiled in SDS-PAGE sample buffer to elute the immunoprecipitated proteins. Proteins were separated by SDS-PAGE in an 8 % gel which was then analysed for radioactive protein bands by fluorography.

5.2.15.1 Radioisotope Detection by Fluorography.

Since [35S] is a β-emitter, much of the energy would be lost in the gel using traditional autoradiography, therefore a fluorographic reagent was used to increase sensitivity. SDS-PAGE gels were fixed in isopropanol:water:acetic acid (25:65:10) for 30 min and then impregnated with Amplify fluorographic reagent by soaking for 30 min with constant shaking. The gels were immediately dried then placed in contact with a sheet of Hyperfilm ML high performance autoradiography film and the cassette stored at -70 °C for 2 weeks before developing.

5.2.16 ISOLATION OF CYTOSOLIC HUVEC PROTEINS WHICH BIND CYTOPLASMIC LRP8.

A peptide column was produced by coupling 10 mg of the 17-mer peptide used to generate αLRP8Ins to a 1 ml NHS-Sepharose HiTrap column as described by the manufacturers. The [35S]-labelled HUVEC cytosol was pre-cleared by passage through a Sepharose 4-B column (10 ml) equilibrated in sonication buffer and then recirculated through the peptide-Sepharose matrix overnight at 4 °C. After washing the column with 10 volumes of PBS, bound proteins were eluted with PBS containing 0.5 M NaCl. The eluate was simultaneously desalted and concentrated to 100 μl using a 15 ml concentrator (10,000 MWCO; Vivaspin). A 20 μl sample was then separated by 12 % SDS-PAGE and any radioactive interacting proteins detected by fluorography (Section 5.2.15.1).
5.2.17 **Statistical Analysis.**

Values in text, tables and figures were expressed as the mean ±SE. Statistical differences between means were determined by Student's t-test or ANOVA test as appropriate and considered significant if P<0.05. Analyses were performed using InStat version 3.01 (GraphPad Software, San Diego, USA) and SigmaPlot for Windows (Jandel Scientific, Erkrath, Germany).

5.3 **Results and Discussion.**

5.3.1 **Expression of eNOS in Cultured Endothelial Cells.**

Primary cells lose their native characteristics with time in culture. Protein expression by primary endothelial cells is lost with repeated passaging including those proteins which are constitutively expressed, such as vWF [424], and those which are induced or superinduced in response to cytokine-stimulation such as CAMs [81]. In addition, the ability of endothelial cells to retain their characteristic gene expression and phenotypic properties is also dependent upon their environment. For HUVEC culture this includes the type of extracellular matrix substratum and cell density, suggesting that cell-matrix and cell-cell interactions have a strong influence on their endothelial behaviour [425]. In cultured bovine endothelial cells, eNOS expression, both in terms of transcript and protein abundance, is sensitive to cell proliferation [426]. Thus, the observation that eNOS expression diminishes when endothelial cells reach confluence has important ramifications for the validity of my hypothesis. Obviously, for a link between apoE and NO to be feasible in HUVECs, eNOS had to be present. There was already indirect evidence that eNOS was present and functionally active in my HUVEC preparations. In Chapter 3 the inhibitory effect of 17β-estradiol on VCAM-1 expression in confluent HUVECs was described. It is known that 17β-estradiol upregulates eNOS activity in endothelial cells [111] with the resulting NO production presumably inhibiting VCAM-1 transcription [112,113]. This suggests that eNOS would need to be expressed in my HUVECs for the effect to occur. However, eNOS-independent mechanisms have also been suggested [113], indicating that eNOS expression may not be obligatory to mediate the effect of 17β-estradiol.

In the light of this, it was prudent to check that my preparations of low passage HUVECs did contain eNOS. HUVECs at the highest passage number used (P*), and grown on a gelatin substratum to confluence, contained eNOS as detected by immunoblotting (Section 5.2.2), proving that both within this time period in culture and under my experimental
conditions HUVECs had not lost their ability to express eNOS protein (Figure 5.3-1). Interestingly, this is the first report that the HUVEC cell line, C11STH, has retained ability to express eNOS (Figure 5.3-1) implying that these immortalized cells may be a useful model to study eNOS.

![Figure 5.3-1 Early passage HUVECs express eNOS.](image)

Confluent HUVECs ($1 \times 10^6$ cells, $P^5$) and cells of the C11STH endothelial cell line ($1 \times 10^6$, $P^5$) were subjected to 8 % SDS-PAGE under reducing conditions, immunoblotted for eNOS and visualized by alkaline phosphatase substrate as described in Section 5.2.2. The HAEC positive control cell lysate (of human arterial endothelial cells and supplied with the eNOS antibody) showed a specific band at 140 kDa corresponding to eNOS. Both HUVEC and C11STH samples contained a band with the same molecular weight.
5.3.2 THE EFFECT OF A NOS INHIBITOR ON APOE-INDUCED DOWNREGULATION OF VCAM-1.

To test the hypothesis that NO mediates the inhibitory effect of apoE, a NOS inhibitor was incubated with the cells in conjunction with CHO cell-conditioned media. Ethyl-ITU is a competitive inhibitor of eNOS (and iNOS) with a more potent action than other L-arginine analogues [427]. Excess inhibitor (final concentration of 100 μM) was used to counteract the concentration of L-arginine (300 μM) present in M199 media. Ethyl-ITU had no effect on VCAM-1 induction when it was added to the control CHO<sup>Δfr</sup> cell-conditioned media (as compared with control media alone, results not shown). However, in the presence of ethyl-ITU, the apoE effect on VCAM-1 using CHO<sup>Δfr</sup> cell-conditioned media was completely abolished as compared to the ethyl-ITU-containing control (Figure 5.3-2), indicating that NOS stimulation and subsequent NO release may mediate the effect. Additional support for this finding came from the observation that the reduction of TNF-α-induced VCAM-1 expression caused by CHO cell-derived apoE was comparable to the suppression by exogenous NO release by the NO donor, GSNO (Figure 5.3-2). Together these data tentatively suggest that release of NO from HUVECs in response to apoE exposure could be responsible for the effect on VCAM-1.

5.3.3 CELL-DERIVED APOE CAUSES AN INCREASE IN INTRAHUVEC cGMP.

The potent intracellular second messenger, cGMP, can be measured as an indicator of NOS activation and subsequent NO production. Levels of cGMP are controlled by the activities of both synthesizing and catabolizing enzymes, soluble guanylate cyclase (SGC) and cGMP phosphodiesterase, respectively [428]. One important cellular mechanism for increasing cGMP is through stimulation of NOS [300]. NO activates heme-containing SGC, a physiological target which is often termed the 'NO receptor' [282,429,430], thus stimulating conversion of GTP to cGMP.

Interestingly, incubating HUVECs for 24 h with CHO<sup>Δfr</sup> cell-conditioned media (containing 5.9±0.9 μg/ml apoE) did increase the level of intraHUVEC cGMP above that detected in the CHO<sup>Δfr</sup> cell-conditioned media controls (analysis kindly performed by Dr D. Riddell). The control level of cGMP of 100.0±15.0 % was increased to 146.5±15.0 % by treatment with apoE-containing media (Figure 5.3-3). An increase in cGMP is indicative of SGC stimulation, presumably by the action of NO, and therefore this data indirectly implies that NO was produced in HUVECs in response to apoE treatment.
Figure 5.3-2 The downregulatory effect of CHO cell-derived apoE on VCAM-1 is blocked by inhibition of NOS and is comparable to the effect of an NO donor.

HUVECs were incubated for 24 h with CHO<sup>apoE</sup> cell-conditioned media (containing 5.5±0.2 µg/ml apoE) and exposed to TNF-α (10 U/ml) for the last 6 h. The downregulatory effect on VCAM-1 by apoE (23.4±2.2% inhibition, *P<0.001, Student’s t-test) was similar to that of the NO donor, GSNO at 200 µM in co-incubation with TNF-α (20.9±3.3% inhibition, *P<0.001, Student’s t-test). When the NOS inhibitor, ethyl-ITU, was preincubated with HUVECs and then also added to CHO<sup>apoE</sup> cell-conditioned media (100 µM ethyl-ITU throughout the incubations), the apoE effect was no longer evident (99.2±6.3% VCAM-1 induction) as compared to the CHO<sup>apoE</sup> cell-conditioned media control (100.1±2.0% VCAM-1 induction, black bar). Results were calculated as described in Section 4.2.3.4 and bars show VCAM-1 induction as a % of the TNF-α control (mean ±SE) for 3 independent experiments performed in quadruplicate and using 3 batches of HUVECs.

However, caution must be taken when interpreting these results, as this assay is an indirect measurement of NOS activation and although NO potently increases cGMP levels, it is not the only stimulator of its production. Indeed, atrial natriuretic peptides have been shown to directly stimulate the particulate form of GC in cultured endothelial cells [431,432]. It should also be stressed that although many of the physiological messenger effects of NO
are exerted via production of cGMP [430], the mechanism by which VCAM-1 is inhibited by NO is reported to be mediated via a cGMP-independent mechanism [101,107]. Hence, in my case the cGMP assay is only being used as an indicator of NOS activation; the rise in intraHUVEC cGMP by apoE treatment is unlikely to directly regulate VCAM-1.

Figure 5.3-3 Levels of intraHUVEC cGMP are increased by incubation with CHO cell-secreted apoE.

Confluent HUVECs in 12-well plates were incubated for 24 h with CHOapoE cell-conditioned media (containing 5.9±0.9 % apoE/ml apoE) or with CHO_7 cell-conditioned media in controls. Intracellular levels of cGMP were measured by commercial radioimmunoassay (Section 5.2.4). Levels of cGMP in controls varied between 45-125 pmol/10^6 cells in the 3 different HUVEC batches used, using 3 preparations of CHO cell-conditioned media. Data for the controls was normalized to 100 % cGMP content (mean 100.0±15.0 %) with the tests in each experiment expressed as a % of the control. Incubation with CHOapoE cell-conditioned media increased cGMP by 46.5±15.0 % to 146.5±15.0 % (*P<0.05, Student’s t-test). Graph shows % cGMP production for 3 experiments using 3 batches of HUVECs (mean ±SE).
5.3.4 **Summary - NO as a Potential Mediator of VCAM-1 Downregulation by CHO Cell-Derived ApoE.**

Taken together, these preliminary data do support the potential scheme that NO could be the mediator of the apoE effect on VCAM-1. However, this is by no means conclusive. I have shown that a NOS inhibitor abolishes the apoE effect on VCAM-1, and indirect measurement of NO supports this hypothesis. However, specific NO involvement has yet to be directly confirmed. To be sure that the activity of HUVEC NOS is increased by CHO cell-secreted apoE, either its enzymatic activity or the NO produced still need to be measured directly, in controlled experiments with and without a panel of NOS inhibitors present. The citrulline assay, the standard assay for measuring NOS activity, monitors the stoichiometric conversion of L-arginine to L-citrulline with the use of a radiolabelled substrate (L-[H]arginine) and allows detection of pmol levels of conversion [429]. Time permitting, this could have been employed to quantify HUVEC NOS activity. Alternatively, as NO is a free radical of lipophilic nature and diffuses randomly from its point of synthesis, any NO released into the culture media could have been measured using an NO analyser with a sensitivity in the pmol range (such as the Sievers NOA 280, Sievers Instruments Inc; Boulder, CO, USA). It is the breakdown products of NO in solution, nitrites ($\text{NO}_2$) and nitrates ($\text{NO}_3$), that the sensitive detector measures; the energy released by a gas-phase chemiluminescent reaction (between NO and ozone) is detected by a photomultiplier tube. Pilot experiments using my M199 media proved that this analysis was unfeasible due to very high levels of background $\text{NO}_2/\text{NO}_3$ (results not shown). However, the possibility of using a differently formulated media, with negligible levels of $\text{NO}_2/\text{NO}_3$, could be explored in the future.

These methodological problems aside there is further circumstantial evidence for an apoE-NO link in endothelial cells. Thus, apoE inhibits endothelial proliferation and migration [277], effects that can be mirrored by NO donors [433-435]. Similarly, as outlined in Chapter 4 (Section 4.3.5), VCAM-1 was significantly reduced by CHO cell-secreted apoE, whereas E-selectin was unaffected. This ties in with the observation that, although NO donors have been shown to downregulate cytokine-induced VCAM-1 expression, E-selectin is not subject to NO modulation in HUVECs [105]. Furthermore, high levels of L-arginine, the precursor for NO biosynthesis, attenuates cytokine-induced monocyte adhesion to HUVECs via the downregulation of VCAM-1, but not E-selectin, by a mechanism that is blocked by NOS inhibitors [359]. Another group have reported that in a different endothelial cell model, HSVEC, E-selectin is suppressed to some degree by NO but not to the same extent as VCAM-1 [101].
If NO is involved in the downregulation of VCAM-1, then how is the signal conveyed by apoE? In platelets, it is thought that apoE binds to its platelet receptor, LRP8 to initiate a tyrosine kinase-dependent signal transduction cascade to upregulate eNOS [214-216,276]. If a similar apoE-LRP8-eNOS link existed in endothelial cells then this could be a potential pathway for the downregulation of VCAM-1 by apoE. Therefore, the following experiments were conducted to probe for the existence of this pathway in my model of the vascular endothelium, HUVECs.

5.3.5 LRP8 AS A RECEPTOR TO POTENTIALLY LINK APOE WITH ELEVATED NO IN THE ENDOTHELIUM.

Human apoE receptor 2 (apoER2), a newly described brain receptor of the LRSF, has now been designated 'LRP8' by the Human Genome Organization Nomenclature Committee (HUGO-NC). LRP8 consists of five domains that resemble those of the LDL-R and the VLDL-R [198,200,211,212] (Figure 5.3-4). Although LRP8 and LDL-R both contain seven class A repeat sequences in their ligand-binding domains, the ligand-binding domain structure of LRP8 is more closely related to that of VLDL-R. There is a high degree of homology between LRP8 and VLDL-R, not only in amino acid sequence (ranging from 45-63% identity per class A repeat) but also in the ligand binding domain architecture [211]. The ligand specificity of LRP8 resembles the VLDL-R; LRP8 binds RAP [218], α2-macroglobulin [436] and apoE-containing lipoproteins (β-VLDL), but not VLDL or LDL [211,212] with high affinity. However, the complete ligand specificity of LRP8 has yet to be determined. To complicate matters, splice variants affecting the extra- as well as the intracellular domains of LRP8 have been demonstrated at the transcript level in brain tissues [211,212,218]. These mRNA variants include transcripts lacking ligand binding repeats 4-6 (LRP8Δ4-6) or 4-7 (LRP8Δ4-7) and LRP8 without a unique cytoplasmic insert (LRP8Δinsert). It has still to be demonstrated whether the multiple forms of LRP8 protein, corresponding to the mRNA variants actually exist in vivo.

Intriguingly, the major difference between LRP8 and the VLDL-R is an insertion sequence of 59 amino acids in the cytoplasmic tail of LRP8. Indeed, this sequence is missing from all other LRSF members and is stated to represent a unique sequence not found in any published protein [211,218]. There is a high degree of conservation between human and mouse LRP8, implying a functional significance. Interestingly, LRP8 could be as a signal transductant (Section 5.3.7); the sequence of the cytoplasmic insert is indicative of cell-signalling potential, with three proline-rich motifs fulfilling minimal consensus sequences for Src Homology 3 (SH3) domain recognition [214-217].
The relevant structural features of LRP8. The LDL-R class A repeats (I-VII), a short linker
linker sequence between V and VI, epidermal growth factor (EGF) homology repeats (A,B & C),
Y/FWxD repeats, O-linked sugar domain with clustered serine and threonine (O), transmembrane region
(TM) and cytoplasmic tail containing a unique 59 amino acid insert (Insert) are indicated.

There is evidence mounting that suggests that apoE binding to LRP8 triggers a signal
transduction cascade leading to eNOS activation [214-217]. As my earlier results implicated
NO in the action of apoE on modulation of VCAM-1 expression, LRP8 would be a serious
candidate for linking apoE to NO in the endothelium. Indeed, the observation that apoE2 is
as effective as apoE3 at VCAM-1 downregulation is consistent with this suggestion (see
Chapter 4). Thus, apoE2 binds poorly to both the LDL-R (only 1 % of apoE3 binding
activity [167]) and LRP (40 % of apoE3 binding [392]), discounting both these receptors
from mediating the VCAM-1 suppression. However, LRP8, like the VLDL-R [437,438], has
a relaxed ligand specificity and binds the different apoE isoforms with equal affinity (personal
communication; Dr T. Yamamoto, Tohoku University, Sendai, Japan). Furthermore, in other
cell types where apoE causes activation of NOS, such as macrophages [304] and vascular
smooth muscle cells [278] mRNA for LRP8 has also been detected, by us (unpublished data)
and by others [230,231].

5.3.5.1 HUVECs have LRP8 mRNA.

To determine whether my HUVEC preparations contain LRP8 mRNA, RT-PCR was
carried out using primers to amplify a specific sequence in the cytoplasmic tail of LRP8
encompassing the insert sequence (Figure 5.3-5). The primers were designed to reveal if this
region was subject to alternative splicing. Messenger RNA from recombinant CHO cells
expressing human full length LRP8 (CHO^{LRP8}) including the cytoplasmic insertion was used
as a control. In HUVECs, a PCR product of the same size as in the CHO^{LRP8} cell control
lane corresponded to cytoplasmic tail of LRP8 with insert. However, a smaller PCR product
was also observed indicating that a variant lacking the insert (LRP8\_\text{Insert}) was expressed.
Our data confirm another report that HUVECs have both full length cytoplasmic tail and LRP8Δinsert mRNA transcripts [231].

Figure 5.3-5 Expression of variant mRNA transcripts in the region encoding the cytoplasmic tail in HUVECs.

mRNA from HUVECs was used for cDNA synthesis with reverse transcriptase (Section 5.2.7). The integrity of the cDNA produced was confirmed by a U1A RT-PCR giving the characteristic band at ~230 bp (Section 5.2.9 for method, data not shown). The resulting viable cDNAs were used for LRP8 PCR amplification with the indicated primer combinations (red arrows). Messenger RNA from recombinant CHO[LRP8] cells expressing human full length LRP8 was used as a control. Amplified products were separated on a 2% agarose gel. The 373 bp fragment corresponds to cytoplasmic LRP8 with insert, while the smaller 196 bp fragment corresponds to a spliced variant lacking the insert (LRP8ΔInsert). The results shown were from one experiment but were reproduced in two other independent reactions using mRNA from different HUVEC batches.
5.3.5.2 Long-Range PCR of HUVEC LRP8.

To determine whether any additional variants are expressed, long-range RT-PCR was carried out on HUVEC cDNA using a pair of primers that flanked the open reading frame of LRP8 (Figure 5.3-6), again using mRNA from CHO^{LRP8} cells as a control for full length LRP8. In HUVECs multiple PCR products were detected (Figure 5.3-6). The major product (~2,500 bp) corresponded to the major transcript found in platelets and human erythroleukaemia (HEL) cells [216,217]. This transcript lacks repeats 4-6 of the ligand-binding domain (LRP8Δ4-6) but contains the 59 amino acid insertion sequence in its cytoplasmic tail. The minor transcripts corresponded to full length LRP8 (expected size, 2,894 bp) and full length LRP8 but without insert (LRP8ΔInsert). However, these products still need to be cloned and sequenced to confirm the true nature of the variants.

![Figure 5.3-6 Long-range PCR of HUVEC LRP8.](image)

*cDNA from HUVECs, HEL and CHO^{LRP8} cells was used for long-range PCR with the indicated primer combinations (red arrows). Amplified products were separated on a 1 % agarose gel. Multiple PCR products were seen in the HUVEC lane. The major product was about 2,500 bp corresponding to a transcript with absence of repeats 4-6 of the ligand-binding domain (LRP8Δ4-6) but with cytoplasmic insert included. Other larger products corresponded to full length LRP8 at ~2,900 bp and possibly full length LRP8 but without the cytoplasmic insert (LRP8ΔInsert, ~2,700 bp).*
5.3.6 **HUVECs Express LRP8 Protein.**

As RT-PCR is extremely sensitive, the identification of LRP8 transcripts in HUVECs by this method does not imply protein expression. Therefore, before LRP8 could be considered as a serious candidate for eliciting the VCAM-1-modulatory effect of apoE, LRP8 protein expression was checked. Earlier work from our laboratory has shown the presence of LRP8 protein in platelets using immunoprecipitation [217]. This technique employed a polyclonal antiserum raised against a 17-mer synthetic peptide within the cytoplasmic insert of LRP8 (designated αLRP8Ins, Figure 5.3-7) and was used to verify protein expression in HUVECs.

Radiolabelled membranes were prepared by metabolically labelling HUVECs with L-[³⁵S]methionine and isolating the membrane fraction by ultracentrifugation. These were then subject to immunoprecipitation with αLRP8Ins (Section 5.2.15). Two specifically precipitated proteins were readily detected confirming that LRP8 was expressed in HUVECs (Figure 5.3-7). One had a reduced molecular mass of 132 kDa, the same size as in platelets, the other being 180 kDa (same as expressed in CHO[LRP8] cells), probably corresponding to LRP8Δ4-6 and full length LRP8, respectively (both with cytoplasmic insert). Although the predicted molecular mass for full length LRP8 is calculated to be 106 kDa [211], the increases were expected and most likely reflect O- and N-glycosylation as is characteristic of other LRSF members [439]. It should also be noted that because αLRP8Ins was commissioned before alternative splicing of LRP8 was recognised, it is only useful for the detection of LRP8 variants containing the cytoplasmic insert and that receptor variants without the insert may also exist in HUVECs.

The functional significance of alternative splicing of HUVEC LRP8 has yet to be determined. Although variants of LRP8 when expressed in recombinant CHO cells bind β-VLDL to the same extent [212,440] there may of course be other ligands such as α₂-macroglobulin [436] which bind LRP8 variants with differing affinity.
\[ ^{35}S \text{-labelled HUVEC membranes were solubilized and subjected to immunoprecipitation with either preimmune sera or antisera raised against a 17-mer synthetic peptide with the cytoplasmic insert of LRP8 (designated } \alpha \text{LRP8Ins). Precipitated cell surface proteins were reduced and separated by } 8\% \text{ SDS-PAGE. Radiolabelled bands were visualized by fluorography; the gel was impregnated with a fluorographic agent, dried and placed in contact with autoradiography film for 2 weeks at -80 °C. Although there were no signals with the HUVEC membrane extract when pre-immune sera was used, } \alpha \text{LRP8Ins specifically immunoprecipitated two proteins with reduced molecular masses of 132 kDa and 180 kDa corresponding to LRP8Δ4-6 and full length LRP8, respectively.} \]
5.3.7 HUVEC LRP8 as a Signal Transductant.

5.3.7.1 The Src Homology 3 (SH3) Motifs.

Many intracellular signalling pathways proceed through a series of modular protein-protein recognition domains. These include Src Homology 2 (SH2) and phosphotyrosine-binding (PTB) domains, which bind specific phosphotyrosine (pY)-containing peptide motifs, and SH3 domains which bind proline-rich motifs [441]. Usually, two or more domains are in the same polypeptide, allowing a network of non-covalently tethered proteins to form and disseminate signalling information to diverse cellular processes [441-444].

Tyrosine (de)phosphorylation is a common consequence of domain-motif binding and evokes positive or negative regulation of specific intracellular signalling cascades [445]. The unique insert in the cytoplasmic tail of LRP8 has three proline-rich motifs, designated P1, P2 and P3 which fulfil minimal consensus sequences for SH3 domain recognition, namely PxxP with each proline usually preceded by an aliphatic residue [441]. In particular, the arginine and additional proline in sequence P1 (REPEDPA P) almost certainly confer high affinity binding [441-443]. For the cytoplasmic insert of LRP8 to be implicated in the signalling cascade to activate NOS in response to apoE binding, the functionality of this protein region needed to be tested by the ability of the P1 sequence to bind HUVEC cytosolic proteins.

The 17-mer peptide selected for antibody production encompasses P1 in cytoplasmic LRP8 (Figure 5.3-8, panel A). To assess whether this motif could interact with HUVEC proteins, the peptide was linked to Sepharose and equilibrated with [35S]-labelled HUVEC cytosol (already passed over Sepharose to eliminate non-specific binding). Two minor proteins (~80 kDa and 29 kDa) and a major 47 kDa protein were bound, implying that this putative SH3 recognition motif is indeed functional (Figure 5.3-8, panel B). The identity of these binding proteins and their possible involvement in the cascade to activate NOS is presently unknown.

5.3.7.2 The Phosphotyrosine-Binding (PTB) Motif.

Interestingly, the so-called internalization motif (ΨxNPxY, where Ψ is hydrophobic, Figure 5.3-8), common to all LRSF members, is also implicated in tyrosine kinase signal transduction [446]. This motif can be recognised by PTB domains present in numerous signalling molecules, including X11 neuronal protein or when tyrosine phosphorylated the docking molecules Shc and IRS-1 [447]. Indeed, these ΨxNPxY motifs are responsible for propagating the outside-in signalling of, amongst others, the insulin receptor and TrkA nerve growth factor receptor (reviewed in [448]). Intriguingly, two observations suggest that this PTB recognition motif in HUVEC LRP8 is functional. Firstly, both the LDL-R and LRP1
FDNPVY motifs bind the PTB domains of signalling molecules, Fe65 and mDab [449], since LRP8 has the same motif within its cytoplasmic tail, this implies functionality. Secondly, although phosphorylation of the conserved LDL-R sequence, FDNPVY, is not required for endocytosis [450], tyrosine-phosphorylation is implicated in regulating receptor degradation [451].

Figure 5.3-8 A putative SH3 recognition motif within cytoplasmic LRP8 binds a 47 kDa protein in HUVEC cytosol.

Panel A, the cytoplasmic domain of platelet LRP8 consists of 115 amino acids with the amino-terminal 25 residues containing the internalization (PTB-binding) motif common to all LRSF members (FDNPVY). Flanking this region are two cGMP/cAMP dependent protein kinase phosphorylation sites. The unique 59 amino acid insert of LRP8 contains three proline-rich motifs (P1-P3) which fulfil the PxxP consensus sequence for SH3 domain recognition. A 17-residue peptide sequence encompassing P1, the proline-rich motif most likely to undergo high affinity binding to SH3 domains within cytoplasmic LRP8 insert was coupled to NHS-Sepharose and equilibrated with [35S] labelled HUVEC cytosol. Panel B, bound proteins were eluted with 0.5 M NaCl, separated by SDS-PAGE and detected by fluorography (for method, see Figure 5.3-7). The autoradiograph shows positions of the minor bands (~80 kDa and 29 kDa) and a major band corresponding to a 47 kDa protein which were all capable of binding the SH3 recognition motif.
5.3.7.3 The cGMP-Dependent Protein Kinases Phosphorylation Sites.

Finally, the Thr_{415} (RKNT) and Thr_{429} (RKTT) residues flanking the PTB motif of LRP8 are potential targets for cGMP-dependent protein kinases [452, 453]. Because one of the biochemical consequences of apoE-induced release of HUVEC NO is a rise in intracellular cGMP, it is tempting to speculate that this constitutes a feedback loop to further activate or inactivate LRP8 signalling potential.

5.3.8 SUMMARY - LRP8 AS A CANDIDATE RECEPTOR TO SIGNAL THROUGH A TYROSINE KINASE PHOSPHORYLATION TRANSDUCTION CASCADE TO LINK APOE TO eNOS ACTIVATION IN THE ENDOTHELIUM.

LRP8, with potential cell-signalling motifs in its cytoplasmic insert and increasing evidence for its involvement in linking apoE with eNOS activation, is a promising candidate to illicit the apoE effect on VCAM-1 expression via an NO-mediated mechanism. Not only was the mRNA for LRP8 found in HUVECs, but more importantly, protein was detected.

As the cytoplasmic domain of LRP8 contains both PTB (ψxNPxY) and SH3 (PxxP) recognition motifs, it is likely that activation of LRP8 by apoE binding to its extracellular domains triggers a signalling cascade which ultimately leads to an increase in eNOS activity. Although it is difficult to predict the precise nature of any downstream signalling cascades from LRP8, a key step forward would be the discovery of cytosolic cell signalling molecules that bind the cytoplasmic tail of LRP8. In this respect, the identification and characterization of the 47 kDa interacting protein may be important.

5.3.9 A HYPOTHETICAL MODEL: LRP8-APOE-eNOS LINK IN THE ENDOTHELIUM DOWNREGULATES VCAM-1.

This model (Figure 5.4-1) can be extrapolated to propose a novel role for locally-secreted apoE as an anti-atherogenic molecule to limit endothelial activation and stem further monocyte recruitment at the lesion site in vivo. The vascular endothelium overlying lesions is exposed to a local source of macrophage-secreted apoE [37, 38]. This apoE may bind and activate LRP8, which has already been detected in the arterial endothelium [230]. A signal transduction cascade could then lead to activation of eNOS and the corresponding increase in NO may alter endothelial redox state, inhibiting NF-κB activation and VCAM-1 transcription. The subsequent downregulation of VCAM-1 may be instrumental in suppressing monocyte recruitment to limit progression of the disease. The downregulation of VCAM-1 by apoE may prove to be more dramatic in arterial cells; these cells have more capacity to generate NO, due to higher levels of eNOS expression, than venous cells like HUVECs [454]. Indeed, a next step may be to repeat experiments in vitro using cultured
arterial cells such as HCAEC, rather than HUVECs, as these would be a better model of vascular endothelium susceptible to atherosclerosis [117].

**Figure 5.4-1** A hypothetical mechanism to explain apoE-induced downregulation of VCAM-1 via LRP8 signalling which ultimately upregulates eNOS in HUVECs.

**HUVEC LRP8** is activated upon binding of cell-synthesized apoE (A), to bind a 47 kDa cytosolic adaptor molecule or protein tyrosine kinase or phosphatase, via its SH3 domain interacting with the PxxP motifs in the cytoplasmic tail of LRP8 (B). This interaction causes a tyrosine phosphorylation of either the PTB motif or the adaptor molecule/kinase itself, which in turn allows other adaptor molecules or tyrosine kinases to bind which ultimately trigger additional (de)phosphorylation events and/or induce Ca$^{2+}$ transients to upregulate eNOS (C). The NO produced stabilizes the NF-κB/I-κB complex in the cytosol and prevents translocation of NF-κB, an important transcription factor for VCAM-1, into the nucleus in response to cytokines (D). Some of the NO released may diffuse across the cell membrane and have paracrine actions on both endothelial cells and other local cell types including smooth muscle cells, platelets and monocytes/macrophages.
Chapter 6
6. GENERAL DISCUSSION

6.1 Cell-Secreted ApoE Inhibits VCAM-1 Upregulation in HUVECs, a Model of Human Vascular Endothelium.

6.1.1 HYPOTHESIS.

Atherosclerosis is a progressive, multicellular disease of the arterial wall which is the principle cause of death in the Western world [1]. There is an emerging concept of atherosclerosis as a chronic inflammatory disease, in which an early cellular event is the localized upregulation of adhesive CAMs on the surface of activated endothelial cells, a necessary prerequisite for the focal recruitment of monocytes and T-lymphocytes to the arterial intima. The importance of VCAM-1 as a key participant in the dynamic inflammatory process of monocyte/lymphocyte accumulation and initiation of early atherogenesis is increasingly being acknowledged. With recent studies inferring atheroprotective roles of apoE at lesion sites, I tested the novel hypothesis that "apoE has an anti-inflammatory/anti-atherogenic action by inhibiting cytokine-mediated upregulation of CAMs."

6.1.2 HUVECS AS A MODEL OF HUMAN VASCULAR ENDOTHELIUM.

To test this hypothesis I established primary HUVEC culture as an in vitro model of human endothelium in which to study CAM upregulation by pro-inflammatory cytokines. Endothelial cells from umbilical veins constitute the most popular in vitro model system with which to study the pathobiology of the human endothelium in processes initiating atherosclerosis, including CAM upregulation. However, HUVECs are derived from a venous endothelium which is not susceptible to atherosclerosis. Furthermore, HUVECs are close to senescence, having been isolated at the end of their natural life. Unfortunately, there is a lack of comparative studies to validate the use of HUVECs as a substitute for primary cultures of HCAECs for coronary arteries, a superior model directly relevant to blood vessels affected by atherosclerosis. It is well known that cultured endothelial cells from different sites show substantial phenotypic and functional heterogeneity, presumably reflecting differences in organ-specific functions [97]. For example, there is variability in endothelial CAM expression [455], CAM upregulation in response to proinflammatory stimuli [98], and hence adhesiveness for monocytes [98]. There is also heterogeneity in the ability of endothelial cells to generate NO [454,456]. However, investigators have shown that HUVECs do represent a relevant model for studying the regulation of endothelial CAM expression on large vessel endothelium in the adult human [82], and suggest that results can be extrapolated to arterial endothelial cells [147]. Indeed, from collaborative experiments
with Dr S. Allen using HUVECs and HCAECs, we found that both cell types gave comparable responses to activation by TNF-α, with similar levels of CAMs being induced.

6.1.3 **CELL-BOUND ELISA is a VALID METHOD FOR the DETECTION OF VCAM-1 IN HUVECs.**

To test the ability of purified plasma apoE to modulate endothelial CAM expression, I established a cell-bound ELISA for semi-quantitation of VCAM-1 expression in HUVECs (see Appendix II). This assay was a useful technique for such analyses, having been fully validated on different preparations of HUVECs for its sensitivity over a wide range of detection while still maintaining low non-specific signals. VCAM-1 was induced by TNF-α to levels consistent with other reports [82-84] although, in rare 'hyper-responsive' batches of HUVECs, higher levels of VCAM-1 were detected. As expected, the ELISA showed that HUVECs were responsive to downregulation by two different inhibitory agents, GSN0 and 17β-estradiol. Furthermore, 96-well plates of confluent HUVECs allow economic use of low passage cells as many different treatments can be compared to controls. Importantly, the ELISA gave comparable results to the more powerful FACS technique which analysed VCAM-1 expression on the surface of individual cells. The ELISA could not provide the individual cell analysis that was necessary to rule out the possibility of responsive subpopulations of cells within the heterogeneous cultures.

6.1.4 **HDL is UNABLE TO SUPPRESS VCAM-1 INDUCTION.**

Using both ELISA and FACS analysis techniques, I was unable to reproduce the original observation of VCAM-1 suppression by HDL [83,146,147]. Our discrepant data were not due to poor cell responsiveness or to HDL lacking biological potency or to donor variation. One difference in experimental set up was the use of heparin supplementation (0.05-20 mg/ml) in all culture media [83,146,147], equating to ~10 to 4,000 times the level in human plasma (~5 μg/ml) [367], whereas my cultures were serially propagated without heparin. Heparin may interact with the endothelial cell growth factor supplement to potentiate its mitogenic effects [317,318], but high doses may cause artefacts [368] and heparin-free media is preferred for some endothelial studies [101,105,107-109,114],[368],[369]. Recent literature has shown that supra-physiological concentrations of heparin alters endothelial function by modulating ICAM-1 transcription in HUVECs [369], by affecting endothelial interactions with leukocytes [370], and by decreasing NO production [371]. Heparin can also bind TNF-α, limiting its availability to interact with target cells [372]. This is relevant to the potential downregulation of VCAM-1 by HDL and hence future studies could test whether high doses of heparin could account for the conflicting results.
6.1.5 **PURIFIED PLASMA APOE FAILS TO DOWNREGULATE CAMS.**

Neither plasma-purified apoE incorporated into phospholipid vesicles (apoE:DMPC), nor isolated apoE-rich HDL, were able to inhibit cytokine-mediated upregulation of CAMs or the process of monocyte adhesion to HUVEC monolayers. I concluded that bulk plasma apoE was unlikely to modulate CAM expression *in vivo*. However, it is known that the biological activity of apoE is sensitive to subtle differences in its conformation [167]. The procedures used to purify apoE from plasma lipoproteins disrupt its endogenous conformation and may also change its oxidation state [360]; conceivably, these may diminish biological activity. Even the isolation of HDL-E, which does not involve denaturation, may cause artefactual results. Thus, prolonged ultracentrifugation can cause apolipoproteins to dissociate from the particle surface [374], which may affect the conformation of retained apoE, while separation by heparin chromatography requires the metal ion, Mn$^{2+}$. Nevertheless, both apoE:DMPC and HDL-E preparations were biologically active, as assessed by their ability to inhibit platelet aggregation [276].

Endothelial cells produce superoxide anions and oxidize LDL in culture [457], and a similar mechanism may occur in the subendothelial space to increase the atherogenic potential of LDL *in vivo* [3],[120]. Moreover, HUVECs can oxidatively modify LDL, thus creating particles capable of inducing both VCAM-1 and ICAM-1 expression in HUVECs [458]. However, it seems unlikely that my apoE:DMPC complexes were oxidized or modified during incubation with HUVECs such that any downregulatory effect of apoE would be counteracted. Thus, apoE is itself an anti-oxidant with apoE2 having greater potency than apoE3 or apoE4 [269], while the fatty acyl chains of DMPC are saturated and therefore not readily oxidized. Furthermore, my experiments demonstrated that neither apoE:DMPC nor DMPC alone induced VCAM-1 above the basal control level (Figure G, Appendix II), nor significantly augmented VCAM-1 induction by TNF-α. Thus, it was unnecessary to add antioxidants to my HUVEC cultures, which in any case would make interpretation of results difficult as such agents suppress NF-κB activation and induction of VCAM-1 in cultured endothelial cells [100],[101].

6.1.6 **CELL-DERIVED APOE INHIBITS VCAM-1 UPREGULATION.**

Utilizing three different models I showed that, in contrast to apoE:DMPC complexes and HDL-E, cell-derived apoE particles suppress VCAM-1 induction by TNF-α.
VCAM-1 is downregulated in HUVECs transiently transfected to secrete apoE.

In the first model, HUVECs were transfected with an apoE expression plasmid to generate a local source of apoE, while control wells were treated with pCMV.0, the same plasmid but without apoE cDNA. VCAM-1 induction inversely correlated with the amount of apoE secreted into the media. This inhibition did not simply reflect cell death i.e. a decrease in VCAM-1 detection because less cells were viable, either because of the lengthy experimental period or because of pCMV.apoE toxicity. Thus, in preliminary experiments the transfection conditions were optimized to minimize cytotoxicity and, moreover, each transfected well (pCMV.apoE and pCMV.0) was assayed for protein to normalize the VCAM-1 measurement, thereby allowing for any slight differences between wells. Prior to the ELISA, cells were washed to exclude any dead cells from the analysis followed by fixation, a procedure that precludes a direct assay for cell viability. Nevertheless, while still permitting VCAM-1 and apoE analyses, protein measurement for individual wells, along side careful microscopic monitoring of changes in cell morphology and cell confluence, demonstrated that cytotoxicity was negligible.

The level of apoE in the media from pCMV.apoE transfected cells varied between wells suggesting that transfection efficiency varied. Another group using similar cationic liposome-mediated lipofection methods, but with different lipid compounds, have reported low efficiency of HUVEC transfection (0.45 % [390,391]), although efficiency may rise to ~20 % when expression is controlled by a strong CMV promoter [390,391]. Unfortunately, beta-galactosidase (β-gal) reporter gene expression was cytotoxic to my HUVECs (using pCMV.β-gal plasmid and my transfection protocol) and so my transfection efficiencies are unknown. Additionally, immunostaining to detect intracellular apoE production could not act as a marker of transfection efficiency since secreted apoE particles may be endocytosed by non-transfected cells. In future studies, the alkaline phosphatase reporter [390,391] may be an alternative to β-gal for such analyses.

Cell-derived recombinant apoE has an inherent ability to downregulate VCAM-1.

Following these initial transfection studies, I was able to verify that cell-derived apoE inhibited VCAM-1 expression, using two further models: HUVECs cocultured with recombinant CHOapoE cells secreting apoE or HUVECs incubated with CHOapoE cell-conditioned media. CHO cell-secreted apoE actively downregulated VCAM-1 regulation at a concentration of ~5 µg/ml, the estimated level of circulating apoE that is contributed by macrophages (~10 % of plasma apoE [235,257]). This implies that downregulation of endothelial VCAM-1 by macrophage apoE in vivo is a realistic mechanism for apoE to exert...
an anti-atherogenic effect. The level of locally-secreted apoE in the bulk media of transfected HUVECs was ~100-fold lower than in the CHO-apoE cell-conditioned media, but still gave greater downregulation of VCAM-1. As discussed in Chapter 4, this potency at low levels of locally-secreted apoE will require further investigation, but may reflect intracellular effects of apoE in the transfected cells, and/or entrapment at the cell surface of secreted apoE by endothelial HSPG. Although native endothelial cells do not synthesize or secrete apoE, this finding may have implications for gene therapy, whereby transfecting endothelial cells to overproduce apoE may reduce endothelial activation in vivo and have anti-atherogenic potential. Such intervention has already been shown to ameliorate atherosclerosis [404] and there is growing interest in the use of endothelial cells as a novel gene therapy platform for the delivery of other gene products.

6.1.7 Active Cell-Derived ApoE Particles Resemble those Secreted by Macrophages.

Due to time limitations, no attempt was made to explain why cell-derived apoE suppresses VCAM-1 upregulation by TNF-α, whereas apoE:DMPC is inactive. It is reasonable to assume that our use of unpurified apoE secreted from cultured cells, minimizing conformational or oxidation alterations [360], was a more physiologically relevant way to study apoE effects on endothelial activation. Indeed, although apoE:DMPC preparations inhibit ADP-induced platelet aggregation, CHO cell-secreted apoE particles are ~100 times more active (Dr D. Riddell, personnel communication). Interestingly, initial characterization revealed that CHO cell-derived apoE particles resembled those secreted by macrophages, although further analyses will be needed to compare their biological potency.

6.2 Potential Mechanisms for the VCAM-1-Modulatory Effect of ApoE.

6.2.1 Is VCAM-1 Reduction by ApoE due to Inhibition of Transcription or Increased Shedding?

I have shown that total VCAM-1 expression in HUVECs is reduced by cell-derived apoE, using an ELISA method that detected both intracellular VCAM-1 (prior to being targeted for expression to the cell surface) and VCAM-1 at the cell surface. Without measuring the cell surface VCAM-1 alone it remains to be seen whether VCAM-1 at the cell surface is reduced. However, since cytokine is necessary to induce VCAM-1, a reduction in intracellular VCAM-1, most likely by suppression of VCAM-1 transcription, would be
reflected by a corresponding reduction in cell surface presentation. An effect at the level of transcription should be confirmed by Northern blots for VCAM-1 mRNA.

Alternatively, the regulation of VCAM-1 levels at the cell surface may be due to increased ‘shedding’ of the extracellular portion of VCAM-1 into the media. This process is poorly understood, but the hypothesis that apoE enhances VCAM-1 shedding is an intriguing alternative anti-inflammatory mechanism to an effect on transcription; VCAM-1 would not only be reduced at the surface but functional sVCAM-1 would negatively regulate monocyte adhesion to endothelium [74].

6.2.2 A Role for ApoE-Induced NO Production in the Suppression of VCAM-1?

My preliminary experiments have indirectly shown that NO could be the mediator of the VCAM-1 inhibitory effect by cell-derived apoE in HUVECs: apoE caused a rise in intraHUVEC cGMP; a NOS inhibitor blocked the downregulation of VCAM-1; and apoE failed to inhibit E-selectin, a CAM not modulated by NO in HUVECs [105,359]. NO is an intracellular signaler and known inhibitor of VCAM-1 transcription [101,105,107-110] and a potent regulator of CAM expression in vivo [293]. Moreover, endogenously produced NO by HUVECs is sufficient to attenuate VCAM-1 expression [107,359]. Intriguingly, NO has already been linked with apoE-induced NOS activation in platelets [276] and arterial cells, including macrophages [304,305] and vascular smooth muscle cells [278,306,307]. A similar link between apoE and NO in the endothelium would be an attractive mechanism to explain the suppressive effect of apoE on endothelial activation.

ApoE has been linked with the activation of both eNOS [276] and iNOS [278,304]. The use of ethyl-ITU, a potent inhibitor of both eNOS and iNOS activity [427], to block the apoE effect on VCAM-1, does not dismiss a contribution from either NOS isoform. Although my HUVECs were confirmed as being positive for constitutive eNOS expression, iNOS may have been induced during exposure to TNF-α but this was not studied. However, the moderate rise in intraHUVEC cGMP, in the absence of TNF-α, is indicative that apoE activated eNOS. This increase in NO may even have suppressed iNOS induction by TNF-α [459]. Furthermore, an apoE effect on eNOS may have been boosted by TNF-α, as indicated by the tendency of TNF-α to elevate cGMP levels (data not shown); cytokines can increase eNOS-derived NO by altering cofactor levels in the face of falling total enzyme [460]. Future experiments using isoform-specific NOS inhibitors will provide the necessary confirmation of my hypothesis that apoE is linked to eNOS activation in endothelial cells.
6.2.3 DOES APOE-INDUCED NO PRODUCTION INHIBIT NF-κB ACTIVATION?

The potential involvement of NO in the apoE-induced downregulation of VCAM-1 suggests that NF-κB is the most likely target for suppressing VCAM-1 induction. NF-κB is an important NO-sensitive transcription factor for VCAM-1 gene expression and is inactivated by either NO donors [101,105,107-110,114] or endogenously produced NO [107,109], while other transcription factors such as AP-1 are not affected [110]. However, NF-κB need not automatically be involved. For example, transcription of VCAM-1 can be suppressed independently of NF-κB inhibition; one novel plant flavonoid selectively inhibits TNF-α-induced VCAM-1 transcription downstream from NF-κB activation and nuclear binding [461]. Electrophoretic mobility shift assays are necessary to check for a decrease in NF-κB activation [107] and should correlate with any decrease in VCAM-1 mRNA levels.

6.2.4 IS LRP8 A SIGNALLING RECEPTOR TO LINK APOE TO NO GENERATION IN ENDOTHELIUM?

Additional indirect evidence implicates an apoE-NO pathway in downregulating VCAM-1. In platelets, binding of apoE by its receptor, LRP8, is thought to initiate a signal transduction cascade to upregulate eNOS [214-217,276]. As LRP8 mRNA and, more importantly, protein were found in HUVECs, a similar apoE-LRP8-eNOS pathway may be functional in endothelium to limit VCAM-1 induction. A role for LRP8 is in agreement with the similar effectiveness of apoE2 and apoE3; apoE2 binds poorly to the LDL-R [167] and LRP1 [392], thereby discounting their involvement in suppression of VCAM-1, whereas LRP8, and its closest mammalian homologue, VLDL-R, bind both isoforms equally well [217,437]. Also relevant is the recent proposal that both LPR8 and VLDL-R, initiate tyrosine kinase signalling cascades to modulate neuronal positioning in the developing brain [205], a process dependent on neuronal CAMs [462]. Since VLDL-R is also present in HUVECs [229] we cannot exclude co-ordinate apoE signalling by the two receptors in HUVECs to activate NOS and limit VCAM-1 induction.

It is generally accepted that eNOS is regulated by Ca^{2+} through its interaction with CaM, with the binding of the Ca^{2+}-CaM complex to a specific region of eNOS increasing its enzymatic activity [463]. In this respect, it is interesting that some lipoproteins, including HDL [464] and LDL [117], cause Ca^{2+} transients in cultured arterial endothelial cells. Furthermore, apoE causes Ca^{2+} fluxes in neuronal cells [465]. It would be prudent, therefore, to check for the ability of cell-secreted apoE to have a similar effect in my cell system.
Recent studies have challenged the concept of eNOS being solely regulated by Ca\(^{2+}\)-dependent mechanisms, implying that post-translational modification and the availability of cofactors are important regulatory mechanisms in response to various stimuli [281]. The regulation of eNOS is poorly understood, but is thought to involve phosphorylation at various sites including tyrosine [281,289,290]. The activation of different kinases and/or phosphatases appears stimulus-dependent so that either down or upregulation of eNOS can occur [289,466]. In endothelial cells, eNOS is reversibly phosphorylated in response to agonists, and there is a correlation between enzyme phosphorylation and cytosolic localization, suggesting that eNOS signalling may be regulated by phosphorylation [289]. Indeed, the stimulus of fluid shear stress has been shown to cause phosphorylation of eNOS in HUVECs via activation of Akt (protein kinase B), a serine/threonine protein kinase, leading to an increase in NO production [467,468]. Although many phosphorylation events are regulated by Ca\(^{2+}\)-dependent signal pathways, fluid flow is a good example of phosphorylation as a discrete signalling mechanism to activate eNOS without an increase in Ca\(^{2+}\) [466-468]. In future experiments it will be interesting to decipher whether activation of Akt and phosphorylation of eNOS, with or without Ca\(^{2+}\) mobilization, can be triggered via an apoE/LRP8-mediated signalling cascade in HUVECs.

### 6.3 Conclusions.

There is an emerging concept that macrophage apoE has local atheroprotective roles within lesions by delaying early stages of atherogenesis [259]. My novel discovery that cell-derived apoE particles, with similarities to those secreted by macrophages, downregulate VCAM-1 in HUVECs, an *in vitro* model of human vascular endothelium, is potentially important. Thus, I propose that apoE, secreted locally by resident cholesterol-loaded macrophages within lesions, suppresses VCAM-1 expression on overlaying endothelium. Moreover, my preliminary evidence suggests that an apoE-NO link, first discovered in platelets, exists also in the endothelium and that this anti-inflammatory action of apoE is mediated by activation of a signalling cascade to generate NO. Suppression of VCAM-1 upregulation, and possibly other NO-responsive endothelial functions, in activated cells may have a profound effect on the recruitment of monocytes initiating atherogenesis. Therefore, understanding the cellular mechanisms whereby apoE-induced NO elicits its protective effects at the lesion site may be vital for the development of novel therapeutic strategies, including apoE gene therapy, for prevention and treatment of atherosclerosis.
This thesis describes the use of a well-established \textit{in vitro} model of human endothelium, HUVECs. Unfortunately, time did not permit the use of other types of endothelial cells including those from coronary arteries (HCAECs), a model directly relevant to the study of blood vessels susceptible to atherosclerosis, to substantiate the VCAM-1-modulatory effect of cell-secreted apoE particles. Furthermore, monocyte adhesion assays under flow conditions to mimic \textit{in vivo} dynamic forces would test for a functional effect of apoE-mediated inhibition of VCAM-1 expression [309]. The establishment of \textit{in vitro} co-culture systems of aortic wall cells [457], where interactions between different cell types and their secreted products presumably mirrors the cross-talk \textit{in vivo}, may also allow the physiological effects of apoE on VCAM-1 downregulation and monocyte adhesion to be studied. Indeed, there is a brief report that locally-secreted apoE reduces monocyte transmigration in this model [311].

The use of increasingly superior \textit{in vitro} models can still not fully replace \textit{in vivo} studies. Macrophage replacement in apoE-deficient mice may provide the best model system to study the effect of locally-secreted macrophage apoE on VCAM-1 modulation. Using this approach, it has recently been shown that arterial macrophage apoE secretion can delay the early stages of atherogenesis [259]. It remains to be tested whether this early intervention of atheroprotective apoE causes downregulation of VCAM-1 and reduction of monocyte recruitment. Interestingly, gene therapy techniques may be harnessed to study the physiological relevance of VCAM-1 downregulation by apoE. One newly documented \textit{in vivo} model has demonstrated the adherence of fluorescently-labelled monocytes to atherosclerotic plaques in apoE-deficient mice [67]. Obviously, gene replacement of apoE and subsequent downregulation of VCAM-1 could be assessed in this model. Furthermore, improved intravital microscopic techniques to analyse large arteries in gene therapy treated mice could be applied [309]. The development of mutant mice that express marker fluorochromes (such as green fluorescent protein) either in monocytes [458] or in VCAM-1 and the crossbreeding with apoE-deficient mice may provide an elegant model with which to study the potential of macrophage apoE to suppress CAM induction and limit monocyte adhesion.

The link between apoE and NO may also be relevant to neurodegenerative diseases. ApoE is a major apolipoprotein of human CSF [166,167,182], being derived almost entirely from the brain where apoE is synthesized by astrocytes [179,180], although microglia and neurones may contribute [180,181]. The endogenous lipoproteins are predominantly HDL-like and, although their role is not known, apoE expression appears to correlate with nerve injury and neuronal remodelling [471-473]. Interestingly, both apoE3 [418] and NO [474]
promote neurite outgrowth, providing indirect evidence for a link between these two molecules in the brain. As a uniquely diffusible mediator, NO has roles in inter- and intra-cellular communication in the nervous system, modulating synaptic plasticity and hence influencing brain development, memory formation and behaviour [475-476]. Of the three major human apoE alleles, apoE4 has a highly significant association with late-onset sporadic and familial Alzheimer's disease (AD) and other chronic neurodegenerative diseases [477]. The role of apoE in the pathology of AD is still unknown although apoE isoform-dependent differential effects on neurite outgrowth may contribute; in contrast to apoE3, apoE4 does not promote neurite outgrowth [418] and may even be inhibitory [403]. NO also has a pathological role in numerous neurological disorders, including AD. Excessive production by activated microglia is neurotoxic [478-479]; NO can react with superoxide to form peroxynitrite, a powerful oxidant. This leads to speculation that apoE4-mediated overproduction of NO via a signal transduction cascade involving LRP8, which is upregulated in AD brain [480], contributes to the pathophysiology in such diseases. My preliminary transfection data give indirect support for a similar hypothesis in endothelial cells; HUVECs transfected with pCMV.apoE4 had very low levels of apoE in their culture media, but still had up to 80% reduction of VCAM-1, suggesting that more NO was generated by this apoE isoform. Future studies to decipher the roles of apoE, NO and LRP8 in neuronal cells and in the metabolism of normal brains should also provide clues to how these factors influence the genesis of AD and other neurodegenerative diseases.


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365. Gillett MP and Owen JS. (1992). Comparison of the cytolytic effects in vitro on *Trypanosoma brucei* brucei of plasma, high density lipoproteins, and apolipoprotein A-1 from hosts both susceptible (cattle and sheep) and resistant (human and baboon) to infection. *J. Lipid Res.* **33**:513-523.


PUBLICATIONS

Original Articles.


Published Abstracts.


**National and International Presentations.**

**Invited Oral Presentations.**


**Poster Presentations.**


# APPENDIX I

## ANTIBODY SOURCES

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Details</th>
<th>Source</th>
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<tbody>
<tr>
<td>apoE</td>
<td>Mouse anti-human apoE monoclonal - clone F5M3/A10</td>
<td>Dr. R. James, Hopital Cantonal, Geneva, Switzerland</td>
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<tr>
<td>biotinylated secondary</td>
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<tr>
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<td>Rabbit anti-human eNOS polyclonal - affinity purified</td>
<td>Transduction Laboratories, Kentucky, USA</td>
</tr>
<tr>
<td>FITC-conjugate</td>
<td>Goat anti-mouse IgG – affinity purified</td>
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</tr>
<tr>
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<td>isotype control</td>
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<td>Dr. D. Riddell, RF&amp;UCMS</td>
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Table A. Antibodies sources.
APPENDIX II

DEVELOPMENT OF A SEMI-QUANTITATIVE CELL-BOUND ELISA FOR THE DETECTION OF VCAM-1 IN HUVECS

Introduction

A cell-bound VCAM-1 ELISA was established as a semi-quantitative assay with which to determine relative levels of VCAM-1 expression in HUVECs adherent to 96-well plates. The ELISA was devised to detect both basal and cytokine-induced levels of VCAM-1 using the methodology for VCAM-1 detection based on the immunocytochemistry protocol described earlier (Section 2.2.8). Cellular VCAM-1 (i.e. both expressed at the cell surface and in the cytoplasm before surface presentation) was detected by fixing the cells prior to antibody treatment steps. Indeed, the ELISA was developed as an extension of this basic immunocytochemical technique and thus was not designed as a conventional fully quantitative ELISA to quantify absolute amounts of antigen. Rather, it was established so that an objective numerical score could be assigned to the levels of VCAM-1 expression that had been visualised by the staining technique. Hence, for the purposes of this type of analysis, it was unnecessary to include ‘VCAM-1 standards’. It was anticipated that once the VCAM-1 ELISA had been validated, both for its ability to detect VCAM-1 induction and for suppression by the use of known VCAM-1 downregulators, the technique would be a sensitive method to test any inhibitory effect of apoE:DMPC.

Specialized Methods

ELISA METHODOLOGY

HUVECs, in complete M199 media supplemented with 20 % (v/v) FBS, were seeded at 3 x 10^4 cells/well in 96-well plates pre-coated with 1 % (w/v) gelatin and were incubated overnight to allow confluent monolayers to form. Cells were not seeded in the first column (reserved for application of the reagent blank). Quadruplicate wells were positioned going across the rows, with the maximum of 8 separate treatments going down columns. Plates were washed twice with pre-warmed PBS and then fixed with 10 % neutral buffered formal saline for 10 min at room temperature. Wells were washed rigorously (5 x 200 µl PBS per well) after fixing and after subsequent reagent additions.
ELISA steps were established from the immunostaining protocol (Section 2.2.8). In brief, endogenous cellular peroxidase activity was quenched with 3 % (v/v) H$_2$O$_2$ in PBS for 15 min. Primary antibodies (monoclonal mouse anti-human VCAM-1), titrated and diluted optimally in PBS containing 0.1 % (w/v) BSA (see below), were incubated with the cells at room temperature for 45 min. Isotype-matched irrelevant antibodies (raised against A. niger glucose oxidase, a protein neither found nor induced in any studied mammalian tissues) were used as negative antibody controls (at equivalent IgG$_1$ protein concentrations). Biotinylated goat anti-mouse antibodies (StreptABCComplex/HRP Duet kit) optimally diluted (see below) in 10 % normal human serum (heat inactivated) in Tris-buffered saline (TBS; 0.05 M Tris and 0.15 M NaCl, pH 7.6) were incubated for 30 min. The degree of antibody binding was amplified by an optimally diluted (see below) streptavidin/biotin-HRP complex (StreptABCComplex/HRP Duet kit) during a 30 min incubation. OPD chromogenic substrate for peroxidase (pH 5.0) containing 0.01 % (v/v) H$_2$O$_2$ was applied for 1 min (100 µl per well, using a multichannel pipette moving across the plate) before stopping the reaction with 1 M sulphuric acid. Absorbances were immediately read at 492 nm (A$_{492}$) in a Titertek Multiskan MCC/340 MK II microtitre plate spectrophotometer against blank substrate plus acid. Note that, in every ELISA assay, one set of quadruplicate wells was treated with primary anti-vWF instead of anti-VCAM-1. This gave a positive signal from the cells, regardless of cytokine-exposure, and also confirmed their endothelial phenotype.

**Antibody Titration**

For the titration of antibodies, HUVECs were first treated with TNF-α (100 U/ml) for 6 h to induce VCAM-1 expression prior to fixation. Other wells were left untreated for the determination of background signal, both from anti-VCAM-1 and isotype-matched irrelevant antibodies (at equivalent IgG$_1$ protein concentrations) binding non-specifically to HUVECs. While the concentrations of secondary antibody (biotinylated goat anti-mouse antibody) and tertiary streptavidin/biotin-HRP complex were fixed at a 1/400 dilution (based on the previously optimized immunostaining protocol), primary anti-VCAM-1 was serially diluted (in doubling dilutions from 1/50 to 1/12,800) in 0.1 % (w/v) BSA in PBS. Quadruplicate wells for each dilution were positioned across the rows, with differing dilutions going down the columns. The optimum anti-VCAM-1 dilution was chosen to be the highest dilution of antibody giving just below maximum specific signal which still gave a low background signal. Then, using the optimum anti-VCAM-1 dilution, the secondary antibody was diluted (1/200 – 1/25,600) in the next ELISA. Similarly, having determined the optimum dilutions of both primary and secondary antibodies, streptavidin/biotin-HRP
was diluted (1/200 – 1/51,200) in the third ELISA in order to find its optimal working concentration. Note that, to maintain these optimum conditions, every new batch of VCAM-1 antibody purchased was titrated and optimized in this way.

Results

ESTABLISHMENT OF THE ELISA

The ‘titre’ of an antibody is defined as the highest dilution of that antibody which results in optimum specific signal with the least amount of background. For a non-competitive ELISA, there should be sufficient antibody to detect all antigen (in this case VCAM-1) [481]. All the components of the ELISA i.e. the primary anti-VCAM-1 antibody, the secondary biotin-conjugated antibody and the streptavidin/biotin-HRP complex in the tertiary step, had been previously titrated for immunostaining of TNF-α-stimulated HUVECs. However, the reagents were ‘retitrated’ in the ELISA to ensure that all cytokine-induced VCAM-1 was effectively detected while non-specific binding was minimized. Furthermore, the signal resulting from the detection of VCAM-1 needed to be within the limit of accurate measurement of the spectrophotometric plate reader. Primary and secondary antibodies and the streptavidin/biotin-HRP complex were each titrated sequentially (starting with the ‘first binding layer’ - primary anti-VCAM-1, Figure A) while keeping the other reagents at constant dilutions (Figures B and C). Antibody titrations were performed on HUVECs exposed to 100 U/ml TNF-α for 6 h, a treatment commonly used to stimulate a high level of VCAM-1 expression in VCAM-1 modulation studies [69],[83,146-148]. In each case the titre, or optimum dilution, of the antibody was the dilution just before the plateau of the titration curve (Figure A, B and C, indicated by dashed line), in accordance with published procedures for establishing non-competitive ELISAs [481]. Chosen dilutions were also shown to give low non-specific binding when the cells were not stimulated with TNF-α (i.e. when they expressed negligible levels of VCAM-1) and when the isotype-matched control was used at the same IgG1 protein concentration (not shown on titration graphs). The optimum dilutions/titres chosen for the primary, secondary and tertiary ELISA steps were 1/200 (corresponding to 5 μg IgG1/ml), 1/400 and 1/400, respectively (note that Figure A, B and C show dilutions as reciprocal values e.g. a dilution of 1/200 is plotted as 0.005, on the x-axis log scale).
Figure A. Primary anti-VCAM-1 titration curve for VCAM-1 ELISA.

Primary VCAM-1 antibody (monoclonal mouse anti-human VCAM-1) was titrated in the VCAM-1 ELISA on HUVECs (3 x 10⁶ cells/well in 96-well plates) after 6 h treatment with 100 U/ml TNF-α to induce VCAM-1 expression (see 'ELISA Methodology' and 'Antibody Titration'). Doubling dilutions of the stock antibody (1 mg IgG/ml) are shown as decimals (e.g. a 1/200 dilution is plotted as 0.005) on the x-axis log scale. The secondary and tertiary reagents in the ELISA were used at 1/400 dilution. ELISA readings are shown as absorbance at 492 nm (A₄₉₂ in OD units). The mean ±SE is plotted for quadruplicate wells in a single ELISA. The optimal dilution is indicated by the dashed line and, after confirmation in a second independent assay, was used for subsequent experiments.
Figure B. Secondary antibody titration curve for VCAM-1 ELISA.

Secondary antibody (biotinylated goat anti-mouse IgG) was titrated in the VCAM-1 ELISA on HUVECs (3 x 10⁴ cells/well in 96-well plates) after 6 h treatment with 100 U/ml TNF-α to induce VCAM-1 expression (see ELISA Methodology and Antibody Titration). Doubling dilutions of the stock antibody are shown as decimals (e.g. a 1/200 dilution is plotted as 0.005) on the x-axis log scale. The primary antibody (anti-VCAM-1) and the tertiary reagent were used at 1/200 and 1/400 dilutions respectively. ELISA readings are shown as absorbance at 492 nm (A₄₉₂ in OD units). The mean ±SE is plotted for quadruplicate wells in a single ELISA. The optimal dilution is indicated by the dashed line and, after confirmation in a second independent assay, was used for subsequent experiments.
Figure C. Streptavidin/ biotin-HRP complex titration curve for VCAM-1 ELISA.

The streptavidin/ biotin-HRP complex (the tertiary ELISA step) was titrated in the VCAM-1 ELISA on HUVECs (3 x 10^4 cells/well in 96-well plates) after 6 h treatment with 100 U/ml TNF-α to induce maximal VCAM-1 expression (see 'ELISA Methodology' and 'Antibody Titration'). Doubling dilutions of the stock streptavidin/ biotin-HRP complex are shown as decimals (e.g. a 1/200 dilution is plotted as 0.005) on the x-axis log scale. The primary and secondary antibodies were used at 1/200 and 1/400 dilutions respectively. ELISA readings are shown as absorbance at 492 nm (A_{492} in OD units). The mean ±SE is plotted for quadruplicate wells in a single ELISA. The optimal dilution is indicated by the dashed line and, after confirmation in a second independent assay, was used for subsequent experiments.
ELISA DETECTS HUVEC VCAM-1 INDUCTION WHICH IS DEPENDENT ON BOTH TNF-α DOSE AND INCUBATION TIME.

In the VCAM-1 ELISA described above, basal HUVEC VCAM-1 expression was negligible with the anti-VCAM-1 signal equivalent to that of the negative isotype-matched control (Figure D, panel A; shown earlier in Chapter 2 as Figure 2.3-5). HUVECs responded rapidly to TNF-α, and after 6 h treatment with 100 U/ml TNF-α (panels A and B), the extent of VCAM-1 induction was similar to that reported by others [82-84]. When stimulated with increasing TNF-α concentrations (0.03–100 U/ml) for 6 h the HUVECs responded in a dose-dependent manner (panel B). Although higher doses of TNF-α were not tested, because of potential cytotoxicity effects [482], the curve suggested that 100 U/ml TNF-α resulted in maximum VCAM-1 induction.

Antibody titration was performed on HUVECs stimulated with 100 U/ml TNF-α (5 ng/ml), a standard dose for inducing VCAM-1 in HUVECs and for testing inhibitory agents including NO donors [69][83,146-148] and HDL [83,146-148]. These published modulators of CAM expression were to be used in the ELISA as positive controls for VCAM-1 downregulation, and so it was important that the same protocol for inducing VCAM-1 with TNF-α was adopted (see Chapter 3). However, in experiments detailed in Chapters 4 and 5, a submaximum dose of TNF-α, 10 U/ml, was used. This induced levels of VCAM-1 to ~90-95 % of the value with 100 U/ml and would have ensured that total VCAM-1 was detected even with the rare ‘hyper-responsive’ batches of HUVECs (illustrated in Figure F).

Time-course studies indicated that induction of VCAM-1 by 100 U/ml TNF-α was detectable after 3 h of exposure, with VCAM-1 expression rapidly and significantly increasing thereafter before reaching a plateau at about 8 h. This high level of expression was then sustained for at least 20 h or more without further addition of cytokine (Figure E). This pattern of induction was comparable to another study in HUVECs [81].
Figure D. VCAM-1 induction by TNF-α in HUVECs as detected by ELISA.

Panel A, confluent HUVECs in 96-well plates were treated with 100 U/ml TNF-α for 6 h; TNF-α was omitted from control wells for unstimulated cells. Basal (unstimulated) cells had a negligible level of VCAM-1 (white bar) while after TNF-α (100 U/ml) treatment VCAM-1 was increased 10-fold (black bar). An isotype-matched irrelevant antibody gave a low background signal (light grey bar). The immunodetection of constitutive vWF was the positive control for the ELISA (dark grey bar). Panel B, HUVECs were exposed to a range (0.03–100 U/ml) of TNF-α concentrations over a 6 h period. VCAM-1 was induced in a dose-dependent manner. Results are from a representative experiment and each bar shows mean ± SE from quadruplicate wells. Note that the x-axis is on a log scale. This Figure also appears in Chapter 2 (Figure 2.3-5).
Figure E. Time-course for VCAM-1 induction by TNF-α in HUVECs.

Confluent HUVECs in 96-well plates were exposed to 100 U/ml TNF-α for 3-30 h before VCAM-1 quantification by ELISA. Before cytokine addition VCAM-1 expression was undetectable, but was rapidly increased in cells activated by TNF-α. Expression plateaued at 8 h and was sustained for at least 30 h. Results are from one representative experiment and each bar shows mean ±SE from quadruplicate wells. This Figure also appears in Chapter 2 (Figure 2.3-6).

VALIDATION OF THE ELISA FOR SEMI-QUANTITATIVE VCAM-1 MEASUREMENT.

Internal controls were incorporated into every VCAM-1 ELISA: 1) to measure the variability between quadruplicate wells within each assay, 2) to test the efficacy of known, or potential, VCAM-1 downregulators and 3) to compare the level of VCAM-1 between assays. The A492 values for the internal controls detecting basal VCAM-1 (without cells being exposed to TNF-α) and TNF-α-induced VCAM-1 were recorded for each assay. Additionally, isotype-matched negative control antibodies (at equivalent IgG concentrations) were used to monitor background signals from non-specific antibody binding, while anti-vWF was used in ‘positive control’ wells to verify the ELISA steps were performed correctly (as vWF is constitutively expressed regardless of cell responsiveness to TNF-α; Figure D, panel A).
Assay Variability

There was low intra-assay variability of the internal standards. Typically, the signals from quadruplicate wells showed low standard errors from the mean absorbance. The VCAM-1 signal after TNF-α treatment gave an A₄₉₂ of ~1.0 OD unit, a value consistent with that reported by others [82-84]. This increase was also within the upper limit recommended for accurate spectrophotometry and allowed a large ‘window’ or dynamic range for VCAM-1 detection. This is essential to ensure that the ELISA could detect small changes in VCAM-1 expression (as compared to the TNF-α control), either when using known CAM inhibitors as positive controls for VCAM-1 downregulation or when adding test substances (including apoE:DMPC and HDL). It was also important that background signals from any non-specific binding was kept to a minimum to maximize the ‘signal-to-noise ratio’ which would affect the sensitivity of the ELISA to detect changes in VCAM-1 (both basal and TNF-α stimulated levels). Thus, isotype control wells gave a background signal of <0.1 OD units A₄₉₂ in every assay and pre-blocking with 1 % BSA (w/v) after cell fixation did not reduce the signal further (results not shown).

The inter-assay variability was relatively large. Over an 8 month period, for 21 independent experiments the VCAM-1 signal after TNF-α treatment was on average 0.9 (±0.3 standard deviation from the mean) OD units above the corresponding basal unstimulated levels in each assay, thus, the coefficient of variation (CV) between assays was 33.3 % (see equation below). This large CV value falls outside the acceptable limits for conventional ELISAs (typically 10-20 % [481]). However, this was expected for a cell-bound assay of this nature using primary endothelial cells that show heterogeneity both between different batches [328] and also passages [81].

\[ CV = \frac{SD}{mean} \times 100 \]

Due to this inter-assay variation in HUVEC VCAM-1 expression, much of the VCAM-1 data presented in Chapters 3, 4 and 5 was ‘normalized’ and expressed as a percentage of the TNF-α control VCAM-1 induction (above basal), before statistical analysis could be performed.
Detection of Variable VCAM-1 Expression

The flexibility of the ELISA to detect a broad range of VCAM-1 induction by treating the cells for 6 h with 100 U/ml TNF-α is illustrated in Figure F. Although the increase in VCAM-1 above basal was on average 8.4-fold (±0.6 SEM, n=21), the range was from a 2.9 to a 15.2-fold increase (Figure F). Thus, the ELISA was capable of detecting VCAM-1 even in the minority of HUVEC batches that responded either poorly to TNF-α stimulation or those that were hyper-inducible (Figure F). Therefore, as expected from using sufficient VCAM-1 antibodies, there was no evidence that an upper limit cut-off for VCAM-1 detection had been reached. However, we can not guarantee that, if in exceptional circumstances, VCAM-1 increased above 15.2-fold, this increase would be detected.

Figure F. The induction of VCAM-1 above basal level varies between assays.

The ELISA ‘internal controls’ were used to calculate the increase in A492 (corresponding to the increase in VCAM-1 expression after TNF-α treatment above the basal level) for each of 21 experiments performed in quadruplicate (21 data points plotted as black dots). The mean 8.4-fold increase in VCAM-1 level is indicated by a line (±0.6 SE not shown).
Testing 'Positive Controls' for VCAM-1 Downregulation

Not only were levels of VCAM-1 expression in the HUVEC ELISA consistent with published data [82-84], but the extent of VCAM-1 downregulation by two known inhibitory agents was also comparable to literature values (Figure 3.3-3) [101,105,107-109,114]. The ELISA demonstrated that the HUVEC cultures were responsive to the NO donor, GSNO, and to 17β-estradiol, with VCAM-1 induction suppressed by 20-25 % and 40-60 %, respectively. Experiments with apoE:DMPC and HDL were repeated many times: a total of 14 different donors of HDL were tested for any VCAM-1-modulatory effect on 10 different preparations of low-passage (P³-P⁶) HUVECs, while 5 different preparations of apoE:DMPC were tested on 7 batches of HUVECs as described in Chapter 3.

Although GSNO or 17β-estradiol consistently reduced expression of VCAM-1 in every case, to the expected degree, neither apoE:DMPC (Figures 3.3-2 and 3.3-3) nor HDL (Figure 3.3-11 and Table 3.3-1) preincubation had a suppressive effect in parallel wells. Thus, GSNO and 17β-estradiol were efficient controls for monitoring the effectiveness of the assay to detect the extent of both VCAM-1 induction and downregulation.

Testing 'Unknowns' for VCAM-1 Downregulation.

Cell-cell interactions have a strong influence on proinflammatory cytokine responses of HUVECs [425] and, therefore, titration of cytokine-inducible antigen i.e. VCAM-1 by reducing cell density was not applicable. However, VCAM-1 expression on confluent HUVEC monolayers could be varied by treating with a range of TNF-α doses (Figure D, panel B). Although the ELISA was established using 100 U/ml TNF-α to induce VCAM-1, suboptimum doses of TNF-α were used to repeat key experiments. TNF-α at 1 U/ml, which limited VCAM-1 induction to 30 % of the standard 100 U/ml amount, was used to test for a suppressive effect of apoE:DMPC (Figure G) as described in Section 2.3.2. Similarly, decreasing doses of TNF-α (100-0.1 U/ml) were used to examine whether an inhibitory effect of total HDL could be revealed (Figure 3.3-11); none was found. Experiments detailed in Chapters 4 and 5 used a submaximum dose of TNF-α, 10 U/ml, to test for VCAM-1-modulatory effect of cell-derived apoE.
Figure G. ApoE:DMPC has no effect on expression of VCAM-1 induced by a suboptimal dose of TNF-α as analysed by ELISA.

HUVEC monolayers were incubated for 16 h with 50 μg protein/ml apoE:DMPC and then stimulated with 1 U/ml TNF-α for 6 h to induce VCAM-1 expression. Unstimulated cells had a negligible level of VCAM-1 (white bar) and preincubating cells with either apoE:DMPC (hatched bars) or DMPC alone at equivalent phospholipid concentrations (grey bar) had no effect on this basal level. Treatment with TNF-α (1 U/ml) for 6 h increased VCAM-1 ~3-fold (black bar) but this was less than half of the level induced by the higher TNF-α dose (100 U/ml, black bar). Preincubating cells with apoE:DMPC (hatched bars) or DMPC alone (grey bar) had no effect on the VCAM-1 expression induced by 1 U/ml TNF-α. The results are from one representative experiment and each bar shows mean ± SE of quadruplicate wells.
Discussion

The HUVEC-bound VCAM-1 ELISA described in this thesis was established as a sensitive technique to detect VCAM-1 induction in response to TNF-α. The technique was optimized with respect to reagent dilutions so that all the VCAM-1 antigen was detected. It was important that total VCAM-1 was measured in TNF-α-treated control wells, as these were used as reference points to determine the extent of VCAM-1 downregulation after treatment with a NO donor, 17β-estradiol, HDL or apoE:DMPC. Underestimating VCAM-1 levels in the TNF-α control would lead to inaccurate detection of downregulation.

Although 100 U/ml TNF-α gave clear VCAM-1 induction at 6 h, it was not until 10 h of exposure that expression reached a plateau when the highest levels of VCAM-1 were detected, comparable to another study [81] (Figure E). Thus, it appears that the ELISA detected total VCAM-1 antigen and had the dynamic range to detect even higher levels of expression. This conclusion was supported by the relatively large batch-to-batch variation in the level of VCAM-1 induction that was detected by the ELISA. In the majority of experiments, VCAM-1 induction levels fell close to the mean 8.4-fold increase (14/21 values fell within a 6.9 to 10.1-fold range), with only 3 cases where VCAM-1 was unusually high (up to 15.2 fold induction, Figure F). Furthermore, the ELISA was able to detect significant increases above the TNF-α-treated control. Indeed, this was observed by pe-incubating HUVECs with total HDL in 10 independent experiments using different batches of cells before TNF-α treatment (see Chapter 3, Table 3.3-1). Lastly, downregulation of VCAM-1 in response to known CAM suppressors (GSNO and 17β-estradiol) was within the range expected from literature values, indicating that the ELISA was indeed efficiently detecting all the VCAM-1 present.

The ELISA had a sufficient sensitivity to detect even weak inhibitory effects on VCAM-1 upregulation by TNF-α. The use of 100 U/ml TNF to stimulate VCAM-1 induction gave an optimum level of VCAM-1 expression at 6 h exposure time, while low background signals ensured that signal-to-noise ratios were maximized. On average, the VCAM-1 signal was 0.9 OD units above the basal level and within the limits of accurate spectrophotometric detection. However, suboptimum doses of TNF-α were also used in key experiments to reduce the final expression of VCAM-1 antigen to below the optimal level induced by 100 U/ml. This ensured that total VCAM-1 was detected even with the rare ‘hyper-responsive’ batches of HUVECs (Figure F). Consistent with the results gained using 100 U/ml TNF-α, the use of decreasing doses of TNF-α failed to demonstrate an inhibitory
effect of either HDL or apoE:DMPC on the induction of VCAM-1. Furthermore, experiments testing the ability of cell-derived apoE to downregulate VCAM-1, used a submaximum dose of TNF-α, 10 U/ml (Chapter 4 and 5).

My ELISA was established using HUVECs and the only other cells to be tested in the assay were C11STH spontaneously transformed HUVEC cells. These had a diminished response to TNF-α with less total VCAM-1 induced when compared with primary HUVECs (data not shown) and hence there was no need to modify the ELISA conditions. Obviously, if other cell lines expressing higher levels of VCAM-1 were to be used, then the ELISA would have to be readjusted, by retitration of antibody steps, to ensure maximum VCAM-1 detection.

It should also be emphasized that the VCAM-1 ELISA is a semi-quantitative method for determining relative VCAM-1 expression. In keeping with other groups, no attempt was made to quantify VCAM-1 expression (e.g. in terms of pmol VCAM-1/10⁶ cells) even though, shortly after my ELISA had been established, a limited supply of recombinant VCAM-1 was commercially available (R&D Systems). Theoretically the use of VCAM-1 standards would permit quantification of VCAM-1 expression. However, this has yet to be described in the literature. Moreover, the interactions of recombinant protein with anti-VCAM-1 have not been fully characterized and extensive validation of such standards would be needed before they could be introduced. Obviously a cell-bound ELISA is a complex system relying on specific antigen detection from amongst a numerous array of other cellular proteins; it is difficult to predict, therefore, how the detection of pure recombinant VCAM-1 would relate to the detection of cell-bound VCAM-1 (both at the surface and within the cell).

Finally, it is important to note that my ELISA findings were verified using FACS as a powerful tool for immunodetection of VCAM-1 on a large number of HUVECs on a cell-by-cell basis. As apoE:DMPC and HDL both failed to downregulate VCAM-1 it was crucial to rule out responsive HUVEC subpopulations within the cultures that could not be discriminated by ELISA. However, although FACS fluorescence profiles showed that, indeed, some HUVECs did appear more sensitive to 17β-estradiol, no evidence was found for a subpopulation of HUVECs that responded to either apoE:DMPC or HDL.
APPENDIX III

STATEMENT OF ORIGINALITY

This thesis contains details of experiments that I designed and performed, with the exception of the Figures listed below, where it is acknowledged that others have contributed.

CHAPTER 2

FIGURE 2.3-1  COMPARISON OF HUVECs AND ECV304 CELLS BY MICROSCOPY ..........................57
The processing of samples for SEM and the taking of electron micrographs (labelled A and B) was undertaken by J. Lewin (Electron Microscopy Unit, RF&UCMS, London, UK).

CHAPTER 3

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These experiments were performed by S. Khan (Department of Cardiothoracic Surgery, Harefield Hospital, Middlesex, UK) using HDL preparations provided by myself.

FIGURE 3.3-13  HDL DOES NOT SUPPRESS TNF-α-INDUCED E-SELECTIN EXPRESSION IN HCAECs ......104
These experiments were performed by S. Khan (Department of Cardiothoracic Surgery, Harefield Hospital, Middlesex, UK) using HDL preparations provided by myself.

CHAPTER 5

FIGURE 5.3-3  LEVELS OF INTRA-HUVEC cGMP ARE INCREASED BY INCUBATION WITH CHO CELL-SECRETED APOE...............................................................156
The analysis of cGMP on my samples was performed by Dr D. Riddell (RF&UCMS).

FIGURE 5.3-7  IMMUNOPRECIPITATION OF HUVEC LRP8.........................................................163
I performed this technique using a protocol established by Dr D. Riddell (RF&UCMS).