THE EFFECTS OF MODIFIED LOW DENSITY LIPOPROTEIN ON CORONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS

Atherogenesis: an in vitro investigation of the interactions between oxidatively modified low density lipoprotein and human coronary artery endothelial and smooth muscle cells

A thesis submitted for the degree of
Doctor of Medicine
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Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential.

Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima.

This thesis is an *in vitro* investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells.

Described herein are:

1. The isolation, culture and characterisation of human endothelial cells from coronary artery and other vessels.
2. The isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells.
3. The isolation and oxidative modification of human LDL of low pyrogenicity.
4. The effects of native and modified LDL on the basic cellular processes of endothelial and smooth muscle cells.
5. An investigation of the adhesion of human monocytes to endothelial and smooth muscle cells stimulated by native and modified LDL, and the role of adhesion molecules in this process.
Acknowledgements

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Finally, my thanks to Jon for his constant encouragement and support.

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For my parents
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Standards were prepared from 1,1,3,3-tetra-ethoxypropane to give 10-400 μM MDA.

Figure 8.10

Page 108 should read:

![Graph showing standard curve for TBA test]

**Standard curve for TBA test**

Correlation = 0.999

Where $c_1$ = absorption at 532nm, and $c_2$ = μM MDA,

linear regression analysis of the standard data yielded the equation predicting:

$$c_2 = 76.5c_1 - 1.2$$
Figure 8.11

Page 109 should read:

The MDA content of copper-oxidised LDL showed a 10-fold increase over that of native LDL. The MDA content of mmLDL rose with up to 9 months of storage to more than twice that of native LDL, but remained less than half that of oLDL.

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SECTION I: INTRODUCTION

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential.

Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima.

This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells.

The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described. In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported.

Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL.

An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells.
The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.

**STATEMENT OF HYPOTHESIS**

Oxidatively modified LDL interacts with human coronary artery endothelial and smooth muscle cells to promote monocyte adhesion and the atherosclerotic process.
ATHEROSCLEROSIS : AN INTRODUCTION

Considering the ubiquity of atherosclerosis in western society, our knowledge of the pathogenesis of this inflammatory, proliferative disease of the arterial intima is surprisingly limited. Only in recent years have new hypotheses advanced upon the theories of Rokitansky (1852) and Virchow (1860).

These 19th century pathologists argued about the nature of the disease. Rokitansky thought that atherosclerosis was due to the incorporation of mural thrombi into the vessel wall following intimal damage. Virchow, however asserted that the disease was due to focal thickening and lipid infiltration at sites of altered blood flow, and that "the view .... defended by Rokitansky ... is refuted".

The response to injury hypothesis (Ross 1986) attempts to encompass recent observations of the cellular and molecular interactions which occur in the course of the disease. The postulate is that the physiological purpose of atherosclerosis is to protect the arterial wall from injury; when injury is repeated and chronic, as with hypertension, smoking, hyperlipidaemia and diabetes, the physiological process becomes a disease.

Arterial occlusion may occur as a result of progressive encroachment on the lumen by the atherosclerotic plaque itself, or by superimposed thrombus. The clinical manifestation of such an event varies according to the artery affected, but since the disease has a predilection for the coronary, carotid and cerebral circulations, the end point is frequently myocardial ischaemia or infarction, stroke, and death.

It follows that the most effective strategies to modify the progression of atherosclerosis should be directed against the early, potentially reversible, stages of
the disease and that the development of such strategies will be facilitated by a greater understanding of the early mechanisms.

This thesis addresses some of the mechanisms involved in atherogenesis. Particular attention is given to the atherogenic effects of oxidatively modified low density lipoprotein on the cells of the human coronary artery wall; endothelial and smooth muscle cells.
SECTION I: INTRODUCTION

CHAPTER 1

THE ENDOTHELIUM

1.1 Introduction
For many years, this large secretory organ, estimated to weigh over 700g (Wolinsky 1980), and lining all blood vessels to cover an area of 1500 m², was considered to be a semi-permeable barrier, in the form of an inert, non-thrombogenic cellular monolayer.

With the development of pure endothelial cell cultures it became clear that the vascular endothelium did not comprise a homogeneous population of cells, but exhibited marked heterogeneity of both form and function.

As the many regulatory functions of the endothelium have been identified, so has the apparent potential of diseases such as atherosclerosis to cause endothelial dysfunction. Furthermore, the contribution of endothelial dysfunction towards the progression of atherosclerosis has been increasingly recognised.

1.2. Endothelial function
As the interface between circulation and tissues, the endothelium is well situated to receive and transduce circulatory signals, both haemodynamic and humoral. It acts as a non-thrombogenic, semi-permeable barrier and a secretory organ. It controls the contractile and proliferative responses of the vascular smooth musculature, and regulates angiogenesis and leucocyte trafficking. An outline of all these endothelial functions follows below.

Coagulation
There are a number of ways in which the endothelium provides an antithrombotic surface allowing the blood to circulate without coagulating. Endothelial cells express
thrombomodulin, a membrane bound ligand with high affinity for thrombin, which it 
inactivates. Endothelial cells also enhance the activation of protein C by 
thrombomodulin, (Maruyama et al 1985), and the action of antithrombin III in 
inactivating thrombin (Busch et al 1982). In addition, the endothelium secretes the 
platelet aggregation inhibitors prostacyclin (Moncada 1982) and EDRF (endothelium-
derived relaxing factor) (Moncada et al 1988), and also has fibrinolytic properties, 
synthesising and releasing tPA (tissue plasminogen activator) (Collen et al 1989). 
Endothelial regulation of coagulation includes pro-coagulant functions, which permit 
repair of vessel damage. vWF (von Willebrand factor) is required for platelet 
adhesion (Meyer et al 1983); the endothelium synthesises and secretes vWF 
constitutively; release is increased in the presence of thrombin (Jaffe et al 1974, de 
Groot et al 1984). Other coagulation factors are also synthesised by the endothelium, 
including factor V (Cerveny et al 1984) and tissue factor, which is also induced by 
thrombin (Galdal et al 1985). In areas of endothelial denudation, subendothelial 
collagen is exposed to the circulation, resulting in platelet aggregation and 
degranulation (Barnes et al 1980), allowing the repair of vessel damage. 

vWF constitutively released to the basement membrane may serve a further function; 
endothelial cells adhere to vWF via integrin adhesion molecules, vWF secreted into 
the basement membrane may therefore maintain endothelial integrity in vivo (Dejana 
et al 1989).

Vasomotor control
The endothelium controls vascular smooth muscle tone by the production of several 
vasoactive substances. As mentioned above, endothelial cells synthesise and release 
prostacyclin, which in addition to its antiplatelet activities is a powerful vasodilator. 
Its actions are balanced by the endothelial derived vasoconstrictor, thromboxane A2 
(Hamberg et al 1975). The phenomenon of endothelium dependent relaxation was 
discovered by Furchgott et al in 1980 and EDRF was subsequently proposed to be
nitric oxide (NO) (Moncada et al 1988). By the constitutive release of NO, the endothelium maintains active, basal vasodilatory tone. In the normal vessel, this overrides the effects of the vasoconstrictors endothelin (Yanagisawa et al 1988) and angiotensin II, which is converted to its active form by angiotensin converting enzyme in the endothelium (Caldwell et al 1976). Endothelin also maintains vasomotor homeostasis by stimulating NO and prostacyclin release from the endothelium (de Nucci et al 1988).

**Growth factors**

The role of the endothelium in regulating smooth muscle cell proliferation is discussed in Chapter 2.

**Leucocyte interactions**

The endothelium controls the migration of leucocytes across the vessel wall by the secretion of chemotactic factors, such as MCP-1 (monocyte chemotactic protein-1), and by the expression of leucocyte adhesion molecules, discussed in Chapters 4 and 5.

**Angiogenesis**

The endothelium is central to the process of angiogenesis. New vessel formation is a tightly regulated phenomenon, only occurring physiologically in growth and development, the female reproductive tract, and in wound repair. Endothelial cell culture studies have shown that angiogenesis requires the induction of endothelial proteases such as tPA, to degrade the basement membrane and allow endothelial migration from the parent vessel, followed by endothelial proliferation to form tubules, and differentiation into a functional vessel (Folkman et al 1980). A variety of growth factors may contribute to this process, including FGF, ECGF, TGF (fibroblast, endothelial cell and transforming growth factors, respectively) and prostacyclin (Gospodarowicz et al 1975, Macaig et al 1979, Schreiber et al 1986, Ben Ezra 1978).
1.3. The endothelium in disease

The role of the endothelium in atherosclerosis is being increasingly appreciated. Despite its morphologically intact appearance, there is accumulating evidence that the endothelium is functionally impaired early in the disease process. In addition, it plays an integral part in early events of atherogenesis, mediating the infiltration of the intima by monocytes and T lymphocytes from the circulation.

Evidence for early endothelial dysfunction came in 1974 when Bell et al demonstrated in vivo, an increase in permeability of the arterial endothelium at sites of predilection for atherosclerosis in young, apparently normal pigs. Once the barrier function of the endothelium is compromised, the intima is exposed to pro-atherogenic circulating growth and chemoattractant factors.

A further sign of endothelial dysfunction is the decreased endothelium-dependent relaxation in atherosclerotic vessels. This is not confined to angiographically detectable atherosclerosis (Chester et al 1990), but is also present in hypercholesterolaemic patients and animal models of hypercholesterolaemia. The return of relaxation to normal by the administration of L-arginine, the substrate for NO synthase, suggests that the underlying abnormality is impaired endothelial NO production (Drexler et al 1991, Cooke et al 1992). In addition, endothelium-dependent vasodilation is impaired in the normal (brachial) arteries of hypercholesterolaemic men without clinical evidence of vascular disease (Chowienczyk et al 1992). The latter observation suggests a generalised endothelial dysfunction in hypercholesterolaemia which precedes the development of clinically apparent atherosclerosis. The resulting reduction in NO production may facilitate the progression of the disease, not only by reducing vasodilatory responses, but also by decreasing basal inhibition of smooth muscle cell proliferation, and promoting monocyte and platelet recruitment (Cooke et al 1992). The mechanism by which
endothelial NO is decreased is not known; the possible role of oxidised LDL (oLDL) is discussed in Chapter 3.

The instrumental roles of the cytokine or oLDL activated endothelium in enhancing monocyte-endothelial adhesion and recruiting monocytes to form foam cells are considered in Chapter 5.

The alignment of the endothelium overlying advanced human atherosclerotic plaques is disordered as a result of turbulent flow. The endothelium shows evidence of activation with increased expression of MHC class II antigens and organelle hyperplasia. Furthermore, activated leucocytes and platelets may be observed adhering to the endothelium (Burrig 1991). In addition, areas of endothelial denudation are frequent and accompanied by adherent monocytes and platelet microthrombi (Davies et al 1988).

Thus the normal endothelium maintains the vessel in a dilated state, and is actively anti-thrombogenic, non-adherent and anti-proliferative. However, endothelial control of vessel wall homeostasis is disrupted early in the atherogenic process, permitting and probably actively contributing towards disease progression.

**1.4 Endothelial heterogeneity**

Differences in morphology, function and dynamic responses to environmental change have been demonstrated between specific endothelial cells (Engelberg 1989). Factors which may determine heterogeneity include species, organ, vessel type or size of origin, and environment.

There is evidence of antigenic heterogeneity between cells. For example, species differences exist in the expression of von Willebrand factor, and human capillary endothelial cell vWF immunoreactivity is weaker than that of large vessels (Piper et
Monoclonal antibodies which differentiate between arterial and venous endothelial cells have been described (Piper et al 1990). Further, immunocytochemical studies (Page et al 1992) have detected MHC class II antigens on the endothelium of cardiac capillaries and normal coronary artery, but not on other large vessels. In addition, vascular cell adhesion molecule-1 (VCAM-1) was detected on normal coronary artery endothelium, but not on other large vessels or capillaries (Page et al 1992). The expression of these antigens by normal coronary artery would favour interactions between the endothelium and T cells or monocytes and might be a factor in the predilection of atherosclerosis for the coronary vasculature.

The activities of the endothelium-derived vasoactive agents EDRF and endothelin have been shown to differ between arteries and veins and between capillary beds of different organs. NO activity is greater in arteries than veins (Vallance et al 1989, Luscher et al 1988), and endothelin is more potent in its actions on veins than on arteries (Lerman et al 1990).

Variations in the metabolic properties of endothelial cells have been reported. There are differences in arachidonic acid metabolism between human microvascular and large vessel endothelial cells, and between human and porcine endothelial cells. In addition, arterial endothelial cells have a greater capacity to produce prostacyclin than those from veins. Other differences between human arterial and venous endothelium include higher ACE activity and greater insulin binding capacity in arteries (Gerritsen 1987).

Such heterogeneity suggests that implications of clinical significance from in vitro studies can only be made when using endothelial cells derived from the vessel and species of interest. Thus in order to improve understanding of human cardiac endothelial cell properties, particularly in the context of atherogenesis, it is important to study these cells directly.
1.5 Endothelial cells in culture

1.5.1. History of endothelial cell culture

Tissue culture had its origins at the beginning of the century. Initial studies involved tissue fragments from which cells were observed to migrate and, occasionally, undergo mitosis (Harrison 1907). Isolated viable endothelial cells were described by Lewis in 1921, and by the 1950's, outgrowths of capillaries derived from bone marrow could be maintained in culture for several weeks (Woodward et al 1953). This was followed by the inception of endothelial cell culture (Pomerat et al 1963). Pure cultures of large vessel human endothelial cells were subsequently established (Gimbrone et al 1974, Jaffe et al 1973), and microvascular endothelial cells were cultured by Folkman et al in 1979.

1.5.2. Tissue culture as a research tool

Tissue culture may offer advantages over other research techniques. It permits the responses of human cells to be studied in diseases such as atherosclerosis, where the validity of animal models has been questioned. Tissue culture allows precise control of the environment: pH, temperature, oxygen and carbon dioxide tensions may all be regulated and physiological conditions kept constant, but not always defined (as in the case of serum constituents). Tissue samples and whole organisms are always heterogeneous, whereas after 1-2 passages, cells in culture assume a uniform constitution, permitting reproducibility of experiments. In addition, cell culture is economical in investigating the effects of reagents; less reagent is generally required to demonstrate its effects on cells in culture than on whole organisms. Moreover, the ethical issues of animal experimentation are avoided.

However, expertise is required in the establishment and maintenance of cells in culture if contamination with unwanted cell types, bacteria and fungi is to be avoided. Furthermore, human cell culture requires a regular supply of normal human tissue, unless transformed cell lines are used. There may be differences between cells in vitro...
and in vivo; in particular, differentiated properties may be lost in late passage or transformed cells. The use of nontransformed, early passage cells largely overcomes this problem.

Cells in culture are devoid of neurohumoral influences and influences from other cell types. However, provided that such considerations are taken into account, the simplicity of cell culture systems may be turned to their advantage. Alternatively, cell culture may be extended to co-culture and organ culture, thus increasing the complexity of the system and allowing interactions between different cell types to be studied.

1.5.3. Sources of endothelial cells for culture

In view of endothelial heterogeneity, endothelial cell culture models require the isolation and culture of endothelial cells from a variety of human tissues. To allow the mechanisms of atherogenesis to be investigated, endothelial cells derived from vessels subject to atherosclerosis are needed. Further, if heterogeneity between human cardiac endothelial cells from "large vessels" (endocardium and coronary artery) through to capillaries could be demonstrated, then investigation of events occurring at a microvascular level would require the culture of microvascular endothelium. Thus microvascular endothelial function during ischaemia and reperfusion, and dysfunction in atherosclerosis might be studied.

Umbilical cords provide a freely available source of endothelial cells, albeit of foetal venous origin. Saphenous vein segments are also readily available, providing adult, but still venous, endothelial cells. Saphenous vein endothelial cells are of particular interest because of the use of the saphenous vein as coronary artery bypass conduits. Cardiac endothelial cells may be obtained from the endocardium of excised mitral valves. Endothelial cells derived from arteries prone to atherosclerosis may be obtained from coronary arteries and aortae from cardiac (and in the case of the aorta,
renal) explants. In addition, left internal mammary arteries deemed unsuitable for coronary bypass grafting may be used as a further source of arterial endothelial cells. The isolation and culture of microvessel endothelial cells from the human heart has not been described previously; they are fastidious cells and difficult to isolate. Furthermore, although culture of animal microvessel cardiac endothelial cells has been reported (Piper et al 1990, Diglio et al 1988, Buderus et al 1989), the techniques described appear flawed (an exception being the description of Gerritsen et al 1982), particularly where a Langendorff perfusion system is utilised. No provision is made to exclude endothelial cells from the aortic root or coronary arteries or from the right atrium or ventricular endocardium. In partial recognition of this, it has been claimed that such endothelial cells comprise only a small proportion of the total mass of cardiac endothelium. Cells from the capillaries are far more numerous, so it is suggested that contamination with non-capillary endothelium should be negligible (Piper et al 1990). Two points strongly mitigate against this argument. Firstly, large vessel and endocardial endothelial cells are considerably less exacting in their growth requirements than those from microvessels (Davison et al 1980, Pearson 1990, Thorne et al 1991), and are therefore likely to overgrow any capillary derived cells. Secondly, where the Langendorff model is used, the heart is perfused with enzyme solution to lyse endothelial cell attachment to the basement membrane. Detached endothelial cells will rapidly occlude the myocardial capillary bed. Thus capillary perfusion will cease and perfusate collected from the coronary sinus will contain endothelial cells predominantly from larger vessels. Therefore studies which utilise cultured animal cardiac "microvessel" endothelial cells (Buderus et al 1989) may be inapplicable to the human heart not only because of interspecies heterogeneity, but also because they may not relate to the cardiac microvasculature at all.
1.5.4. Purification of endothelial cell cultures

Primary endothelial cell cultures contaminated with non-endothelial cells require further purification.

Magnetic cell sorting is a sensitive and specific technique to exclude non-endothelial cells from cell populations. Dynabeads® M450 (Dynal (UK) Ltd, Wirral, UK) are magnetisable polystyrene beads 4.5μm in diameter. They may be coated with proteins or carbohydrates directed against endothelial-specific cell surface antigens or glycoproteins and glycolipids, and used to isolate endothelial cells from mixed cell populations.

The lectin *Ulex europaeus* agglutinin type-1 (UEA-1, Sigma), binds to endothelial cell surface L-fucose, but not to smooth muscle cells (Holthofer et al 1982). The lectin may be bound to Dynabeads and incubated with mixed population cells: endothelial cells bound to the lectin coated beads may be separated from non-endothelial cells.

CD31 (cluster designation antigen 31, or PECAM: platelet endothelial cell adhesion molecule) is a member of the immunoglobulin superfamily, expressed by all endothelial cells and by platelets and myeloid cells (Albelda et al 1991). The major endothelial cell culture contaminants are CD31 negative smooth muscle cells and fibroblasts. Mouse monoclonal antibody to CD31 (British Bio-technology Products Ltd, Abingdon, UK) may be bound to endothelial cells which may subsequently be extracted with Dynabeads coated with anti-mouse immunoglobulins.
1.5.5. Characterisation of endothelial cells

The endothelial nature of cultured cells may be confirmed by the following characteristics:

i) Development of cobblestone morphology.

ii) \textit{in vitro} differentiation: capillary tubule formation

iii) Uptake of fluorescent acetylated LDL

iv) Binding to \textit{Ulex europaeus} agglutinin-1

v) CD31 expression (PECAM)

vi) CD34 expression

vii) Immunohistochemical localisation of von Willebrand factor.

viii) Detection of von Willebrand factor secretion

ix) Induction of E-selectin expression

Endothelial cells in culture are contact inhibited. When confluent, they form a characteristic "cobblestone" pattern monolayer. However some endothelial cells, in particular those derived from the microvasculature, may demonstrate a more irregular pattern, and the cobblestone pattern can occasionally occur in non-endothelial cells (van Hinsberg \textit{et al} 1990).

Additional criteria, listed above, and detailed in Chapter 6, should therefore be employed to confirm the endothelial nature of cells. The use of these methods enables the endothelial nature of cells to be identified, but no method currently exists to allow one to distinguish reliably endothelial cells from vessels of different origins. Chapter 6 therefore describes studies of the isolation, culture and characterisation of human endothelial cells from a variety of sources.
2.1 Introduction

In the normal adult artery, vascular smooth muscle cells are generally of a contractile phenotype, modulating vessel tone in response to vasomotor stimuli (Thyberg et al 1990). They contain smooth muscle contractile proteins such as $\alpha$-actin and smooth muscle myosin and are metabolically quiescent, with a low proliferative capacity. The other, synthetic smooth muscle cell phenotype (Schwartz et al 1986) has enhanced secretory and proliferative capacities, and diminished contractile proteins, with a corresponding loss of contractile function (Thyberg et al 1990). It predominates in atherosclerotic plaques (Campbell et al 1990), restenotic lesions (Kocher et al 1991) and, in vitro, in subculture (Chamley-Campbell et al 1979).

2.2 Regulation of vascular smooth muscle cells in atherosclerosis

As will be discussed in Chapter 5, key events in the development of the intimal atherosclerotic plaque are the migration into the intima of medial smooth muscle cells, and their transformation to the proliferative, matrix-secreting phenotype. Factors controlling smooth muscle cell proliferation and migration are considered below, and outlined in Table 2.i.

In the normal vessel, smooth muscle cells are protected from mitogenic and chemotactic factors by the barrier of the intact endothelium, the short plasma half life of many growth factors (2 minutes for PDGF: platelet derived growth factor) (Raines et al 1990), their low basal expression of PDGF receptors, and the absence of activated monocytes, lymphocytes and platelets. However, if the endothelium is breached, the intima is exposed to activated platelets, and becomes infiltrated with activated monocytes and lymphocytes, which cause the release of growth and
migratory factors in the immediate environment of the smooth musculature.

Although not a true model of atherosclerosis or restenosis, the response to injury of the normal rat carotid artery has suggested a mechanism by which some of these factors may act (Clowes et al 1991). In this model, PDGF, released from endothelial and smooth muscle cells, platelets, monocytes and T lymphocytes appears to provide a potent and early stimulus for migration of medial smooth muscle cells to the intima.

The predominant smooth muscle mitogen within the first two days of injury is basic fibroblast growth factor (bFGF), released from damaged vessel wall cells (Reidy et al 1991). The roles of the various growth factors in subsequent neointimal smooth muscle hyperplasia remain to be elucidated.

The mitogenic activities of IL-1, TNFα and TGFβ (transforming growth factor β) are mediated by the induction of PDGF expression (Raines et al 1990), and LDL also enhances PDGF production by smooth muscle cells (Stiko-Rahm et al 1992). In addition, PDGF upregulates its own synthesis and the expression of the smooth muscle PDGF receptor (Raines et al 1990). This is counterbalanced by the down regulation of the PDGF receptor by TGFβ (Raines et al 1990). A number of vasoactive substances have also been implicated in the regulation of smooth muscle cells. Endothelin enhances transcription of the proto-oncogenes c-fos and c-myc; both it and another vasoconstrictor, serotonin, have mitogenic actions (Änggard et al 1990, Cascells 1992). Angiotensin II induces smooth muscle hypertrophy, as does vessel wall stretch (Cascells 1992). Prostacyclin and some other vasodilatory eicosanoids maintain the smooth muscle contractile phenotype, capable of responding to vasoactive but not mitogenic stimuli; nitric oxide is also anti-proliferative (Raines et al 1993).
<table>
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<tr>
<th>FACTOR</th>
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<th>Migration</th>
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<tr>
<td><strong>Cytokines and growth factors</strong></td>
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<tr>
<td>PDGF</td>
<td>+</td>
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<td>TGFβ</td>
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<td>MCP-1</td>
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<td><strong>Vasoactive agents</strong></td>
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<td><strong>Extracellular matrix</strong></td>
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<td>fibronectin</td>
<td>↑ synthetic</td>
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<td>laminin</td>
<td>↑ contractile</td>
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<td>plasmin/ PLG activators</td>
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Table 2.1. Control of smooth muscle cell proliferation and migration

Agents affecting smooth muscle cell migration or proliferation, and their sources, are tabulated.

EC = endothelial cell, S = smooth muscle cell, M = monocyte, T = T cell, P = platelet, PLG = plasminogen. (Modified from Raines et al 1993).
Extracellular matrix components also influence smooth muscle cell regulation. Heparin inhibits both migration and proliferation by binding PDGF (Wight 1989), and fibronectin promotes conversion from the contractile to the synthetic phenotype, whereas laminin promotes the contractile state (Thyberg et al 1990). Plasmin and plasminogen activators enhance smooth muscle migration by degradation of the extracellular matrix. The effects of LDL on smooth muscle cell function are discussed in Chapter 3.

A considerable body of work has focused on animal models and smooth muscle cells in the investigation of atherogenesis (Fallier-Becker et al 1990). However, atherosclerotic lesions in human vessels differ from those in animals. In addition, unlike the arterial intima of the rat and rabbit, the normal human intima contains smooth muscle cells (Stary et al 1989). Since changes in smooth muscle cell activity during these early stages appear to be confined to the intima, the culture of human intimal smooth muscle cells may therefore provide further relevant models for the study of atherosclerosis.

As will be discussed in Chapter 5, intimal smooth muscle cell proliferation and matrix secretion contribute to the body of the atheromatous plaque and the formation of its fibrous plaque. In addition, intimal smooth muscle cells may be more than mere effector cells in atherogenesis: they may play a role in monocyte adhesion. An investigation of this hypothesis is the subject of Chapter 11.

2.3 Vascular smooth muscle cells in restenosis
Restenosis following percutaneous coronary intervention is a major problem, both clinically and economically, occurring in 30-50% of patients 6 months post angioplasty (Holmes et al 1984, Nobuyoshi et al 1988). The restenotic lesion differs from the primary atherosclerotic plaque, in that intimal smooth muscle cell proliferation is the major component (Giraldo et al 1985).
The mechanisms involved in the intimal hyperplasia of restenosis are not fully understood; they include the actions of smooth muscle mitogens and chemoattractants, discussed above. Some animal data on restenosis are derived from animal models which closely represent both the primary atherosclerotic and restenotic processes in humans, namely the pig and non-human primate (DW Muller et al 1992, Nabel et al 1993). However, the financial cost of using such models in screening potential compounds which may prevent restenosis is prohibitive.

Much information has therefore been derived from the response of normal rat or rabbit arteries to injury. In these models, proto-oncogene expression has been observed, and restenosis reduced, by the administration of anti-mitogenic agents such as heparin or angiotensin converting enzyme inhibitors (Bauters et al 1992, Hanke et al 1992, Powell et al 1989). Although these models approximate coronary angioplasty, causing endothelial denudation and medial injury, followed by migration and proliferation of smooth muscle cells, there are a number of caveats.

Firstly, a number of factors which reduce smooth muscle hyperplasia and restenosis in animals have not been shown to reduce the incidence of restenosis in humans. These include angiotensin converting enzyme inhibitors, colchicine and aspirin (MERCATOR Study Group 1992, O'Keefe et al 1992, Harker et al 1992).

Secondly, the morphology of the muscular coronary artery differs from the elastic arteries (carotid, iliac and aortic) studied in many animal models. There are other differences in arterial morphology: rat arteries, unlike those of humans, do not have intimal smooth muscle cells. and have no vasa vasorum (DW Muller et al 1992).

Thirdly, animal models are often used to study the response of a normal artery to injury, whereas human angioplasty is performed on an atheromatous substrate. Even where the animal model is susceptible to "atheroma", such as in the rabbit, the nature
of the disease has been described as more typical of a lipid storage disease than atherosclerosis (DW Muller et al 1992), so that the obstructive lesion is of a different consistency to the human atheromatous plaque.

Whilst it is clear that observations made in vitro cannot be extrapolated directly to clinical practice, human smooth muscle cell culture provides a relevant model for the screening of agents in the investigation of atherosclerosis and restenosis. The culture of human smooth muscle cells should also allow the series of events leading to restenosis to be unraveled. For example, recent evidence suggests that the smooth muscle cells of patients who develop restenosis have abnormal growth regulatory responses (Chan et al 1993) and that their primary lesions have a higher proportion of activated smooth muscle cells of the synthetic phenotype than those who do not restenose (Simons et al 1993).

There is no consensus yet as to the role of lipids in restenosis (Austin 1992). Whether native or oxidatively modified LDL affects smooth muscle proliferation is addressed in Chapter 9.

2.4 Vascular smooth muscle cells in culture

2.4.1 Culture of vascular smooth muscle cells
The development of smooth muscle cell culture paralleled that of endothelial cells, beginning early this century. Isolated and viable smooth muscle cells were described in 1921 (Lewis), but culture techniques were not established until the 1970's (Thyberg et al 1990).

Smooth muscle cells de-differentiate to the synthetic phenotype after prolonged culture (Chamley-Campbell et al 1979), so although they may continue to grow through several passages, such cells may be less representative of the smooth muscle cells of the normal artery in vivo. In addition, since it is the smooth muscle cells of the
subendothelial intima which are implicated early in atherogenesis, attempts to isolate and culture smooth muscle cells from the whole vessel wall will yield a mixed, and therefore perhaps not representative, population of intimal and medial cells.

2.4.2 Characterisation of vascular smooth muscle cells

Although smooth muscle cells exhibit a high cytoplasmic to nuclear ratio and prominent nucleoli, and form a typical "hill and valley" pattern at confluence, these features are not specific. Furthermore, smooth muscle cells in culture show considerable polymorphism (Smirnov et al 1990). Additional means of characterising these cells depend on their specific cytoskeletal properties: smooth muscle cells show immunoreactivity for the intermediate filament desmin (specific for myogenic cells), and for α-smooth muscle actin (specific to smooth muscle cells) (Alberts et al 1989).

Studies on the culture and characterisation of human coronary artery and aortic smooth muscle cells are described in Chapter 7.
SECTION I: INTRODUCTION  
CHAPTER 3  
LOW DENSITY LIPOPROTEIN

3.1 Lipoprotein metabolism
The plasma lipoproteins are responsible for the transport of the insoluble lipids cholesterol, cholesterol esters and triglycerides, and amphipathic phospholipids. Lipoproteins comprise a core of lipid and fat soluble vitamins (A and E) surrounded by a surface of phospholipids and apolipoproteins (apolipoproteins). The apoproteins, A to F, function as ligands for cellular receptors and co-factors for cellular enzymes (Havelet et al 1989). The lipoproteins are: chylomicrons, VLDL, LDL, IDL and HDL (very low, low, intermediate and high density lipoprotein); their metabolism under physiological conditions is outlined below.

Chylomicrons
These are synthesised in the mucosa of the small intestine during fat absorption and contain fat soluble vitamins and lipid, mainly in the form of triglyceride. Their apoprotein moieties include apo B, C and E. Chylomicrons are taken up by liver, adipose tissue and muscle. Here, activation of lipoprotein lipase by apo C results in the rapid hydrolysis of triglycerides to fatty acids, which are used directly as a source of energy, or reconverted to triglyceride and stored.

VLDL
VLDL is synthesised in the liver, its triglyceride rich core being derived mainly from plasma free fatty acids. As with chylomicrons, VLDL's apoproteins include apo B, E and C, the latter determining that the site of breakdown is in tissue rich in lipoprotein lipase (Goldstein et al 1977).
IDL
Higher density VLDL and IDL remnants from the catabolism of chylomicrons and VLDL are transported to the liver where uptake by hepatocytes is mediated by receptors on the cell surface which recognise apo E. These particles are metabolised to form LDL as a major end product.

LDL
Elevated plasma LDL is an independent risk factor for atherosclerotic disease (Goldstein et al 1977). LDL may be defined as the lipoprotein fraction with a density range between 1.019-1.063g/ml. (Esterbauer et al 1992). The LDL molecule is a large, spherical particle with a diameter of 19-25nm, and a molecular weight of 1.8-2.8 million. Its main protein constituent is apo B. The structure of LDL is represented schematically in Figure 3.1.

Rich in cholesterol esters, especially the polyunsaturated fatty acid, linoleate, LDL transports cholesterol from the liver to peripheral tissues where it regulates de novo cholesterol synthesis. Approximately 2/3 of LDL is catabolised by the liver; the remainder is catabolised in peripheral cells including adipocytes, endothelial cells, mononuclear cells, smooth muscle cells and fibroblasts. The cell membrane of these cells contains pits coated with receptors which recognise the apo B moiety of LDL. LDL enters the cell by endocytosis and the apoprotein is hydrolysed to free amino acids. Cholesterol esters are hydrolysed by lipoprotein lipase to cholesterol which is either stored or re-esterified to monounsaturated fatty acids by ACAT (acyl coA cholesterol acyl transferase).

The process of cholesterol synthesis is regulated in two ways: firstly the uptake of LDL is controlled by down regulation of the cell surface apo B receptors in the presence of elevated intracellular cholesterol. Secondly, high levels of intracellular cholesterol inhibit the synthesis of HMG coA reductase (3-hydroxy-3-methylglutaryl coA
reductase), the rate limiting enzyme in \textit{de novo} cholesterol synthesis (Goldstein \textit{et al} 1977).

**Figure 3. LDL structure: schematic representation**

LDL comprises a hydrophobic core of cholesterol ester molecules surrounded by an amphipathic shell of unesterified cholesterol, phospholipid, and apolipoprotein B. ApoB is responsible for the binding of LDL to the LDL receptor.

**HDL**

Plasma HDL concentration is inversely related to the risk of atherosclerosis (Wolinsky 1980). This lipoprotein is secreted by the liver and small intestine. It is responsible for the reverse transport of cholesterol from the tissues to the liver, where it can be excreted intact into the bile. Unlike LDL, HDL is inversely related to the risk of atherosclerosis. HDL acquires its unesterified cholesterol core from peripheral tissues and plasma; its apoprotein components include apo A-1 (and excludes apo B). In the circulation, apo A-1 acts as a cofactor for the enzyme LCAT (lecithin-cholesterol acyltransferase), resulting
in the esterification of cholesterol and its transport to the liver and subsequent excretion (Havel et al 1989).

3.2 Oxidatively modified LDL

3.2.1 The oxidation of LDL

As discussed in Section 3.3, oxidatively modified LDL is implicated in the pathogenesis of atherosclerosis; it readily undergoes oxidative modification, resulting in the esterification of the cholesterol core. In addition, the polyunsaturated fatty acids (mainly linoleic and arachidonic acids) undergo peroxidation, a process of oxidative degradation. This is a chain reaction initiated by free radical species, which then self propagates due to the formation of unstable lipid peroxide radical intermediates and conjugated dienes, as illustrated in Figure 3.2. The end products of this reaction include short chain aldehydes such as malonaldehyde and 4-hydroxynonenal which may themselves modify LDL to a cytotoxic form (Esterbauer et al 1990a). In addition, intrinsic phospholipase-A2 activity results in the degradation of lecithin (phosphatidylcholine) to lysolecithin (lysophosphatidylcholine) (Parthasararthy et al 1990a). Furthermore, the protein moiety, apo B, is oxidised by the reactive intermediates from the peroxidation of lipids. This results in the modification of the lysine residues on apo B to a form recognised by the scavenger, but not the native LDL, receptor (Esterbauer et al 1990a).

Oxidation of LDL only takes place once its endogenous antioxidants are exhausted (Esterbauer et al 1990b). The main antioxidants within the LDL particle are α-tocopherol (vitamin E) and carotenoids. Vitamin E protects the LDL particle from oxidation by breaking the self propagating chain reaction of lipid peroxidation. It reacts with peroxide radicals to form a stable vitamin E phenoxy radical which does not react further with the lipid. in vitro studies suggest that the role of the antioxidant ascorbate is to regenerate vitamin E from its phenoxy radical, enabling it to retain its chain breaking properties (Burton et al 1985). β-carotene also acts as a quencher of singlet oxygen and a radical trapping agent (Burton et al 1985).
Figure 3.2  Lipid peroxidation in LDL: a chain reaction

During the lag phase, reactive oxygen species (ROS) are quenched by antioxidants within the LDL particle. Once these are consumed, unstable lipid peroxide radicals are formed, and the peroxidation reaction becomes self-propagating. Finally, conjugated dienes decompose to short chain aldehydes and ketones. Phospholipase A2 within the LDL particle also degrades phosphatidylcholine to lysophosphatidylcholine. In addition, lipid peroxide radicals and the aldehydes malonaldehyde and 4-hydroxynonenal modify the lysine residue on apoB.
Therefore, as illustrated in Figure 3.3, the peroxidation of LDL to form conjugated dienes follows a curve with an initial lag phase during which intrinsic antioxidants are consumed, and the time of which is proportional to the vitamin E content of the LDL (Esterbauer et al. 1990b). This is followed by a propagation phase during which the reaction is self-generating. Lastly, the decomposition phase is entered as the conjugated dienes decompose to aldehydes.

Figure 3.3 Spectrophotometric monitoring of conjugated dienes during peroxidation of LDL
LDL (0.1mg/ml) oxidised by incubation with 1.66μM CuSO₄. The lag phase is followed by the propagation phase. After reaching a plateau, there follows the decomposition phase as the dienes decompose to aldehydes.
3.2.2 Mechanisms of LDL oxidation

A variety of mechanisms provide an initiating radical species for the oxidation of LDL in vitro. Incubation with transition metal ions such as copper results in the oxidative degradation of LDL (Esterbauer et al 1990a). In addition, LDL may be oxidatively modified by several cell types cultured in medium containing traces of iron salts: endothelial cells, macrophages, lymphocytes and smooth muscle cells. The mechanisms by which cells oxidise LDL are not fully elucidated, although reactive oxygen species including the superoxide radical appear to be involved (Morel et al 1984, Hiramatsu et al 1987, Steinbrecher 1988, Leake et al 1990, Lamb et al 1992a).

Cellular lipoxygenases have also been implicated in the oxidative modification of LDL. This family of enzymes may initiate the oxidation of LDL either directly by the action of a peroxyl radical intermediate formed in the oxidation of cellular lipids to lipid hydroperoxides, or indirectly by the oxidation of cell membrane lipids which may then react with LDL. Although in vitro studies have demonstrated the ability of cellular lipoxygenases to oxidise LDL (Parthasarathy et al 1989), a mandatory role for these enzymes remains controversial (Jessup et al 1991, Sparrow et al 1992a), since lipoxygenase inhibitors may protect LDL from oxidation by non-specific antioxidant actions. Phospholipase A2 also plays a role in the oxidation of LDL by endothelial cells (Parthasarathy et al 1985).

LDL may also be minimally modified by storage for 3-6 months at 4°C, or by mild iron oxidation (Berliner et al 1990). This mild oxidation produces little oxidation of the apo B moiety, and so minimally modified LDL (mmLDL) is recognised by the native, not the scavenger LDL receptor. The initial oxidation of LDL in vivo probably involves the interaction of native LDL with only a few cells in the vessel wall intima, resulting in the formation of minimally modified rather than highly oxidised LDL, as illustrated in Figure 3.4. The biological activities and characterisation of modified LDL are discussed below.
Figure 3.4 Minimal modification of LDL by cells of the arterial intima
Circulating LDL enters the intima via the native LDL receptor. The intima provides a microenvironment protected from circulating antioxidants, in which endothelial and intimal smooth muscle cells mildly oxidise the LDL. The minimal modification of LDL may involve cellular lipoxygenases (LO), phospholipase A2 (PLA2) and the superoxide radical (O$_2^-$). The apoB moiety of the minimally modified LDL (mmLDL) remains intact; it therefore continues to be recognised by the native rather than the scavenger LDL receptor. The mmLDL may subsequently be further oxidised as monocytes are recruited to the intima.
3.2.3 Assessment of oxidatively modified LDL

Methods to assess LDL oxidation reveal heterogeneity between LDLs modified by different methods. For example, mmLDL shows little increase in conjugated diene formation compared to the more completely copper-oxidised LDL, nor does it demonstrate enhanced electrophoretic mobility, reflecting its intact apoB protein moiety. MmLDL differs from native LDL in its increased lysophosphatidylcholine content and raised malonaldehyde content (Berliner et al 1990); again these indices are more markedly elevated in copper-oxidised LDL.

Modified LDL may also be assessed by cellular degradation. Oxidation of LDL which results in modification of its apo B moiety may be assessed by a macrophage degradation assay. Such oxidatively modified LDL demonstrates increased scavenger receptor mediated uptake and degradation by macrophages. Radiolabeling of the LDL allows the quantification of this process (Leake et al 1990). MmLDL, with its intact apoB moiety, is not recognised by the scavenger receptor and is taken up and degraded to a lesser extent, via the native LDL receptor (Berliner et al 1990).

It follows that this biochemical heterogeneity may be accompanied by different biological activities in the different forms of modified LDL. The oxidation of LDL by copper and macrophages, and minimal modification are therefore the subject of Chapter 8.

3.3 Oxidatively modified LDL and atherogenesis

3.3.1 Atherogenic properties of oxidatively modified LDL

Oxidatively modified LDL is implicated in the pathogenesis of atherosclerosis from the earliest stages of foam cell formation. Its proposed roles are outlined below, and the process of atherogenesis discussed in more detail in Chapter 5.
Macrophages do not accumulate native LDL to form foam cells. The uptake and metabolism of native LDL by endothelial cells and macrophages occurs via a cell surface receptor which recognises the apo B moiety of LDL. This is a regulated process which prevents overloading of macrophages and consequent foam cell formation. Goldstein et al (1979) were the first to demonstrate that modified LDL is avidly internalised by macrophages via a separate, unregulated scavenger receptor, expressed both on monocyte/macrophages and endothelial cells. It is the oxidative modification of the apo B moiety of LDL which renders it recognisable by the scavenger, and not the native, LDL receptor (Esterbauer et al 1990a). It is the scavenger receptor mediated avid uptake of oLDL which results in the formation of macrophage-derived foam cells.

There are at least two distinct scavenger receptors (Kodama et al 1991): both contain N terminal cytoplasmic, transmembrane, spacer, $\alpha$ helical coiled coil, and collagenous domains. The type I receptor also contains a C terminal cysteine rich domain which is absent from the type II receptor. The latter domain is not required for the binding of modified LDL, whether it confers distinct functions on the type I receptor is unknown. It is unlikely that the scavenger receptor would have evolved if its only effect was detrimental. It is therefore interesting that it may also play a significant protective physiological role in endotoxic shock: bacterial lipopolysaccharide (endotoxin) is a ligand for the receptor which mediates hepatic uptake and clearance of the endotoxin (Krieger 1991).

Oxidatively modified LDL possesses a number of other properties which native LDL does not, and which may contribute to atherogenesis.

Both mm-LDL and oLDL are chemotactic for circulating monocytes, which cross the endothelium to become tissue macrophages where they are retained by the presence of oLDL (Quinn et al 1987). This effect is thought to be due to the lysophosphatidylcholine component of modified LDL (Esterbauer et al 1990a) and the induction of gene
expression for monocyte chemotactic protein-1 (MCP-1) in endothelial and smooth muscle cells (Berliner et al 1990, Cushing et al 1990, Liao F et al 1991). The mechanism by which monocyte adhesion to the endothelium is selectively increased by oLDL (Berliner et al 1990, Frostegård et al 1990) remains to be elucidated, as discussed in Chapter 5. In addition, modified LDL induces the expression and secretion of M-CSF and GM-CSF (macrophage, and granulocyte macrophage colony stimulating factor, respectively) from endothelial cells (Rajavashisth et al 1990). Such CSFs are not only chemotactic for monocytes, but induce macrophage proliferation within the atherosclerotic plaque. Whether macrophages are stimulated to enter the mitotic cycle once resident within the plaque, or whether the CSFs act upon a subset of macrophages which had already entered the cell cycle in the bone marrow, is not known (Ross 1993).

The lipid component of oLDL is cytotoxic to a variety of cell types, including endothelial cells (Morel et al 1983, Cathcart et al 1985). It may thus play a role in breaching the endothelium as the atherosclerotic process progresses, resulting in the exposure of intimal smooth muscle cells to platelet derived and circulating growth factors. Furthermore, oLDL has been reported to be chemotactic for smooth muscle cells in culture (Autio et al 1990) and may thus play a role in recruiting medial cells to the intima in atherogenesis. LDL may also induce smooth muscle cell proliferation (Stiko-Rahm et al 1992, Harris Hooker et al 1992).

oLDL also causes detrimental vasomotor effects, inducing endothelin synthesis (Boulanger et al 1992), and impairing endothelium dependent relaxation responses (Simon et al 1990, Flavahan 1992). The mechanism by which the latter phenomenon occurs is not fully understood. It may be hypothesised that oLDL might block basal NO release, or interfere with its action on vascular smooth muscle. Alternatively, the lipid hydroperoxides within the oLDL may react with NO and inactivate it. OLDL may therefore contribute to the impaired endothelium dependent vasodilatory responses observed in early atherosclerosis and hypercholesterolaemia, described in Chapter 1.
OLDL may mediate a local thrombotic tendency both by inducing plasminogen activator inhibitor in the endothelium (Latron et al 1991), and by the stimulation of tissue factor expression in the endothelium (Drake et al 1991).

Furthermore, the scavenger receptor shares homology with an epitope of collagen (Krieger 1992) and it may be this which accounts for the binding of oLDL to collagen (Kalant et al 1991), providing a mechanism whereby oLDL is deposited in the vessel wall.

3.3.2 Evidence for a role for oxidised LDL in atherogenesis

An increasing body of evidence supports a role for oLDL in atherogenesis. Biologically active oLDL is associated with atheromatous changes in the vessel wall of both humans and animal models of atherosclerosis (Ylä-Herttuala et al 1989). In addition, oLDL in human fatty streaks is co-localised with mRNA for 15-lipoxygenase (Ylä-Herttuualae et al 1991), suggesting a mechanism by which the LDL may be oxidatively modified. Furthermore, oLDL is immunogenic; not only have circulating autoantibodies to specific epitopes of oLDL been demonstrated, but it has also been suggested that high titres of such antibodies are predictive of the progression of atherosclerotic disease (Salonene et al 1992). It might therefore be that such autoantibodies are not only markers, but may also contribute to the inflammatory process of atherogenesis.

3.3.3 Antioxidants and LDL in atherosclerosis

A number of antioxidants have been shown to increase the resistance of LDL to oxidation in vitro. These include LDL samples isolated following the administration of probucol and vitamin E, but not β-carotene, to humans (Reaven et al 1991, Princen et al 1992), and N, N'-diphenyl-phenylenediamine to rabbits (Sparrow et al 1992b). The susceptibility of LDL to oxidation is also reduced by the in vitro addition of phenolic extracts from red wine, or the anti-inflammatory compound, leumedin (Frankel et al 1993, Navab et al 1993). The presence of HDL also inhibits LDL oxidation, perhaps
explaining the inverse correlation between serum HDL and atherosclerosis (Parthasararthy *et al* 1990 b).

LDL isolated from patients with hypercholesterolaemia or overt coronary artery disease shows increased susceptibility to oxidation compared with normal controls; this may be explained by the corresponding lower levels of vitamin E present in the patients' LDL (Lavy *et al* 1991, Liu *et al* 1992). To date, there is conflicting evidence regarding the susceptibility of smokers' LDL to oxidation (Harats *et al* 1990, Princen *et al* 1992).

There is also epidemiological evidence to suggest that plasma levels of antioxidants such as vitamin E are inversely related to the incidence of coronary artery disease (Gey et al 1989). Furthermore, antioxidant therapy with probucol and N,N'-diphenyl-phenyldiamine has been shown to inhibit atherogenesis in animal models (Kita *et al* 1987, Sparrow *et al* 1992b).

The artery may have a further means of protecting itself from the oxidant stress which results in, and from, the oxidative modification of LDL. The expression of heat shock, or stress, proteins is induced in tissues by a variety of insults, including oxidant stress. Several stress proteins have been implicated in protecting the heart during episodes of post-ischaemic reperfusion (Thorne *et al* 1992a), and such a response might provide the cells of the arterial intima with protection against the lipid radical species of oxidised LDL.

It is clear that oxidised LDL is a heterogeneous entity, containing a variety of intermediate and end products. Some components may become polarised so that they are able to leave the oLDL particle and may mediate biological effects independently. Heterogeneity may also depend on the presence of intrinsic antioxidants, and on the manner in which the LDL has been oxidised (by different cell types, enzymes or different metal ions). It is therefore not surprising that apparently conflicting results
regarding the biological activities of modified LDL have arisen, even within single laboratories (Liao F et al 1991, Witzum et al, 1991). In view of this, it is important that researchers delineate clearly the methods used in the isolation and oxidation of LDL.

3.4 Isolation of LDL

LDL may be isolated from human plasma by density gradient ultracentrifugation (Havel et al 1955). The quality of the isolate obtained is important if its biological effects are to be demonstrated reproducibly. To avoid spontaneous oxidation during separation, LDL should be isolated rapidly under controlled temperature conditions in the presence of antioxidants which may subsequently be removed by dialysis. Contamination with other lipoprotein fractions also affects the biological properties of the preparation: β-VLDL induces monocyte-endothelial cell adhesion (Territo et al 1989) and HDL inhibits the oxidative modification of LDL (Navab et al 1991, Parthasararthy et al 1990b). Furthermore, contamination with bacterial lipopolysaccharide would result in spurious induction of cellular adhesion molecules (Bevilacqua et al 1987).

It is necessary to determine the protein content of isolated LDL, not only to quantify experiments, but also to allow comparison between the concentrations to which cells would be exposed and normal human plasma LDL levels of 0.6-0.7mg LDL protein/ml (Havel et al 1989).

3.5 LDL - cell interactions

The biological effects of differently modified LDL preparations on cytotoxicity, migration and growth may be quantified, and a toxic effect on the cells excluded for adhesion assays.

3.5.1 Assessment of cytotoxicity

As discussed in Section 3.3.1, oLDL may be cytotoxic; its effects may be quantified by a radiolabeled chromium release from damaged cells. The assay is based on the principle
that chromium, as trivalent sodium chromate, due to its positive charge, readily crosses the intact plasma membrane of viable cells. Once within the cell, it is covalently bound to basic amino acid components of intracellular proteins. If the cell becomes damaged or lysed, the increase in cell membrane permeability allows chromate to leak out into the surrounding medium. The rate of leakage is proportional to the amount of damage. $^{51}$Cr decays with the release of gamma radiation, the amount of leakage, and hence the degree of cell lysis, can therefore be quantified using a gamma counter. Since there is natural leakage of chromium from undamaged cells, the useful period of the assay is limited to about 6 hours.

### 3.5.2 LDL and cell proliferation

Some reports have suggested that both native and oLDL enhance smooth muscle cell proliferation, a phenomenon which may further contribute to its atherogenic actions (Stiko-Rahm et al 1992, Harris Hooker et al 1992). An investigation of the effect of native and modified LDL on human arterial smooth muscle cell and endothelial cell growth by 5-bromo-2'-deoxyuridine (BrDU) is the subject of Chapter 9.

The assay is based on the principle that BrDU is taken up by cells in the S phase of the cell cycle and incorporated into newly synthesised DNA in place of thymidine. Thus incubation of cells with BrDU allows the identification of those undergoing proliferation.

### 3.5.3 LDL and cell migration

Modified LDL appears to have diverse effects on cell migration, having been reported to be chemotactic for smooth muscle cells, but to inhibit migration in endothelial cells (Autio et al 1990, Murugesan et al 1993). Its effects on cell migration, using a method described by Thorne et al (1992b), are detailed in Chapter 9.
Following the demonstration of a subset of apparently migrating endothelial cells which express CD34 (described in Chapter 6), it may be hypothesised that CD34 might be acting as an adhesion molecule, allowing the migration of cells over the endothelial monolayer. The effects of native and modified LDL on endothelial CD34 expression are discussed in Chapter 9.
SECTION I: INTRODUCTION

CHAPTER 4

ADHESION MOLECULES

4.1 Introduction

Atherosclerosis is an inflammatory disease, and the role of leucocytes, in particular that of the monocyte, is becoming increasingly recognised. Early events in atherogenesis involve the accumulation of monocytes at the vascular endothelium and their transmigration to the subintima where they take up oxidised LDL to form foam cells.

Adhesion of leucocytes to the vessel wall, their subsequent migration from the circulation into the extracellular matrix and their communication with other cells is mediated by adhesion molecules. Adhesion molecules and their ligands comprise several classes; their classification, regulation and functions are discussed below.

4.2 The Selectins

This family of transmembrane adhesion molecules comprises E-selectin, P-selectin and L-selectin.

The nomenclature of the selectin family has been recently simplified; E-selectin was previously referred to as ELAM-1 (endothelial leucocyte adhesion molecule-1), and P-selectin, or CD62, has also been referred to as PADGEM and GMP-140 (granule membrane protein-140). L-selectin has been known by a number of names: LECAM-1 (leucocyte endothelial cell adhesion molecule-1), GP-MEL-14, LAM-1, LY22, LEU-8, TQ-1 and DREG 8.

All 3 molecules are encoded within a short stretch of chromosome 1 (Watson et al 1990). Their structure is schematically illustrated Figure 4.1.
Figure 4.1 Selectin structure: schematic representation.
Binding occurs at the extracellular lectin-like N-terminal.
CRP: complement regulatory protein, EGF: epidermal growth factor motif
(Adapted from Springer 1990)

P-selectin is present in the α-granules of platelets and in endothelial cell storage organelles, Weibel Palade bodies. Endothelial cell degranulation is stimulated in vitro by rapidly acting agonists (thrombin, histamine) and hydrogen peroxide, resulting in the immediate transient translocation of P-selectin to the endothelial cell membrane (McEver 1990, Patel et al 1991).

E-selectin is expressed exclusively by activated endothelial cells; its in vitro expression is induced de novo by cytokines (TNFα, IL-1) or bacterial
lipopolysaccharides, being detectable within 2 hours of stimulation, and with maximal expression occurring at 8 hours (Bevilacqua et al 1987).

The lectin domain of the selectins binds to sialylated fucosylated oligosaccharide ligands by a calcium dependent process. The molecular identities of the selectin ligands remain largely undefined, although the Sialyl-Lewis^ antigen acts as a ligand for E-selectin, and vascular addressins are ligands for L-selectin (Pardi et al 1992). Ligands for P-selectin are expressed on neutrophils, monocytes and platelets, and for E-selectin, on neutrophils and monocytes (Springer 1990).

The function of the selectins appears to be in mediating the initial rolling and low affinity adhesion of unactivated leucocytes to the endothelium (Lawrence et al 1991, Williams 1991, Zimmerman et al 1992).

**4.3 The integrins**

The integrin family of adhesion molecules comprise 2 non-covalently linked protein sub-units: an α and a β chain. The associations of the several different β and α subunits may confer specificity of function on these molecules. The subunits of an integrin molecule associate to form a small cytoplasmic C-terminal, a transmembrane segment and a large extracellular N-terminal domain (Albelda et al 1990), as illustrated in Figure 4.2.
Integrins are expressed on many cell types, including unstimulated leucocytes and endothelial cells. They bind to two groups of ligands: the immunoglobulin superfamily of adhesion molecules, and to extracellular matrix components, thus mediating both cell-cell and cell-substratum interactions.

In facilitating cell-substratum adhesion, integrins mediate an interaction between the cytoskeleton and extracellular matrix components, including fibronectin, collagen, and von Willebrand factor (Albelda et al 1990). The extracellular N-terminal of the integrin β sub-unit binds to the extracellular matrix resulting in changes in the
cytoplasmic C-terminal of the integrin which cause an alteration of the cytoskeleton. This interaction is required for the initiation and maintenance of endothelial spreading on extracellular matrix, preservation of endothelial monolayer integrity and control of vascular permeability (Dejana et al 1990, Lampugnani et al 1991).

Leucocyte integrins mediate adhesion to endothelial cells by binding to the immunoglobulin superfamily of adhesion molecules, as shown in the Table 4.i. The immunoglobulin binding site is distinct from that which binds to extracellular matrix components. Although constitutively expressed, the integrins may be transiently functionally upregulated, or activated, by phorbol esters, chemotactic factors and PAF (platelet activating factor). In addition, activated leucocytes produce an integrin modulating factor, which is thought to be involved in the activation of the MAC-1 integrin receptor (Hermanowski-Vosatka et al 1992). The mechanism by which functional upregulation occurs has been studied in the CD11a/CD18 integrin; activating factors cause conformational change in the cytoplasmic domain of the integrin via a calcium-dependent protein kinase C (PKC) pathway. PKC mediates phosphorylation of both the integrin cytoplasmic domain and of cytoplasmic proteins, resulting in polymerisation of actin and the polarisation of the leucocyte cell membrane which is in contact with the apposing cell membrane. This chain of events produces enhanced avidity of the integrin for its immunoglobulin ligands, ICAM-1 and -2 (intercellular adhesion molecule-1 and 2) (Pardi et al 1992, Zimmerman et al 1992).

Integrins of particular interest to this discussion are LFA-1 (lymphocyte function associated antigen-1: CD11a/CD18), expressed by leucocytes (Marlin et al 1987), Mac-1 (CD11b/CD18), present on monocytes, neutrophils and some lymphocytes (Diamond et al 1991), and VLA-4 (very late activation antigen: CD49d/CD29), present on mononuclear cells (Hemler et al 1987). The interactions of the integrins and their counter receptors are detailed in Table 4.i.
4.4 The immunoglobulin superfamily

These adhesion molecules share structural homology with antibodies (Springer 1990). ICAM-1, -2 and -3 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule) and PECAM (platelet endothelial cell adhesion molecule) are discussed below; the class also includes LFA-2 and -3 (lymphocyte function associated antigen; CD2 and CD58, respectively), CD4, CD8, and the major histocompatibility complex classes I and II. The structure of the immunoglobulin adhesion molecules is illustrated in Figure 4.3.

As mentioned above, 3 forms of ICAM have been cloned. ICAM-1 (CD54) is expressed on endothelium and upregulated by cytokines (Simmons et al 1988). It has also been demonstrated on keratinocytes, fibroblasts, haemopoetic cells and vascular smooth muscle in atherosclerotic plaques (Poston et al 1992). Its ligands are the integrins LFA-1 and Mac-1 which bind to separate immunoglobulin domains on ICAM-1 (Marlin et al 1987, Diamond et al 1991).

ICAM-2 (Staunton et al 1989) is constitutively expressed by the endothelium and mononuclear cells, is not upregulated by cytokines, and binds to the ligand LFA-1. ICAM-3 has been described recently; it appears not to be inducible on HUVECs; it is present on haemopoetic cells, and probably on the endothelium of some tumours; its ligand is LFA-1 (de Fougerolles et al 1992).

VCAM-1 exists in 2 forms: full length (Polte et al 1991), and alternatively spliced: VCAM-1 (alt) (Cybulsky et al 1991a). Both forms are expressed by activated, but not quiescent, endothelium, and bind to the ligand VLA-4. The expression of VCAM-1 on smooth muscle cells is the subject of Chapter 11.
N terminal extracellular domain

immunoglobulin domain:
anti-parallel β pleated sheets with central disulfide bond

C terminal cytoplasmic domain

Figure 4.3. Immunoglobulin superfamily adhesion molecule structure: schematic representation
Binding to integrin ligands is mediated by the immunoglobulin domains
(adapted from Springer 1990)

Cytokines and bacterial lipopolysaccharide induce the synthesis and expression of ICAM-1 and VCAM-1. In addition, the expression of ICAM-1, VCAM-1 and E-Selectin is differentially regulated by IFN-γ (interferon-γ) and IL-4 (interleukin-4): IFN-γ and IL-4 selectively induce ICAM-1 and VCAM-1 respectively, thus allowing selective control of leucocyte adhesion (Thornhill et al 1991).

As discussed in Chapter 1, PECAM (CD31) is constitutively expressed by the endothelium, and by platelets and myeloid cells (Albelda et al 1991). Its possible role
in the migration of circulating leucocytes to the intima is discussed below. A ligand for PECAM has not yet been defined, but it is speculated to capable of binding both to itself and to a separate ligand (Muller WA et al 1992). Table 4.i summarises the cells on which these adhesion molecules are expressed, and the integrin ligands to which they bind.

<table>
<thead>
<tr>
<th>INTEGRIN</th>
<th>INTEGRIN DISTRIBUTION</th>
<th>IMMUNOGLOBULIN LIGAND OR OTHER COUNTER RECEPTOR</th>
<th>IMMUNOGLOBULIN DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1 (CD11a/CD18)</td>
<td>leucocytes</td>
<td>ICAM-1</td>
<td>endothelial cells, mononuclear cells smooth muscle cells keratinocytes chondrocytes</td>
</tr>
<tr>
<td>Mac-1 (CD11b/CD18)</td>
<td>monocytes neutrophils some lymphocytes</td>
<td>ICAM-1</td>
<td>endothelial cells monocytes some lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>lymphocytes</td>
</tr>
<tr>
<td>VLA-1&amp;2</td>
<td>mononuclear cells</td>
<td>ICAM-1 Factor VIII Lipopolysaccharide</td>
<td></td>
</tr>
<tr>
<td>(CD49a/CD29, CD49b/CD29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-3</td>
<td>mononuclear cells</td>
<td>Laminin Collagen</td>
<td></td>
</tr>
<tr>
<td>(CD49c/CD29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-4</td>
<td>mononuclear cells</td>
<td>VCAM-1 Fibronectin</td>
<td>endothelial cells macrophages epithelial cells myoblasts</td>
</tr>
<tr>
<td>(CD49d/CD29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-5</td>
<td>mononuclear cells</td>
<td>Fibronectin</td>
<td></td>
</tr>
<tr>
<td>(CD49e/CD29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLA-6</td>
<td>mononuclear cells</td>
<td>Laminin</td>
<td></td>
</tr>
<tr>
<td>(CD49f/CD29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD51/CD61</td>
<td>leucocytes</td>
<td>Vitronectin Factor VIII Fibrinogen Thrombospondin</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.i. Leucocyte integrins and their counter receptors

The integrin family mediates leucocyte adhesion both to other cells, via immunoglobulin superfamily receptors (designated in **bold**), and to extracellular matrix components (designated in *italics*).
4.5 Mechanisms of leucocyte adhesion

The migration of leucocytes from the circulation to the tissues is mediated by the dynamic interaction between adhesion molecules expressed by leucocytes and the endothelium. The process involves 4 steps: margination, rolling, firm adhesion, and extravasation (Hogg 1992).

Within minutes of endothelial stimulation by early inflammatory mediators, such as thrombin, leucocytes leave the main stream of flow and marginate. Marginating leucocytes are rapidly captured and start to roll along the vessel wall, a process requiring the continual adhesion to, and detachment from, the endothelium. This is thought to be mediated by the selectins, initially P-selectin (Lorant et al 1991), the expression of which does not require protein synthesis. Thrombin induces the translocation of P-selectin to the endothelial surface within minutes, resulting in the initial arrest and rolling of leucocytes on the endothelium. Endothelial expression of P-selectin is transitory, its maximum expression occurring between 10 and 30 minutes. The interaction is continued by the induction of E-selectin by inflammatory cytokines; its induction is maximal at 2-4 hours, and extends to 6 hours. The rapid binding kinetics of the selectin receptors enable the selectins to mediate the initial rolling of leucocytes (Williams et al 1991).

The downregulation of selectins by proteolytic cleavage is required before the tighter adhesion of leucocytes to the endothelium is mediated by integrin-immunoglobulin interactions, also resulting in flattening of the leucocyte on the endothelial surface (Lawrence et al 1991).

For T lymphocytes and monocytes, the main integrins involved are VLA-4 and LFA-1, functionally upregulated by inflammatory mediators. The former interacts with the constitutively expressed immunoglobulin, ICAM-2, which may be important for initiating firm adhesion. ICAM-1, and VCAM-1, the ligand for VLA-4, are
upregulated over 12-24 and 4-10 hours, respectively. Thus rolling leucocytes are arrested and adhere firmly to the activated endothelium.

The leucocyte then migrates between endothelial cells into the intima. The molecular mechanism for extravasation is unknown. However, the ICAM-1/LFA-1 interaction appears necessary (Hakkert et al 1991), and CD31 (PECAM) may also be involved, being expressed with high density at endothelial cell junctions (Albelda et al 1991).

4.6 Monocyte adhesion and atherosclerosis

The possible role of endothelial cells adhesion molecules in recruiting monocytes and T lymphocytes to the intima in atherogenesis, and their interactions with oxidatively modified LDL is the subject of Chapter 10. The expression of adhesion molecules on vascular smooth muscle and the implications for monocyte adhesion in atherogenesis is discussed in Chapter 11.

The adhesion molecules implicated in monocyte adhesion are illustrated in Figure 4.4.
Figure 4.4. Monocyte-endothelial interactions

The figure represents a monocyte within a blood vessel. The endothelial immunoglobulin ligands, VCAM-1 and ICAM 1-3 are shown to interact with their corresponding integrin ligands expressed on the monocyte, and the endothelial selectins with their oligosaccharide ligands. The ligand for PECAM, expressed by both monocytes and endothelial cells, may be PECAM itself.
SECTION I: INTRODUCTION
CHAPTER 5
ATHEROSCLEROSIS

5.1 Natural history of atherosclerosis

From infancy, the human coronary arterial intima contains smooth muscle cells, unlike many of the species used as animal models of atherosclerosis (Stary 1989). This finding must be considered normal; it does not represent the early stages of atherosclerosis. However the presence of smooth muscle cells within the intima may provide a background on which atheroma can develop in later life.

The earliest identifiable lesion in the atherogenic process is the fatty streak, an intimal lesion present in elastic and muscular arteries from childhood. It comprises smooth muscle cells, T lymphocytes and macrophages; the latter cell type also exists as lipid laden foam cells (Stary 1989, Tsukada et al 1990). Fatty steaks have a predilection for sites of low shear stress, as do more atheromatous advanced lesions (Caro et al 1971). However, controversy remains as to whether fatty streaks are the precursor of frankly atherosclerotic lesions; it is likely that at least some fatty streaks may regress, whereas others progress to become atheromatous plaques.

The next stage in the development of the atherosclerotic plaque is the accumulation of extracellular lipid, probably as a result of foam cell death, and the plaque becomes raised. Subsequently smooth muscle cells migrate and proliferate in the intima, secreting an extracellular collagen matrix to form an elevated fibrolipid plaque. The endothelium often remains at least morphologically intact, covering an extracellular lipid core surrounded by foam cells and smooth muscle cells in a collagen matrix, separating the plaque from the media.
The response to injury hypothesis (Ross 1986) states that endothelial damage, whether resulting in denudation or more subtle injury causing dysfunction or activation, as discussed in Chapter 1, is a key event in the initiation of atherosclerosis. In addition, the atherosclerotic process may be considered to have a physiological purpose in protecting the arterial wall from injury, whether haemodynamic, as in hypertension, or from smoking, hyperlipidaemia or diabetes. Thus macrophage foam cell formation provides a mechanism whereby the artery is protected from toxic lipids, platelet thrombi overlying focal areas of endothelial denudation maintain haemostasis, and the migratory and proliferative behaviour of smooth muscle cells strengthens the intima at sites of haemodynamic stress and lipid deposition. It is under the onslaught of repeated and chronic insult that atherosclerosis progresses, and is manifest as clinical disease.

As the lipid content of the plaque increases, the fibrous cap may become less resistant to mechanical stress, and rupture, exposing the thrombogenic plaque contents to the circulation (Richardson et al 1989). The subsequent formation of intravascular thrombus may result in an episode of unstable angina or myocardial infarction.

The contributions to the development of the plaque by its constituent cell types and lipid (which includes oxidised LDL) are discussed below. Particular emphasis is placed on the formation of foam cells: perceived as a key event in atherogenesis.

5.2 Foam cell formation

The formation of foam cells is one of the initiating, albeit reversible, events in atherogenesis. Lipid laden foam cells are present in the intima from the earliest stages of the disease; they derive mainly from circulating monocytes, but also from smooth muscle cells (Stary 1989, Tsukada et al 1990).
There are four stages to consider in the formation of foam cells: chemoattraction of circulating monocytes, their adhesion to the endothelium, transmigration to the intima, and the uptake of modified LDL.

5.2.1 Monocyte chemoattraction

The attraction of circulating monocytes to the intima is the first step in the formation of foam cells. Monocytes are borne passively in the circulation, and in regions of high shear stress are unlikely to have enough contact time with the vessel wall for cell-cell interactions to occur. The chances of monocyte-endothelial cell contact are increased in regions of low shear stress, such as vessel bifurcations, and it may be this which is one of the most important factors in permitting monocyte recruitment and determining the site of plaque formation.

Monocyte chemoattraction appears to be a process specific to monocytes, and experimental evidence suggests it may be due to the action of modified LDL on the endothelium. Lysophosphatidylcholine, a component of oLDL and mmLDL, specifically induces monocyte migration to, and reduces egress from, the intima (Quinn et al 1988). Modified LDL mediates these actions by inducing the expression of MCP-1 (monocyte chemotactic protein-1) in endothelial and smooth muscle cells (Cushing et al 1990, Navab et al 1991). Endothelial cells, monocytes and smooth muscle cells also secrete MCP-1 in response to cytokines and LPS (Rollins et al 1990, Colotta et al 1992). Supporting evidence for the role played by MCP-1 in monocyte recruitment includes the demonstration of its mRNA in human atheromatous plaques (Nelken et al 1991).

Other factors are involved in monocyte attraction. These include TGFβ (Wahl et al 1987), the extracellular matrix component, fibronectin (Norris et al 1982), and glycosylation end products which accumulate in the vessel wall of diabetic patients (Kirstein et al 1990). As discussed in Chapter 1, the endothelium also regulates
leucocyte-vessel wall interactions by expressing inhibitors of chemotaxis such as NO and prostacyclin.

5.2.2 Monocyte adhesion

Monocyte adhesion to the vascular endothelium has been observed in human post mortem sections early in the atherogenic process (Faruqui et al 1993), and in animal models. The endothelium can be activated by a variety of substances to induce monocyte adhesion in vitro. These include bacterial lipopolysaccharide, the cytokines IL-1 and TNF (Bevilacqua et al 1985), thrombin (Di Corleto et al 1989) and certain viruses (Span et al 1989). The latter observation suggests a possible role for viral infection in the pathogenesis of atherosclerosis.

A variety of modifications of LDL cause monocyte adhesion to endothelial cells. These include copper oxidised LDL (Frostegård et al 1990) and the more physiologically minimally modified LDL (Berliner et al 1990). High ('atherogenic') concentrations of native LDL have also been reported to enhance the adhesion of monocytes to the endothelium (Pritchard et al 1991); however this phenomenon may be due to the cellular modification of LDL. In addition, enhanced leucocyte adhesion to arterioles in the presence of oLDL has been observed by intervital microscopy (Lehr et al 1991).

Several lines of investigation implicate modified LDL in the adhesion of monocytes to the endothelium in atherosclerosis. As discussed in Chapter 3, oLDL is present in human atherosclerotic plaques, and plasma LDL concentrations are directly related to the risk of atheromatous disease. Similarly, circulating antioxidants, which may protect native LDL from oxidative modification to its more atherogenic form, are inversely related to the risk of atherosclerosis. In addition, it is mononuclear cells (monocytes and T lymphocytes), not polymorphonuclear leucocytes, which are recruited to the intima in atherosclerosis. Therefore, modified LDL, which induces
mononuclear but not polymorphonuclear leucocyte adhesion (Berliner et al 1990), is more likely to be involved in promoting monocyte adhesion than inflammatory mediators such as lipopolysaccharide and cytokines, the actions of which are non-specific.

The mechanism by which modified LDL enhances monocyte adhesion to the endothelium is not known; the role of adhesion molecules is under investigation. The adhesion molecules E-selectin, VCAM-1 and ICAM-1 are inducible on the endothelial surface by cytokines, and can support monocyte adhesion (Carlos et al 1991). In addition, the adhesion molecules ICAM-1 and VCAM-1 are expressed in atherosclerotic plaques (Poston et al 1992, Cybulsky et al 1991b). Furthermore, lysophosphatidylcholine, a component of oLDL, stimulates endothelial ICAM-1 and VCAM-1 expression and monocyte adhesion in cell culture systems (Kume et al 1992). An investigation of endothelial adhesion molecule expression and monocyte adhesion is the subject of Chapter 10.

Since some endothelial denudation occurs early in atherogenesis (Davies et al 1988), intimal smooth muscle cells may be exposed to the circulation. ICAM-1 expression has been demonstrated in the smooth muscle cells wall of human atherosclerotic plaques (Poston et al 1992). Furthermore, cytokines have been shown to induce smooth muscle cell adhesion molecule expression both in vitro and in vivo. Stemme et al (1992) demonstrated that cytokine-induced adhesion of T lymphoblasts to cultured uterine artery smooth muscle cells was partly mediated by the induction of ICAM-1 expression on the smooth muscle cells. Briscoe et al (1992) reported the in vivo induction of VCAM-1 expression in the smooth musculature of baboon skin.

Whether human coronary artery smooth muscle cells are capable of supporting monocyte adhesion, or can be induced to express adhesion molecules, and the role of LDL in these processes, is the subject of Chapter 11.
5.2.3 Monocyte transmigration

Monocyte transmigration appears to take place at intercellular junctions, and occurs over a period of about an hour \textit{in vitro} (Valente \textit{et al} 1992). The mechanism is not fully understood, it may involve MCP-1 and CD18, the ligand for ICAM-1 (Navab \textit{et al} 1991, Hakkert \textit{et al} 1991).

5.2.4 Macrophage foam cell formation

As described in Chapter 3, once resident in the intima, the macrophage takes up oLDL via the scavenger receptor until it becomes a foam cell. It secretes smooth muscle cell growth and migratory factors, further oxidises intimal LDL, and when it dies, as a result of the cytotoxic effects of oLDL, releases atherogenic lipid to the extracellular compartment.

5.2.5 Summary

The possible interactions between LDL and the vascular endothelium in the development of foam cells are schematically represented in Figures 5.1 and 5.2, and summarised as follows. Native LDL enters the intima via the endothelial LDL receptor. Its lipid moiety is "minimally" modified by oxidants from endothelial cells and intimal smooth muscle cells. This process is inhibited by HDL (Navab \textit{et al} 1991) and the mmLDL is not recognised by the scavenger receptor, its protein moiety being unchanged. MmLDL induces endothelial expression of MCP-1 and a specific monocyte adhesion molecule. Monocytes subsequently enter the intima where they are activated by the mmLDL and differentiate into macrophages. The activated macrophages release reactive oxygen species, which further oxidise LDL. The highly oxidised LDL is taken up by the macrophage scavenger receptor to form foam cells. Other atherogenic actions of oLDL are considered in Chapter 3. The migration of smooth muscle cells to the intima, their proliferation and secretion of extracellular matrix, are discussed in Chapter 2, as is their role in coronary restenosis following intervention.
monocyte adhesion to endothelium

Figure 5.1. Minimally modified LDL and monocyte recruitment to the arterial intima. The actions of mmLDL include: (i) the induction of MCP-1 expression by endothelial and smooth muscle cells, resulting in chemoattraction of circulating monocytes. (ii) The induction of as yet undefined monocyte adhesion mechanisms on the endothelial surface. (iii) The release of smooth muscle cell migration and growth factors. MmLDL retains an intact apoB moiety and is therefore not recognised by the scavenger receptor. However, reactive oxygen species (ROS) released by macrophages, endothelial and smooth muscle cells further oxidise the LDL.
The properties of oLDL include: (i) a cytotoxic effect which may contribute to the focal endothelial denudation of early atherosclerosis and exposure of intimal smooth muscle cells to the circulation. (ii) Continuing attraction of circulating monocytes by endothelium and smooth muscle derived MCP-1 (not shown). (iii) The induction of undefined monocyte adhesion mechanisms on the endothelium and possibly on intimal smooth muscle cells. (iv) The recognition of the oxidatively modified apoB moiety of oLDL by macrophage scavenger receptors, resulting in the avid uptake of oLDL, and foam cell formation.
SECTION II: EXPERIMENTAL

CHAPTER 6

THE ENDO THEL IUM IN CULTURE

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells.

The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described.

In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported. Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL. An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells. The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 6
THE ENDOTHELUM IN CULTURE

6.1. Methods
All reagents used in this chapter were obtained from the Sigma Chemical Co., Poole, UK unless otherwise stated.

6.1.1 Isolation and culture of human endothelial cells
The techniques for the isolation and culture of both macro- and microvessel endothelial cells are described below. All cell culture techniques were performed using aseptic techniques in a Class II laminar airflow cabinet.

6.1.1.1. Umbilical vein endothelial cells
Human umbilical vein endothelial cells (HUVEC) were isolated by a modification of the method described by Jaffe et al in 1973.

Umbilical cords, stored dry at 4°C, were collected daily from the labour ward. The exterior of the cord was cleaned with chlorhexidine, and any sections damaged by needle punctures or clamps discarded. The vein was cannulated with two consecutive luer adapters and sealed with two cable ties and flushed gently with sterile phosphate buffered saline (PBS, Unipath Ltd, Basingstoke, UK) to remove blood. The other end of the cord was then sealed with a cable tie. The vein was filled with 0.05% type II collagenase solution (Sigma Chemical Co., Poole, UK) in HBSS (Hank's balanced salt solution, calcium and magnesium free, Gibco (Life Technology), Paisley, UK) and incubated for 15 minutes at 37°C. The collagenase solution and dissociated cells were subsequently removed by repeated passage in and out of the vein. The cell-containing solution was mixed 1:1 with Medium 199 (M199), supplemented with 20% foetal calf serum (FCS) (see Appendix I for full contents) and centrifuged at 400G for 5 minutes.
to sediment cells. The cell pellet was resuspended in M199 with 20% FCS, plated into a 25cm\(^3\) tissue culture flask and incubated at 37\(^\circ\)C in 95% air 5% carbon dioxide.

After 24 hours, the cells were washed 3 times with HBSS to remove debris, and the medium replaced. The medium was replaced every 3 days.

Confluent primary cultures were passaged as follows. Cells were washed 3 times in HBSS to remove traces of serum and then incubated for 2-3 minutes in 0.05% trypsin/0.02% EDTA solution (Gibco (Life Technology), Paisley UK). Trypsin is a non-specific protease which degrades the extracellular matrix resulting in cell detachment. Once cells had detached, the enzyme was inactivated by the addition of M199 with 20% FCS, and the cell suspension centrifuged at 400G for 5 minutes. Cells were resuspended in medium and split in a ratio of 1:4.

6.1.1.2 Aortic and endocardial endothelial cells
Sections of aorta obtained as operative specimens or from cardiac explants were incubated at 37\(^\circ\)C in type II collagenase solution. The luminal surface was gently scraped with a flamed Pasteur pipette to remove endothelial cells. The cell suspension was centrifuged, and further steps were performed as described above, except that the primary culture was plated onto a 35mm diameter tissue culture dish to allow weeding of non-endothelial cells, as described below. The technique to isolate endocardial endothelial cells from mitral valve leaflets or segments of ventricle was the same as that for aortic endothelial cells.

6.1.1.3 Coronary and internal mammary artery, and saphenous vein endothelial cells
Sections of coronary artery and saphenous vein obtained as operative specimens or from cardiac explants were dissected open along their long axis. Left internal mammary artery (LIMA) specimens dissected, but not used during coronary artery
bypass grafting were dissected free of muscle and connective tissue and also opened along their long axis. Further processing was performed as described for aortic endothelial cell isolation and culture.

6.1.1.4. Cardiac microvascular endothelial cells

This method to isolate and culture human cardiac microvascular endothelial cells utilised modifications of methods previously described (Diglio et al 1988, Gerritsen et al 1982, Marks et al 1985, Piper et al 1990) for animal cardiac and human non-cardiac microvessel endothelial cell culture, including a method devised within the Inflammation Research Unit for the isolation and culture of human synovial microvascular endothelial cells (Abbot et al 1992).

Fresh tissues were obtained from operative specimens (mitral valve papillary muscle, right atrial appendage and whole cardiac explants). Endocardial and epicardial surfaces, fibrous tissue and macroscopic blood vessels were excised. Right atrial appendage endocardium was rendered non-viable by immersion in absolute alcohol, followed by washing in Hank's balanced salt solution. Dissected tissue was minced into 2mm$^3$ and incubated for 60 minutes at 37°C in a 0.5% crude collagenase solution containing 100mM CaCl$_2$ to kill contaminating myocytes (see Appendix I). Capillary segments were expressed from the digest with a microspatula and the material pipette disaggregated. Following filtration through a 250μm mesh, the filtrate was centrifuged for 5 minutes at 400G and the pellet resuspended in M199 containing 40% FCS (see Appendix I), and plated out onto a 35 mm$^3$ culture dish. After 24 hours, the cells were washed and the medium replaced, thus removing debris and leaving the adherent endothelial cells.

6.1.2 Purification of endothelial cells in culture

Care was taken during the primary isolation procedure to avoid contamination with non endothelial cells. In addition, the routine additions of endothelial cell growth
factor (ECGS) and smooth muscle cell inhibitors such as heparin, to culture media, provided growth media selective for endothelial cells. However, intimal smooth muscle cell contamination sometimes occurred in cultures of endothelial cells derived from arteries.

Such cells were either weeded out manually, using flamed capillary tubing and an inverted microscope, or removed using Dynabeads. Dynabeads were used to purify mixed cell populations in conjunction with either the lectin *Ulex europaeus agglutinin-1* (UEA-1, Sigma), (Jackson *et al* 1990, Abbot *et al* 1992) or CD31.

Both methods are described below.

**6.1.2.1. Endothelial cell sorting using *Ulex europaeus agglutinin-1***

UEA-1 was covalently bound to Tosyl activated Dynabeads M-450 as follows. 4x10^8 beads ml^-1 were washed, using a magnet, in BBS pH 9.5 (Appendix II), and resuspended in 2ml BBS. 2ml UEA-1 (0.2mg/ml) were added to the beads and incubated for 24 hours at room temperature with end over end rotation. The beads, now coated, were collected with a magnet, the supernatant discarded and the beads washed for 4 x 15 minutes in sterile PBS. The beads were incubated overnight in 0.01M PBS containing 0.1% BSA (bovine serum albumin (Sigma)) before being collected using a magnet. Finally, the beads were resuspended in PBS/0.1% BSA and stored at 4°C in aliquots of 200μl (8x10^7 beads).

The application of UEA-1 coated Dynabeads to cultured cells was as follows. All solutions were maintained at 4°C, and incubations performed on ice throughout the procedure. Cells were trypsinised, resuspended in HBSS, counted using a haemocytometer, and beads added in a ratio of 5 beads to 1 cell. The cell/bead suspension was incubated on ice for 10 minutes, inverting every 5 minutes. Endothelial cells with bound beads were then retained in a magnetic field, and non-
endothelial cells decanted. The endothelial cells attached to the beads were subsequently plated down and cultured.

Although a method to detach the beads from endothelial cells has been described (Jackson et al 1990), it was not found to be reproducible. Therefore the cells were cultured with beads still attached. In order to assess whether the continued presence of beads resulted in endothelial cell activation, E-selectin expression was quantified in cells both with, and without, attached beads, using the ELISA technique described in Chapter 10.

6.1.2.2. Endothelial cell sorting using antibody to CD31
To purify cell populations using anti-CD31 antibody, cells were trypsinised, washed, and resuspended in 1ml HBSS. All solutions were maintained at 4°C, and incubations performed on ice throughout the procedure. 1μl/100 000 endothelial cells of mouse monoclonal anti-human CD31 IgG immunoglobulin (1mg/ml, British Biotechnology Products Ltd, Abingdon, UK) was added and the cell suspension incubated for 30 minutes, gently inverting every 10 minutes. The volume was increased to 10ml with HBSS, and the suspended cells washed twice before being resuspended in 1ml HBSS. Dynabeads® M-450 coated with sheep anti-mouse IgM were added to achieve a ratio of approximately 5 beads: 1 endothelial cell. The subsequent incubation and separation of attached endothelial cells from non attached, non endothelial cells were performed as described above.
6.1.3 Characterisation of endothelial cells

The methods for the following criteria for endothelial cell characterisation are detailed below.

i) Development of cobblestone morphology.

ii) *in vitro* differentiation: capillary tubule formation

iii) Uptake of fluorescent acetylated LDL

iv) Binding to *Ulex europaeus* agglutinin-1

v) CD31 expression (PECAM)

vi) CD34 expression (QB-END/10 immunocytochemistry)

vii) Immunohistochemical localisation of von Willebrand factor.

viii) Detection of von Willebrand factor secretion

ix) Induction of E-selectin

6.1.3.i. Development of cobblestone morphology.

Cultured cells were allowed to reach confluence and observed for the formation of the characteristic cobblestone morphology of confluent endothelial cells.

6.1.3.ii. *in vitro* differentiation

Endothelial cells may also be characterised by *in vitro* differentiation to form capillary-like tubules (Folkman *et al* 1980) when cultured on basement membrane preparations such as Matrigel™ (Beckton Dickenson, Cowley, UK). Matrigel is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma. This tumour is rich in extracellular matrix proteins; Matrigel contains laminin, type IV collagen, heparan sulphate proteoglycans, growth factors and tissue plasminogen activators (Kleinman *et al* 1982, McGuire *et al* 1989).

Cells were incubated on a thick film of Matrigel, as follows. Matrigel gels rapidly and irreversibly at 22°C; frozen aliquots were therefore thawed and prepared at 4°C, with
equipment at 4°C. 100μl/cm² was added to tissue culture wells. After incubation for 30 minutes, endothelial cells were plated onto the gel and incubated overnight to allow in vitro differentiation (tubule formation) to occur.

6.1.3.iii. Uptake of fluorescent acetylated LDL
Endothelial cells show scavenger receptor-mediated uptake of oxidised or acetylated LDL, providing a useful method to identify the nature of cells growing in culture (Wharton et al 1990).

Fluorescently labeled acetylated LDL (Ac-dil-LDL, Biogenesis Ltd, Bournemouth, UK) was diluted to 10μg/ml in standard medium. Putative endothelial cells were incubated in the medium for 12 hours at 37°C, and HUVEC included as a positive control. The cells were then washed 3 times in HBSS at 37°C and probe-free medium replaced. Cells were visualised by fluorescence microscopy using a rhodamine excitation: emission filter.

6.1.3.iv. Binding to Ulex europaeus agglutinin-1
A further reliable means of identifying vascular endothelium is by binding to the lectin Ulex europaeus 1 (UEA 1) which allows for sensitive immunolocalisation of the lectin to L-fucose on the endothelial cell membrane (Wharton et al 1990). As discussed in Section 6.1.2.1, when bound to magnetic beads, this lectin may be used to extract and characterise endothelial cells in culture.

6.1.3.v. CD31 expression (PECAM)
All endothelial cells express CD31 (PECAM) (Page et al 1992); they bind monoclonal antibodies directed against CD31 in culture, allowing the extraction of pure, characterised endothelial cells as described in Section 6.1.2.2.
6.1.3. vi. CD34 expression (QB-END/10 immunocytochemistry)

QB-END/10 is a monoclonal antibody raised against HUVECs stressed to induce membrane blebbing; it recognises the CD34 antigen. CD34 is expressed by haemopoetic progenitor cells and is also present on the abluminal surface of endothelium: it is used as an endothelial marker in tumour histology (Anthony et al. 1991).

Endothelial cells were studied at different stages of confluence, and after stimulation by TNFα, native and modified LDL, wounding of the monolayer (all detailed in Chapter 9).

Cells on Thermanox (Nunc Inc., Naperville, Illinois, USA) coverslips were fixed for 10 minutes in 10% formol saline at 37°C, washed in TBS (tris buffered saline, see Appendix II) and fixed for a further 30 seconds in acetone. Cells were washed 3 times between each stage, in TBS. It was found to be unnecessary to quench endogenous peroxidase in endothelial monolayers, for this or any immunocytochemistry procedures discussed in this thesis. Non-specific binding sites were blocked by 20 minutes incubation in normal goat serum (Dakopatts, High Wycombe, UK) diluted 1:5. The primary mouse monoclonal antibody to CD31: QBEND/10 (Serotec, Kidlington, UK), was applied in a 1:50 dilution overnight. This was followed by amplification using a biotinylated secondary antibody (goat anti mouse IgG, 1:50 dilution) and avidin/biotin horseradish peroxidase complex (both Dakopatts). Coverslips were developed in nickel-diaminobenzidine (DAB, Sigma) peroxidase substrate solution (Appendix II), counterstained in Carazzi's haematoxylin, dehydrated and mounted.

6.1.3. vii. Immunocytochemical localisation of von Willebrand factor.

A method frequently used in the characterisation of endothelial cells is the demonstration of von Willebrand factor (vWF) (Jaffe et al. 1974) immunoreactivity.
vWF is synthesised in the endothelium and stored in organelles known as Weibel-Palade bodies which produce a characteristic granular staining pattern on light microscopy. vWF is also present in platelets, megakaryocytes and mast cells, unlikely contaminants of endothelial cell cultures. Although it may also be expressed by cultured mesothelial cells, the staining pattern exhibited is not granular, but faint and diffuse (van Hinsberg et al 1990). However, although endothelial cells derived from human large vessels contain Weibel-Palade bodies, they are frequently not demonstrable in human microvessel endothelial cells (Piper et al 1990).

Cells were fixed for 10 minutes in 10% formol saline at 37°C. Cells were washed 3 times, as between each between each stage, in TBS. They were incubated in primary antibody: rabbit polyclonal immunoglobulin to vWF (Dakopatts, High Wycombe, UK) diluted 1:1200 in 1.5% normal goat serum/TBS, followed by biotinylated goat anti-rabbit IgG (Vector Laboratories, Peterborough, UK). Following amplification with avidin-biotin horseradish peroxidase complex (Vector Laboratories), cells were developed in DAB peroxidase substrate solution (Appendix II).

6.1.3.viii. Detection of von Willebrand factor secretion

The functional characteristics of endothelial cells in culture were also assessed by their constitutive release of vWF, mediated by Weibel-Palade body exocytosis, a process enhanced by stimuli such as thrombin and PMA (phorbol 12-myristate 13-acetate, Sigma) (Jaffe 1988). Secretion of vWF by resting and stimulated endothelial cells in culture was quantified by ELISA, thus characterising cells functionally as well as by immunocytochemistry.

In addition, a cell blot technique to assess vWF secretion by individual cells was performed. Cell blot analysis employs the principle of Western blotting to detect peptide secretion by individual cells. Thus cell populations may not only be assessed for purity by the secretion of characteristic peptides, but also for functional integrity.
a) By enzyme linked immunosorbent assay (ELISA)

Stimulation of cells by phorbol 12-myristate 13-acetate

The phorbol ester PMA was used to stimulate vWF release from endothelial cells. Cells were incubated in fresh medium or 200nM PMA for 2-6 hours before the assessment vWF released by ELISA. Statistical analyses were performed by one way analysis of variance (ANOVA).

ELISA

A 96 well plate was coated with 100μl per well of rabbit polyclonal immunoglobulin to vWF (Dakopatts) diluted 1:1000 in 0.05M carbonate buffer, pH 9.6 (see Appendix II). After 1 hour incubation in a moist chamber at room temperature, the plate was washed 3 times with BBS pH 7.5/tween 0.5ml/l (see Appendix II), as between each stage, and tapped dry. Standards were prepared from normal pooled human plasma diluted in BBS/tween 1ml/l: 1:40-1:320, and applied to the coated plate. Medium was aspirated from each well of the cell containing plate and also transferred to the antibody coated plate. After 1 hour incubation in a moist chamber at room temperature, the plate was washed (as between each stage) with BBS/tween 0.5ml/l and tapped dry. The plate was then incubated for 1 hour in 100μl 1:1000 peroxidase conjugated rabbit immunoglobulins to vWF (Dakopatts). After washing, wells were rinsed once in 0.1M citrate buffer pH 5 (see Appendix II) and tapped dry before being developed in OPD (1,2 ortho phenylenediamine dihydrochloride, Sigma) substrate solution. Dual wavelength readings were performed at 405nm (reference) and 492nm.

b) By cell blot assay

Cell blot analysis was performed in collaboration with Prof JM Polak (Histochemistry Unit, Royal Postgraduate Medical School, London). The cell blot assay was originally described for the detection of prolactin release from mixed cell systems of isolated rat anterior pituitary cells (Kendal et al 1989). The assay has been further developed to detect a variety of other anterior pituitary derived peptides (Cimini et al 1991).
translucent hydrophobic polyvinylidene difluoride (PVDF) membrane was used, allowing the immunostained "halo" of absorbed peptide around each secreting cell to be visualised microscopically and quantified by densitometry (McBride et al 1990). The size and optical density of the halo is proportional to the quantity of peptide released and bound to the membrane.

Dot blots of purified antigen were used as standards for the cell blot assay using a modification of the technique described by Arita et al 1991. Briefly, purified human plasma vWF (a gift from Dr Harrison, Rayne Institute, St Thomas' Hospital, London) was diluted to 50-250 pg/μl in distilled water and 2μl dots incubated on the PVDF membrane for 1 hour. Following air-drying, the membranes were processed as described below for the cell blots.

Strips of hydrophobic polyvinylidene difluoride (PVDF) membrane (Pluskal et al 1986) were placed onto glass slides in humidified Petri dishes. Endothelial cells were passaged and resuspended in M199 with the addition of 20% complement inactivated foetal calf serum. Fibroblasts in DMEM provided a negative control. Cells were plated onto the membrane in 80μl drops, containing 1000-3000 cells per drop.

Two alternative methods of applying cells to the PVDF membrane were also investigated. To this end, the membrane was pre-wetted by immersion in methanol followed by washing in distilled water and equilibration for 1 hour in medium prior to the application of cells. A further method, a modification of that described by Arita et al in 1991, avoided the direct application of cells to the membrane. The membrane was pre-wetted and equilibrated as described above. Subconfluent cells on Thermanox coverslips were placed on top of the membrane such that the cells, and 20μl of medium, were in contact with the membrane. Thus vWF secreted by the cells would be absorbed by the membrane.
Cells were incubated overnight at 37°C in 5% CO₂, 95% air to allow adhesion. Shorter incubation times (6 hours) resulted in poor cell adhesion to the membrane, and no difference in subsequent background staining. Following this pre-incubation period, control cells were subject to a further 2-4 hours incubation, or stimulated with 0.1-1 IU/ml bovine thrombin (Diagnostic Reagents Ltd, Thame, UK) to increase vWF secretion.

Subsequent steps were performed with care to avoid detachment of cells. The medium was aspirated off, and the cells washed thrice with PBS, as between each step. Endogenous peroxide was quenched with 0.15% phenylhydrazine in PBS for 30 minutes and cells incubated for 4 hours in 6% bovine serum albumin BSA/PBS to block non-specific binding sites.

Cells were incubated overnight in rabbit polyclonal immunoglobulins to human vWF (Dakopatts) diluted 1-16 000 in 3% BSA/PBS. Omission of the primary antibody, or antibody absorbed with 1nmol purified human vWF provided negative controls. They were then incubated in 1:500 goat anti-rabbit immunoglobulins for 2 hours, followed by a further 2 hours in 1:1000 rabbit peroxidase anti-peroxidase (PAP) (both Dakopatts). After washing in 0.1M acetate buffer pH6, they were developed using nickel-DAB substrate solution (Appendix II) and air dried.

6.1.3.ix. Induction of E-selectin

The adhesion molecule E-selectin is expressed exclusively by activated endothelial cells; its in vitro expression is induced by cytokines and bacterial lipopolysaccharide (Bevilacqua et al 1987). The activation state of endothelial cells in culture may therefore also be assessed by the measurement of E-selectin expression.

Endothelial cells were grown to confluence on a 96 well plate in standard medium. Arterial smooth muscle cells provided a negative control. Cells were incubated for 4
hours at 37°C in 10% serum containing medium or lipopolysaccharide (0.01-10μg/ml Sigma), with dilutions being performed in triplicate. The medium was removed and the cells fixed immediately, and the ELISA performed as described in Chapter 10.

6.2 Results

6.2.1 Isolation and culture

High levels of purity (~99% endothelial cells) were achieved in primary cultures of HUVECs. Routinely cultured HUVECs therefore provided a readily available source of pure endothelial cells.

Surgical handling of vessels obtained during vascular surgery sometimes resulted in removal of the endothelium. Success rates for the isolation of endothelial cells, from saphenous vein and aorta in particular, were therefore lower than for the umbilical vein. Cells isolated from saphenous vein showed endothelial purity comparable to that of the umbilical vein. Isolation of endocardial endothelial cells was achieved from >90% of specimens.

Primary cultures from endocardium, coronary artery and aorta frequently comprised mixed cell populations. In the case of the endocardium, contaminating cells were usually fibroblasts, especially when the source tissue was valvular. Coronary arterial and aortic cultures were frequently contaminated with smooth muscle cells, even when there was no macroscopic evidence of atherosclerosis. Results of purification procedures are discussed below.

The growth requirements of umbilical and saphenous vein, endocardial and aortic endothelial cells were similar. Coronary artery endothelial cells in culture were more fastidious, being unable to withstand short periods (<4 hours) in serum free conditions
without detaching. Therefore all experiments were performed in at least 10% serum containing medium.

Although no morphological signs of senescence were observed in endothelial cell cultures up to the 5th passage, cultures beyond the 3rd passage were not used. Thus cells which might have begun to senesce were excluded from adhesion and LDL studies.

A total of 6 left internal mammary artery (LIMA) specimens were obtained for endothelial cell culture, but the yield of endothelial cells was insufficient for their establishment in culture.

The isolation of microvascular endothelial cells from papillary muscle, left ventricle and right atrial appendage had a low success rate. An endothelial cell yield sufficient to become established in culture was achieved for only 2 papillary muscle and 1 ventricular specimens. The success rate for atrial appendages was higher, with ~30% of specimens providing enough cells to culture and characterise. However, the cell yield was low: 100-300 endothelial cells per atrial appendage.

Microvascular endothelial cells had more fastidious growth requirements than those derived from large vessels or endocardium, needing 40% foetal calf serum and three fold higher concentrations of endothelial cell growth factor. Such growth requirements are typical of capillary endothelial cells in culture (Davison et al 1980).

6.2.2. Purification

Manual weeding of non endothelial cells and magnetic cell sorting both achieved endothelial cell cultures of >90% purity. Magnetic cell sorting using the monoclonal antibody against CD31 was found to be more specific than that using UEA-1, and was therefore used in preference to the lectin.
It was found that cells coated with more than 10 magnetic beads had reduced adherence to the tissue culture plate, remaining in suspension. Cells attached to fewer beads rapidly became adherent, spread and proliferated as normal. In addition, cells to which beads were allowed to remain attached showed no evidence of activation as quantified by an ELISA to demonstrate E-selectin expression. Figure 6.7 illustrates endothelial cells isolated form a mixed cell population by magnetic cell sorting.

Figure 6.1. Coronary artery endothelial cells 2 hours after magnetic cell sorting using monoclonal antibody directed against CD31.
The cells have become adherent and begun to spread, the latter process appears to be inhibited by the presence of >10 beads per cell (*). Phase contrast photomicrograph; original magnification x25.

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6.2.3. Characterisation

6.2.3.i. Development of cobblestone morphology.

Figures 6.2, 6.3 and 6.4 demonstrate the typical cobblestone morphology of confluent endothelial cell monolayers.

Figure 6.2. Confluent human umbilical endothelial cells: cobblestone morphology
Phase contrast photomicrograph; original magnification x25.
Figure 6.3. Confluent human coronary artery endothelial cells
Phase contrast photomicrograph; original magnification x50.

Figure 6.4. Human cardiac microvascular endothelial cells derived from atrial appendage
Phase contrast photomicrograph; original magnification x25.
6.2.3.ii. *in vitro* differentiation: capillary tubule formation

Figure 6.5 shows *in vitro* differentiation to form capillary-like tubules by coronary artery endothelial cells on a Matrigel substrate. Although all endothelial cells formed tubules on Matrigel, coronary artery endothelial cells differentiated more rapidly than endothelial cells derived from other large vessels including aorta and umbilical vein. Furthermore, coronary artery endothelial cells allowed to remain at confluence on uncoated tissue culture plastic for longer than 48 hours also showed evidence of differentiation, a phenomenon not observed until at least a week for other large vessel endothelial cells.

Figure 6.5. *in vitro* differentiation. tube formation by coronary artery endothelial cells on Matrigel

Phase contrast photomicrograph, original magnification x25.
6.2.3. iii. Uptake of fluorescent acetylated LDL

The uptake by endothelial cells of fluorescently labeled acetylated LDL by the scavenger receptor is illustrated in Figure 6.6.

Figure 6.6  Uptake of fluorescently labeled acetylated LDL by subconfluent coronary artery endothelial cells

(A): phase contrast photomicrograph; original magnification x100.
(B): the same field of view, using a rhodamine excitation/emission filter.
6.2.3.iv & v. Binding to Ulex europaeus agglutinin-1 and CD31 (PECAM) expression
The endothelial nature of cells could be confirmed during magnetic cell sorting using either UEA-1 or antibody against CD31, as demonstrated in Figure 6.1.

6.1.2.3.vi. CD34 expression (QB-END/10 immunocytochemistry)
Only a subpopulation of endothelial cells in culture expressed the CD34 antigen, despite having been confirmed as endothelial by simultaneous vWF immunocytochemistry (Figure 6.7). As shown in Figure 6.7, endothelial cells expressing CD34 had the appearance of migrating cells, extending fine microprocesses. In addition, such cells were only to be found overriding the monolayer, and never in direct contact with the tissue culture plastic on which the cells were cultured.

The proportion of CD34 positive cells was independent of endothelial cell type and was not affected by prior stimulation of the cells for 4 hours with TNFα 0.1-100 IU/ml, native or oxidised LDL 1-100μg/ml. Furthermore, after wounding the cell monolayer (as described in Chapter 9), cells migrating directly on tissue culture plastic were not shown to express CD34.

The only factor found to increase the number of cells expressing CD34 was the degree of confluence. Thus the number of positive cells rose from ~2% in subconfluent cultures, to over 40% in cultures allowed to remain at confluence for several days. This phenomenon is illustrated in Figure 6.8.
Figure 6.7. HUVEC, simultaneous CD34 and vWF immunocytochemistry. The endothelial nature of the cells is confirmed by characteristic granular cytoplasmic vWF immunoreactivity (*). The CD34 positive cell (**) is overriding the monolayer, and appears to be migratory, extending fine microprocesses. Photomicrograph; original magnification x160

Figure 6.8. Saphenous vein endothelial cells. CD34 immunoreactivity, demonstrating the effect of confluence. To the left, cells are confluent, with a high proportion of CD34 positive cells overriding the monolayer. To the right, where the cells are subconfluent, the proportion of CD34 positive cells is diminished. Photomicrograph; original magnification x10.
6.2.3. vii. Immunocytochemical localisation of von Willebrand factor.

Endothelial specific localisation of vWF is illustrated in Figure 6.9. The characteristic cytoplasmic granules represent Weibel Palade bodies containing vWF.

Figure 6.9. Endothelial cell vWF immunoreactivity

The cytoplasmic granular staining pattern, specific to endothelial cells is illustrated.

A. Photomicrograph of human coronary artery endothelial cells. Original magnification x160.

B. Photomicrograph of human cardiac microvascular endothelial cells derived from atrial appendage. Original magnification x100.
6.2.3.viii. Detection of von Willebrand factor secretion

a) ELISA

PMA significantly increased vWF release from HUVECs with respect to baseline (p<0.001), as shown in Figure 6.10. Using the standard curve obtained from normal pooled plasma, vWF release was calculated as a percentage of normal pooled human plasma.

![Graph showing vWF release from HUVECs with PMA](image)

**Figure 6.10. Stimulation of vWF release from HUVEC by PMA**

PMA significantly enhanced vWF release after both 2 and 6 hours of incubation. Experiments (n=4) were performed in triplicate. Error bars are standard deviation from the mean, and statistical analysis performed by one way ANOVA.
b) *Cell blot assay*

*Dot Blots*

Positive vWF staining occurred in an antigen and antibody concentration-dependent manner. Absorbed anti-vWF antibody produced negative results against purified vWF.

*Cell blots*

Although the endothelial cells were adherent to the membrane, they remained rounded and did not spread or divide. The endothelial cells demonstrated characteristic cytoplasmic granular vWF immunostaining, but no secretion haloes were observed after 22 hours incubation, or after stimulation with thrombin.

Results for the alternative methods for applying cells to the membrane were as follows. Cells applied to pre-wetted membrane did not adhere. Cells on Thermanox coverslips remained adherent throughout the experiment and stained for vWF; however, no corresponding secretory haloes were observed on the membrane.

6.2.3.ix. *Induction of E-selectin*

Unstimulated endothelial cells did not express E-selectin. Stimulation with lipopolysaccharide or TNF-α induced a dose dependent increase in endothelial E-selectin expression. No expression was seen in non-endothelial (smooth muscle) cells. The results are illustrated in Figures 6.11 and 6.12.
Figure 6.77. Induction of E-Selectin expression in HUVEC by lipopolysaccharide

Significant E-selectin expression was induced from very low basal levels by LPS in concentrations of 0.001μg/ml and above. Experiments (n=5) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.
Figure 6.12. Induction of E-Selectin expression in HUVEC by TNFα

Significant E-selectin expression was induced from very low basal levels by TNFα in concentrations of 0.1 IU/ml and above. Experiments (n=6) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.
SECTION II: EXPERIMENTAL

CHAPTER 7

VASCULAR SMOOTH MUSCLE CELLS IN CULTURE

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells. The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described.

In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported.

Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL. An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells. The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 7
VASCULAR SMOOTH MUSCLE CELLS IN CULTURE

7.1 Methods

All reagents used in this chapter were obtained from Sigma Chemical Co, Poole, UK unless otherwise stated.

7.1.1 Culture of human arterial smooth muscle cells

There are two principal methods of processing tissue for the culture of smooth muscle cells, by enzymatic digestion, or by an explant technique. The former method allows the selective digestion of specific layers of the vessel wall, but requires larger tissue samples than the explant method. Since samples were often as small as 3mm in diameter (discs of aorta from graft implants), the latter method was used.

Smooth muscle cells were cultured using a modification of an explant method (Smirnov et al 1990). Specimens of human aorta and coronary artery were obtained at operation or from cardiac explants and transported in sterile HBSS at 4°C. Tissues were processed aseptically using a class II laminar airflow cabinet. The endothelium was removed by collagenase digestion, and the adventitia excised. The medial and intimal layers were separated using fine forceps and a dissecting microscope. The tissue was finely minced, and plated into a 25cm³ sealed tissue culture flask in 10% FCS-containing Dulbecco's Modified Eagle Medium (DMEM) (see Appendix I). The tissue was incubated undisturbed at 37°C for 10 days, and thereafter the medium changed thrice weekly. Confluent cultures were passaged by trypsinisation in 0.05% trypsin, 0.02% EDTA (Gibco (Life Technology), Paisley UK).

Cells were used for experiments at or before the third passage. Cells were discarded if evidence of senescence was observed; ie if peri-nuclear vacuolation or granulation appeared, or if growth rates decreased.
7.1.2 Characterisation of human arterial smooth muscle cells

The cells were characterised as smooth muscle cells by the following criteria.

i) High cytoplasmic: nuclear ratio and prominent nucleoli

ii) Typical "hill and valley" pattern at confluence.

iii) Desmin immunoreactivity

iv) $\alpha$-smooth muscle actin immunoreactivity

Demonstration of desmin immunoreactivity

Smooth muscle cells were cultured on Thermanox coverslips (Nunc Inc., Naperville, Illinois, USA), fixed for 2 minutes in ice cold 50% methanol / 50% acetone and washed in TBS. After blocking non-specific binding sites with normal rabbit serum (1:5 dilution), the coverslips were incubated for 1 hour in mouse monoclonal anti desmin antibody (1:30 dilution) (Serotec, Kidlington, UK). Cells incubated in TBS served as controls. Following washing, cells were incubated for 30 minutes with 1:200 biotinylated rabbit anti-mouse IgG diluted 1:50, followed by avidin/biotin horseradish peroxidase complex (both Dakopatts, Denmark). Coverslips were developed in DAB (di-amino benzidine) peroxidase substrate solution, counterstained in Carazzi's haematoxylin, and mounted.

Demonstration of $\alpha$-smooth muscle actin immunoreactivity

Smooth muscle cells were cultured on Thermanox coverslips and fixed in 10% formal saline for 10 minutes followed by 1 minute in acetone, then washed in TBS. After blocking non-specific binding sites by incubation in normal goat serum (1:5 dilution), cells were incubated for 1 hour in mouse monoclonal anti-$\alpha$-smooth muscle actin antibody (1:50 dilution) (Dakopatts). Cells incubated in TBS acted as controls. Subsequent stages were performed as described above for desmin immunocytochemical staining.
7.2 Results

7.2.1 Culture of human arterial smooth muscle cells

Outgrowths from explanted tissue were observed at approximately 10 days of culture, as illustrated in Figure 7.1, and cells became confluent in 3-4 weeks.

Signs of senescence were noted in some cultures before or just after the 1st passage, whereas other cultures continued to proliferate for several (>4) passages. Such signs included slowing and cessation of proliferation and perinuclear vacuolation. In general, the early senescing cells were derived from the more atherosclerotic arteries. Senescent cells were not used in experiments, and no cells were used beyond the 3rd passage.

7.2.2 Characterisation of human arterial smooth muscle cells

i) The typical high cytoplasmic: nuclear ratio and prominent nucleoli of vascular smooth muscle cells is demonstrated in Figure 7.1.

ii) The characteristic "hill and valley" pattern was observed in confluent cultures of smooth muscle cells.

iii) Smooth muscle desmin immunoreactivity is shown in Figure 7.2.

iv) α-smooth muscle actin immunoreactivity, specific to smooth muscle cells is illustrated in Figure 7.3.
Figure 7.1. Human coronary artery smooth muscle cells.
Phase contrast photomicrographs.
A: Original magnification x25
B: Illustrates a high cytoplasmic to nuclear ratio and prominent nucleoli. Original magnification x50.
Figure 7.2. Desmin immunoreactivity in human coronary artery smooth muscle cells.

Photomicrograph, original magnification x40.
Figure 7.3. α-actin immunoreactivity in human coronary artery smooth muscle cells.

Photomicrographs.
A. Original magnification x25
B. Same field of view, original magnification x40
SECTION II: EXPERIMENTAL

CHAPTER 8
LOW DENSITY LIPOPROTEIN: ISOLATION AND MODIFICATION

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells. The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described. In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported.

Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL.

An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells. The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 8
LOW DENSITY LIPOPROTEIN: ISOLATION AND MODIFICATION

8.1 Methods

Unless otherwise stated, all reagents used in this chapter were obtained from Sigma Chemical Co., Poole UK.

8.1.1 Isolation of LDL

The method used was a modification of that described by Havel et al (1955) and performed at 4°C. Blood (100ml) was taken from a healthy volunteer into 0.4M EDTA (ethylenediaminetetraacetic acid), and plasma removed by centrifugation for 10 minutes at 700G. Allowing for the serial dilutions of plasma which occurred during the isolation procedure, the initial concentration of 0.4M EDTA ensured that EDTA was present at concentrations greater than 1mM throughout the isolation procedure, thus preventing oxidation of low density lipoprotein (LDL).

Lipoprotein fractions were separated by potassium bromide (KBr) density gradient ultracentrifugation; the densities of the lipoproteins are as follows:

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (kg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons/VLDL</td>
<td>1.006</td>
</tr>
<tr>
<td>IDL</td>
<td>1.019</td>
</tr>
<tr>
<td>LDL</td>
<td>1.063</td>
</tr>
<tr>
<td>HDL</td>
<td>1.215</td>
</tr>
</tbody>
</table>
Initial centrifugation was required to remove very low and intermediate density lipoprotein (VLDL and IDL) fractions. Potassium bromide (KBr) was therefore prepared as follows:

where equal volumes of plasma and KBr solution are used:

\[
\text{density of final combined solution} = \frac{\text{plasma density} + \text{KBr density}}{2}
\]

\[
\Rightarrow 1.019 = \frac{0.979 + \text{KBr density}}{2}
\]

\[
\Rightarrow \text{KBr density} = 1.059
\]

Therefore, from Figure 8.1, constructed from Wolf et al 1989, the concentration of KBr required is 84.6g/l.
Equal volumes of plasma and KBr solution were pipetted into 13.5ml disposable Quickseal ultraclear centrifuge tubes (Beckman Instruments Inc., Palo Alto, USA), and the tubes heat sealed. Following centrifugation at 55 000 rpm, 4°C for 5 hours (or 65 000rpm for 2½ hours), using a near vertical rotor (Beckman NVT 65), the tubes were cut open. The visible supernatant band containing VLDL and IDL was removed. Using the above calculation, the concentration of the remaining VLDL-free solution was raised to 1.063 and centrifugation repeated, as above.

The resulting supernatant band of LDL was removed and dialysed (Spectra/Por cellulose ester sterile membrane, Spectrum Medical Industries, Inc., Los Angeles, USA) against PBS (Unipath Ltd, Basingstoke, UK) or PBS/1mM EDTA at 4°C. The dialysate was changed 3 times over 24 hours.

LDL preparations had not only to be sterile, but also free of pyrogen for use in endothelial cell assays, in order to prevent stimulation of adhesion molecule expression by endotoxin. The above procedure was therefore performed where possible, in a laminar air flow cabinet, using equipment treated as follows. All glassware was depyrogenated by thorough washing in 2.5% deoxycholate and rinsing in deionised, pyrogen free, double distilled water, followed by baking for 10 hours at 180°C (Ludwig et al 1990). Solutions were prepared in sterile pyrogen free water (Baxter Healthcare Ltd, Thetford, UK) and filtered through a 0.22μm mesh. Centrifuge tubes were sterilised by ethylene oxide. Spectra/Por cellulose ester sterile membrane (Medicell, London, UK) was used to dialyse the LDL. In addition, handling of dialysis tubing was minimised, but where necessary, pyrogen free sterile gloves (Biogel, LRC Products Ltd, London) were used.
8.1.2 Characterisation of LDL

The isolated LDL was characterised as follows. The protein and endotoxin content was estimated, and contamination with other lipoprotein fractions excluded by a double immunodiffusion technique, by agarose gel electrophoresis, and by electron microscopy.

8.1.2.1 Protein estimation

The protein content of LDL preparations was determined by a modification of Lowry et al (1951). The assay is based on the formation of a complex between peptide bonds and and alkaline cupric tartrate reagent. On addition of Folin's phenol reagent a purple-blue colour forms, and absorbance is measured spectrophotometrically at 500nm. LDL concentrations throughout this thesis are expressed in μg/ml of protein.

8.1.2.2 Endotoxin testing

The endotoxin content of isolated LDL was assessed by the Limulus amoebocyte lysate assay, a sensitive test, capable of detecting endotoxin at levels of between 0.003-0.0075 endotoxin units/ml (equivalent to 3-7.5 pg endotoxin/ml). The principle of the assay is based on the reaction of a lysate of the circulating amoebocytes of the horse shoe crab, Limulus polyphemus, with minute quantities of endotoxin, to form a gel.

E-Toxate® (amoebocyte lysate) was added to test samples and to endotoxin standards prepared from E. coli 055:B5 lipopolysaccharide, and gel formation assessed after incubation for 1 hour at 37°C.
8.1.2.3 Immunodiffusion

A 1% agarose gel (type IIa) in barbital buffer (see Appendix II) was formed. 7µl of each lipoprotein fraction to be tested was added around a central well containing 7µl goat polyclonal antibody to LDL (cross reacts with VLDL), HDL, or whole human serum. The gel was incubated overnight in a moist atmosphere at room temperature. Following squashing, drying and washing, the gel was incubated in a triple stain (Crowle 1973, see Appendix II) for 5 minutes, then destained in 2% acetic acid.

8.1.2.4 Agarose gel electrophoresis

Lipoprotein electrophoresis was performed using the Paragon® electrophoresis system (Beckman Instruments Inc., Palo Alto, USA). Plasma fractions were loaded onto 0.5% agarose electrophoresis film, run for 30 minutes at 100V, and fixed (see Appendix II for fixative). Gels were squashed and dried before staining for 2 minutes with Beckman Paragon Lipostain and destaining in ethanol/deionised water (v/v 9/11).

8.1.2.5 Electron microscopy

LDL at 0.1mg/ml was placed onto a copper coated electron microscopy grid, followed by 2% aqueous sodium/potassium phosphotungstate pH 5.6. The grid was viewed using a scanning electron microscope.

8.1.3 Oxidative modification of LDL

LDL was oxidatively modified by 3 techniques: copper oxidation, macrophage modification, and minimal modification by prolonged storage.

8.1.3.1 Copper oxidation

LDL was oxidised by the addition of 1.66µM copper sulphate (BDH Ltd, Dagenham, UK) to 0.1mg/ml LDL followed by overnight incubation at room temperature and spectrophotometric monitoring of conjugated diene formation at
234nm. LDL preparations for use in cell culture were oxidised under sterile conditions by the addition of 16.6μM CuSO₄ to 1mg/ml LDL.

8.1.3.2 Macrophage modification

This was performed in collaboration with Dr D Leake, University of Reading. Immediately prior to use, LDL was diluted to 0.1mg LDL protein/ml in Ham's F10 Medium (Flow Laboratories Ltd, High Wycombe, UK) with the additions of FeSO₄ to a final concentration of 6μM, and EDTA to a final concentration of 1mM or 5μM. The former concentration of EDTA would completely inhibit, and the latter concentration enhance, the macrophage oxidative modification of LDL (Lamb et al 1992b). LDL preparations in which oxidation was to be assessed by the macrophage degradation assay were radiolabeled with Na¹²⁵I using iodine monochloride, as described by Leake et al (1990).

Mouse peritoneal macrophages were isolated according to the method of Cohn et al 1965. Macrophages were harvested from the peritoneal fluid of mice by centrifugation at 250g for 10 minutes at 4°C. Macrophages were allowed to adhere for 4 hours at 37°C to 22mm diameter wells at 10⁶ cells per well, in DMEM containing 10% FCS. After 4 hours, the macrophages were washed to remove serum and detached cells and 1ml/well of LDL added. Each concentration was performed in duplicate, with macrophage-free or LDL free wells as negative controls. In addition, 2 wells contained I¹²⁵ labeled LDL 0.1mg/ml with 5μM EDTA, one with, and one without, macrophages.

After 18 hours incubation, media were removed and centrifuged at 1500g for 10 minutes to remove cell debris. The medium containing I¹²⁵ labeled LDL was retained to assess LDL oxidation, as described below.
8.1.3.3 Minimal modification

LDL preparations were minimally modified by prolonged (>2 months) storage at +4°C.

8.1.4 Assessment of oxidatively modified LDL

The oxidation of LDL was monitored by the formation of conjugated dienes, β-carotene depletion, changes in electrophoretic mobility, malonaldehyde content (by the thiobarbituric acid test), and macrophage degradation.

8.1.4.1 Conjugated diene formation

As described above, copper oxidation of LDL is accompanied by a rise in conjugated dienes; this was monitored spectrophotometrically at 234nm.

8.1.4.2 β-carotene depletion

Depletion of the antioxidant β-carotene in LDL preparations subject to oxidant stress was measured by spectrophotometry between 370 and 700nm.

8.1.4.3 Agarose gel electrophoresis

LDL and oLDL were separated by agarose gel electrophoresis using the Beckman Lipogel system, as described above.

8.1.4.4 Thiobarbituric acid test

The thiobarbituric acid test was used to detect malonaldehyde (MDA) formation in minimally modified LDL preparations, in which other changes were not detectable. The method used was a modification of Yagi (1982).

Standards were prepared from 1,1,3,3-tetra-ethoxypropane to give 0.025-5mM MDA. LDL preparations were at 50-200μg/ml. 250μl aliquots of samples and standards were incubated for 15 minutes at 100°C, with 0.5ml 1M HCl and 0.5ml
1% 2-thiobarbituric acid (BDH). Formation of MDA was assessed by spectrophotometry at 532nm.

8.1.4.5 Determination of modified LDL degradation by macrophages

This assay was performed in collaboration with Dr D Leake, University of Reading. Degradation of $^{125}$I LDL which had been oxidatively modified by incubation with mouse peritoneal macrophages was assessed by incubation with cells of the J774 monocyte-macrophage cell line. The assay was performed as described by Leake et al (1990); the method is described below.

J774 cells were plated down at a density of $10^5$ cells/3.8cm$^2$. They were incubated for 22 hours in macrophage-modified or control $^{125}$I labeled LDL, diluted to 10μg/ml in 10% foetal calf serum containing DMEM. At the end of the incubation period the medium containing intact $^{125}$I LDL and $^{125}$I peptide LDL degradation products was removed, and centrifuged to remove cell debris. Intact $^{125}$I LDL was then precipitated out with trichloroacetic acid, leaving soluble $^{125}$I peptide degradation products in the supernatant. The total protein content of the supernatant was assessed by the Lowry technique, and the $^{125}$I degraded LDL quantified using a gamma counter. Results were assessed as μg degraded LDL per mg of total protein, and statistical analysis performed by one way analysis of variance.
8.2 Results

8.2.1 Isolation of LDL

In this isolation procedure the donor was not fasting, so the VLDL supernatant also contained chylomicrons. After the second centrifugation, the LDL supernatant, coloured yellow by its β carotene content, was removed.

8.2.2 Characterisation of LDL

8.2.2.1 Endotoxin testing

As discussed in Chapter 10, the endotoxin content of LDL preparations had to be below 100pg/ml (1 endotoxin unit (Eu)/ml) in order to avoid endotoxin stimulation of endothelial adhesion molecules.

Initial sources of contamination were traced to standard cellulose dialysis tubing which contained large amounts of endotoxin not inactivated by sterilisation procedures such as boiling, and to the starch content of sterile surgical gloves. The use of Spectra/Por cellulose ester sterile membrane and starch and pyrogen free
sterile gloves (Biogel, LRC Products Ltd, London) eliminated this contamination.

Endotoxin levels were found to be <30pg/ml in all LDL preparations, comparing favourably with those of other groups (Berliner et al 1990).

8.2.2.2 Immunodiffusion

An Immunodiffusion gel of isolated lipoprotein fractions is illustrated in Figure 8.3.

![Immunodiffusion gel](image)

**Figure 8.3. Immunodiffusion gel**

LDL = pure native LDL, oLDL = pure oxidised LDL.

VLDL = supernatant from first centrifugation, it therefore also contains IDL and some LDL. HDL = infranatant from second isolation, it therefore also contains non-lipoprotein plasma proteins. The upper left gel shows lipoprotein fractions around antibody against LDL which cross reacts with VLDL. The LDL, oLDL and VLDL fractions, but not the HDL fraction show immunoreactivity. The lower right gel shows lipoprotein fractions around antibody against HDL to which only the HDL fraction reacts. The LDL preparation is therefore not contaminated with HDL.
8.2.2.3 *Agarose gel electrophoresis*

An agarose gel of isolated lipoprotein fractions is illustrated in Figure 8.4.

![Figure 8.4: Agarose gel electrophoresis of lipoprotein fractions](image)

**Figure 8.4: Agarose gel electrophoresis of lipoprotein fractions**

Lanes 2 & 6 = LDL. Only one band is visible, indicating that no contamination by other lipoprotein fractions has occurred. Lanes 1 & 5 = VLDL. This fraction was obtained from the supernatant from the first centrifugation, and therefore also contains LDL. There are therefore 2 visible bands, that which remains nearest the origin represents LDL. Lanes 1 & 2 are from the same isolation, as are lanes 5 & 6.
8.2.2.4 Electron microscopy

LDL visualised by scanning electronmicroscopy is illustrated in Figure 8.5.

Figure 8.5 Scanning electronmicrograph of LDL
The LDL particles are of a uniform diameter (18-30nm). There is no contamination by VLDL (35-85nm), or HDL (7-10nm). Original magnification x 120 000.
8.2.3 Assessment of oxidatively modified LDL

8.2.3.1 Conjugated diene formation

The oxidation of LDL, catalysed by copper, resulted in a rise in conjugated diene formation, illustrated in Figure 8.6.

![Figure 8.6. Copper oxidation of LDL: conjugated diene formation](image)

**Figure 8.6.** Copper oxidation of LDL: conjugated diene formation

LDL 0.1mg/ml, EDTA 1mM, CuSO₄ 1.66μM

The addition of CuSO₄ 1.66μM resulted in the oxidation of 0.1mg/ml LDL. The curve followed the typical lag, propagation and plateau phases described in Chapter 3. LDL alone underwent some auto-oxidation. Both auto- and copper catalysed oxidation was completely inhibited by the addition of 1mM EDTA.
8.2.3.2 β-carotene depletion

Figure 8.7. illustrates the typical β-carotene spectra observed for LDL in different oxidation states.

Figure 8.7. Spectrophotometric analysis of β-carotene content of LDL.

The characteristic spectrum of β-carotene, with several major absorbance bands in the visible region (Gurd 1960) was diminished in LDL which had been stored (minimally modified) for 2 months in the absence of EDTA, compared with freshly isolated LDL. It was virtually undetectable in LDL which had undergone copper-catalysed oxidation.

8.2.3.3 Agarose gel electrophoresis

As illustrated in Figures 8.8 and 8.9, copper oxidised LDL showed enhanced electrophoretic mobility on agarose gels, as compared with native and mmLDL. This indicated that the protein moiety of oLDL had been modified, whereas that of mmLDL remained intact.
Figure 8.8. Agarose gel electrophoresis of native and copper oxidised LDL.
Lane 5 = native LDL. The copper oxidised LDL (lane 4) has migrated further from the origin, reflecting the oxidative modification of its protein moiety.

Figure 8.9. Agarose gel electrophoresis of native, minimally modified and copper oxidised LDL.
Lane 5 = copper oxidised, 6 = mm, 7 & 8 = native LDL.
There is no increase in electrophoretic mobility by mmLDL compared to that of native LDL, reflecting the intact nature of its protein component. In comparison oLDL shows enhanced mobility.
8.2.3.4 Thiobarbituric acid test

The results of a typical assay are shown below. Figure 8.10 shows the graph constructed from the standard data, from which the MDA concentrations of the LDL preparations were calculated.

![Graph showing standard curve for TBA test](image)

**Figure 8.10. Standard curve for TBA test**

Correlation = 1.000

Where $c_1$ = absorption at 532nm, and $c_2 = \mu$M MDA,

linear regression analysis of the standard data yielded the equation predicting:

$$c_2 = 0.939 c_1 - 0.00414$$

Therefore, the MDA concentration could be calculated from the absorbance values.

The results are demonstrated graphically in Figure 8.11.
The MDA content of copper-oxidised LDL showed a 10-fold increase over that of native LDL. The MDA content of mmLDL rose with up to 9 months of storage to more than twice that of native LDL, but remained less than half that of oLDL.

8.2.3.5 Macrophage degradation assay

Mouse peritoneal macrophages oxidatively modified LDL. $^{125}$I LDL modified by incubation with mouse peritoneal macrophages showed increased uptake and degradation by J774 macrophage/monocytes compared to $^{125}$I LDL which had been incubated in the absence of peritoneal macrophages. Results from a typical experiment are illustrated in Figure 8.12.
Macrophage modified \(^{125}\)I LDL underwent significantly greater degradation by J774 macrophage/monocytes than did control \(^{125}\)I LDL (** p=0.002). Experiments (n=2) were performed in duplicate. Error bars are standard deviation from the mean.

Statistical analysis was by one way ANOVA.
SECTION II: EXPERIMENTAL

CHAPTER 9

LDL-CELL INTERACTIONS

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells. The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described. In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported. Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL.

An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells.

The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 9
LDL-CELL INTERACTIONS

9.1 Methods

Studies of the cytotoxic, migratory and proliferative effects of native and modified LDL on endothelial and smooth muscle cells are described in this chapter. The effects of LDL on cell adhesion are described in Chapters 10 and 11.

All reagents used in this chapter were obtained from Sigma Chemical Co, Poole, UK unless otherwise stated.

9.1.1 Chromium release cytotoxicity assay

Endothelial or smooth muscle cells were grown to confluence on 96 well plates (approximately 10^5 cells per well). The growth medium was replaced with 100μl per well of fresh growth medium with the addition of 300μSv sodium ^{51}CrO_4 per well, and the cells incubated for 90 minutes at 37°C. The sodium chromate-containing medium was removed and the cells washed 3 times in HBSS (see Appendix I). 100μl per well of fresh medium was added, containing 0.001-400 μg/ml of the various LDL preparations, a positive control (1% Triton), or the negative control (medium alone). Dilutions were performed in triplicate and the cells incubated for 5 hours.

Cytotoxic effects were quantified by measuring ^{51}Cr release from cells. ^{51}Cr released into the medium was quantified using a Beckman 550 gamma counter. Differences in ^{51}Cr release between the various LDL preparations were analysed by one way ANOVA. Since the positive control resulted in 100% cell lysis, and the negative control was assumed to represent 100% viability, results could also be expressed as the percentage of cells lysed.
9.1.2 Morphological assessment of LDL cytotoxicity

During the above 51Cr release assays, morphological changes in the cells were noted. Therefore photomicrographs were taken at 3 and 5 hours.

9.1.3 LDL and proliferation: 5-Bromo-2'-deoxyuridine assay

Proliferating cells were considered to be those undergoing DNA synthesis during the course of the assay.

Cells were seeded onto a 96 well plate at a density of ~5000 cells/well in their usual growth medium, with the addition of native or modified LDL (1-100 μg/ml). After the addition of 10^-5M BrDU (5-Bromo-2'-deoxyuridine), they were incubated for 10 hours (endothelial cells) or 18 hours (smooth muscle cells). The different incubation times were found to be necessary due to the slower proliferation rates in even early smooth muscle cell cultures. Following 30 minutes fixation in 70% ethanol in PBS/0.5% Tween, the cells were hydrolysed for 30 minutes in 4M HCl, and washed. The cells were incubated in mouse monoclonal antibody to BrDU (Dako) diluted 1:50, and developed in DAB after avidin-biotin amplification. Counterstaining was performed with Toluidine Blue. Cells with positive staining nuclei were in the S (DNA synthesis) phase of the cell cycle during the assay. Random quadrat sampling was used to assess the percentage of positive cells in each well. Statistical analysis was performed by one way ANOVA.

9.1.4 LDL and migration

Previous studies had demonstrated enhanced coronary artery endothelial cell migration following wounding of the cell monolayer, in response to the thrombolytic agents tPA and streptokinase (Thorne et al 1992b). This had resulted in the development of a quantifiable migration assay, which was subsequently used in these studies of the effects of LDL on endothelial cell migration.
Briefly, endothelial cells were grown to confluence onto fibronectin coated Thermanox® coverslips. The coverslip undersurface had been scratched with a cross previously, as an orientation marker. The monolayer was wounded with a 1.4mm resin scraper and washed to remove detached cells. Cells were incubated with medium M199 with 10% FCS, or with additions of a range of concentrations of native and modified LDL. During the incubation period, migration was assessed by sequential photography.

Migration was quantified by measuring the change in wound width during the incubation period, from the photomicrographs. For each wound, 7 width measurements were taken at each time point, and the mean percentage change in width calculated. Statistical analysis was performed by one way ANOVA.

The technique described above was also applied to cultures of smooth muscle cells.

9.1.5 LDL and CD34 expression

Endothelial cells were incubated with 1-100μg/ml of native, mm, or oLDL for 6 hours, prior to quantification of CD34 expression by immunocytochemistry with the monoclonal antibody QB-END/10, as detailed in Chapter 6. CD34 expression was quantified by random quadrat sampling, and statistical analysis performed by one way ANOVA.
9.2 RESULTS

9.2.1 Chromium release cytotoxicity assay

Endothelial cells

For endothelial cells there was no significant release of $^{51}$Cr above basal levels for any concentration tested of native or minimally modified LDL, nor for oxidised LDL in a range of concentrations up to 200µg/ml. 400µg/ml oxidised LDL caused a significant increase in $^{51}$Cr release. The results are illustrated in Figure 9.1.

![Graph illustrating chromium release cytotoxicity in HUVEC by 51 chromium release. 0.4mg/ml caused a significant (** p<0.002) increase in $^{51}$Cr release compared with the negative control. This was calculated to be due to 29% cell lysis. In contrast, neither native or mmLDL induced significant cytotoxicity, with 0.2 and 0.82% cell lysis, respectively. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean, and statistical analysis was by one way ANOVA.](image-url)

Figure 9.1. Quantification of LDL cytotoxicity in HUVEC by 51 chromium release. 0.4mg/ml caused a significant (** p<0.002) increase in $^{51}$Cr release compared with the negative control. This was calculated to be due to 29% cell lysis. In contrast, neither native or mmLDL induced significant cytotoxicity, with 0.2 and 0.82% cell lysis, respectively. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean, and statistical analysis was by one way ANOVA.
**Smooth muscle cells**

As illustrated in Figure 9.2, for aortic smooth muscle cells there was no significant release of $^{51}$Cr above basal levels for native or oxidised LDL in a range of concentrations up to 200μg/ml. 400μg/ml of both native and oxidised LDL caused significant increases in $^{51}$Cr release, although oLDL was significantly more cytotoxic than LDL.

![Figure 9.2](image)

**Figure 9.2.** Quantification of LDL cytotoxicity in aortic smooth muscle cells by $^{51}$chromium release.

400μg/ml of both native and oxidised LDL caused significant (*p=0.045 and **p<0.0001, respectively) increases in $^{51}$Cr release. $^{51}$Cr release from cells incubated with oLDL was significantly greater than from native LDL at 400μg/ml (p=0.013). These data corresponded to 23% and 4% cell lysis, respectively. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean, and statistical analysis was by one way ANOVA.
9.2.2 Morphological assessment of LDL cytotoxicity

A qualitative assessment of morphological changes in endothelial cells was made from the photomicrographs, as shown in Figure 9.3. No morphological abnormalities were observed for any of the dilutions of native LDL throughout the incubation time. At three hours, rounding up of cells had occurred, most markedly for oxidised LDL 400μg/ml, followed by oxidised LDL 200μg/ml, then minimally modified LDL 400μg/ml. By 5 hours, rounding up had progressed, and had also become apparent for minimally modified LDL 200μg/ml.

A comparison between morphological change and $^{51}$Cr release is shown in Table 9.i.

<table>
<thead>
<tr>
<th>LDL</th>
<th>significant 51Cr release</th>
<th>cell rounding at 5hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>native LDL 400</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mmLDL 400</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>mmLDL 200</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>oLDL 400</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>oLDL 400</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 9.i. Morphological change and 51chromium release in HUVEC in response to LDL

The morphology of aortic smooth muscle cells was assessed in the same manner as for endothelial cells. Only cells incubated with oLDL at a concentration of 400μg/ml showed evidence of morphological change, beginning to round up at the end of the 5 hour incubation period.
Figure 9.3 HUVEC: morphological change after 5 hours incubation with 400 μg/ml LDL preparations

Phase contrast photomicrographs; original magnification x50

A. Native LDL. There is no visible disruption of the confluent monolayer.

B. mmLDL. Some rounding of cells has occurred.

C. oLDL. Rounding of cells is more marked and also associated with $^{51}$Cr release (Figure 9.7).
9.2.3 LDL and proliferation: BrDU assay

Endothelial cells:

Native LDL had no effect on coronary artery endothelial cell proliferation as assessed by DNA synthesis, in concentrations up to 100 μg/ml. In contrast, both mm- and oLDL significantly inhibited proliferation at 100μg/ml. In addition, morphological changes were observed in cells incubated in oLDL at this concentration: some cells had begun to round up at the end of the incubation period.

Table 9.ii shows results from a typical experiment. Proliferating cells were defined as those which took up BrDU and were therefore in the DNA synthesising S phase of the cell cycle.

<table>
<thead>
<tr>
<th>Replicating cells %</th>
<th>Standard deviation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (medium)</td>
<td>11.89</td>
<td>4.71</td>
</tr>
<tr>
<td>oLDL 100 μg/ml</td>
<td>4.29</td>
<td>3.01</td>
</tr>
<tr>
<td>10</td>
<td>9.15</td>
<td>4.59</td>
</tr>
<tr>
<td>mmLDL 100 μg/ml</td>
<td>7.40</td>
<td>3.67</td>
</tr>
<tr>
<td>10</td>
<td>11.05</td>
<td>4.06</td>
</tr>
<tr>
<td>mmLDL 100 μg/ml</td>
<td>10.42</td>
<td>3.94</td>
</tr>
</tbody>
</table>

Table 9.ii. Effect of LDL on coronary artery endothelial cell proliferation

Both oLDL and mmLDL 100μg/ml significantly inhibited proliferation, quantified by the percentage of nuclei which had taken up BrDU. oLDL inhibited proliferation significantly more than did mmLDL (p 0.021). Native LDL did not influence proliferation. Experiments (n=4) were performed in triplicate. Data were obtained by random quadrat sampling, and statistical analysis was by one way ANOVA.
Smooth muscle cells:
Native LDL had no effect on proliferation as assessed by DNA synthesis in smooth muscle cells derived from coronary artery or aorta, in concentrations of up to 100μg/ml. 100μg/ml oLDL significantly inhibited proliferation. Concentrations of oLDL of 10μg/ml or less had no effect on smooth muscle cell proliferation. No morphological changes were observed during the incubation period. Table 9.iii shows results from a typical experiment.

<table>
<thead>
<tr>
<th>Replicating cells</th>
<th>Standard deviation</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Control (medium)</td>
<td>10.72</td>
<td>6.95</td>
</tr>
<tr>
<td>oLDL 100 μg/ml</td>
<td>3.56</td>
<td>2.81</td>
</tr>
<tr>
<td>10</td>
<td>7.85</td>
<td>5.47</td>
</tr>
<tr>
<td>LDL 100</td>
<td>8.75</td>
<td>4.35</td>
</tr>
<tr>
<td>10</td>
<td>10.29</td>
<td>5.65</td>
</tr>
</tbody>
</table>

Table 9.iii. Effect of LDL on aortic smooth muscle cell proliferation
oLDL at a concentration of 100μg/ml significantly inhibited proliferation, quantified by the percentage of nuclei which had taken up BrDU. Native LDL did not influence proliferation. Experiments (n=4) were performed in triplicate. Data were obtained by random quadrat sampling, and statistical analysis was by one way ANOVA.

9.2.4 LDL and migration

Endothelial cells
Qualitative assessment of photomicrographs showed that individual cells did not migrate from the wound edge of endothelial monolayers after incubation for 5 - 36 hours in 100μg/ml oLDL. No morphological changes suggestive of cytotoxicity were observed in these cells. In contrast, photomicrographs of cells incubated in medium alone, in concentrations of up to 100μg/ml of native or mmLDL, or up to 10μg/ml of oLDL, showed individual cells migrating along the length of the wound. These results are illustrated in Figure 9.4.
Quantitative assessment of changes in wound width from a typical experiment are illustrated in Table 9.iv. 100µg/ml oLDL significantly inhibited endothelial cell migration; native and mmLDL had no effect.

<table>
<thead>
<tr>
<th></th>
<th>7 hr % reduction in wound width</th>
<th>Standard deviation</th>
<th>p value</th>
<th>29 hr % reduction in wound width</th>
<th>Standard deviation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (medium)</td>
<td>30.3</td>
<td>6.69</td>
<td></td>
<td>86.3</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>oLDL 100µg/ml</td>
<td>7.3</td>
<td>4.71</td>
<td>&lt;0.0001</td>
<td>7.4</td>
<td>3.96</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>mmLDL 100µg/ml</td>
<td>32.3</td>
<td>6.05</td>
<td>NS</td>
<td>88.4</td>
<td>4.56</td>
<td>NS</td>
</tr>
<tr>
<td>native LDL 100µg/ml</td>
<td>27.4</td>
<td>4.75</td>
<td>NS</td>
<td>81.6</td>
<td>10.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 9.iv. The effects of LDL on endothelial cell migration.
Reduction in wound width was due to migration of cells into the wound space. Endothelial cell migration was significantly (p<0.0001) inhibited at both 7 and 29 hours. Native and mmLDL did not effect migration. Experiments (n=4) were performed in duplicate. Statistical analysis was by one way ANOVA.
Figure 9.4. HUVEC migration study
Phase contrast photomicrographs; original magnification x25.
A. Immediately after wounding the confluent monolayer. The orientation marker is scored on the undersurface of the coverslip.
B. Same field of view, after 7 hours incubation in M199 + 10% FCS + 100μg/ml mmLDL. Cells have migrated into the denuded area.
Smooth muscle cells

It did not prove possible to apply the assay used to investigate endothelial cell migration to the study of smooth muscle cells. It was not possible to remove a strip of cells to leave a "wound edge" which could either be monitored photographically, or from which measurements could be made. Attempts to modify the assay by the use of a blunted razor to remove a strip of cells from the monolayer not only resulted in damaged cells at the wound edge, but also scored the tissue culture plastic surface, thus creating an obstacle over which cells had to migrate.

9.2.5 LDL and CD34 expression

Neither native or modified LDL increased expression of CD34 in endothelial cells above that of control cells incubated in normal growth medium alone, as shown in Table 9.v.
<table>
<thead>
<tr>
<th></th>
<th>Mean % CD34 positive cells</th>
<th>Standard deviation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (medium)</td>
<td>6.2</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>native LDL 100μg/ml</td>
<td>5.3</td>
<td>1.9</td>
<td>NS</td>
</tr>
<tr>
<td>native LDL 10μg/ml</td>
<td>5.7</td>
<td>1.8</td>
<td>NS</td>
</tr>
<tr>
<td>oLDL 100μg/ml</td>
<td>6.1</td>
<td>2.7</td>
<td>NS</td>
</tr>
<tr>
<td>oLDL 10μg/ml</td>
<td>5.7</td>
<td>1.5</td>
<td>NS</td>
</tr>
<tr>
<td>mmLDL 100μg/ml</td>
<td>5.4</td>
<td>2.0</td>
<td>NS</td>
</tr>
<tr>
<td>mmLDL 10μg/ml</td>
<td>5.6</td>
<td>1.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 9.v. The effects of LDL on endothelial cell CD34 expression

Neither native or modified LDL had an affect on CD34 expression in HUVEC. Experiments (n=2) were performed in duplicate, and statistical analysis was by one way ANOVA.
SECTION II: EXPERIMENTAL

CHAPTER 10
ENDOTHELIAL CELL ADHESION STUDIES

SUMMARY
Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells. The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described. In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported. Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL. An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells.

The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 10
ENDOTHELIAL CELL ADHESION STUDIES

10.1 Introduction
As discussed in Chapters 3 and 5, oxidised LDL is implicated early in the atherogenic process, mediating the adhesion of monocytes to the endothelium prior to their passage into the intima. The effect of native and modified LDL on monocyte adhesion to human coronary artery endothelial cells was therefore investigated by an adhesion assay, as was the effect of LDL on endothelial cell adhesion molecule expression, by ELISA (enzyme linked immunoabsorbent assay). Both assays are described in this chapter.

LDL was used in its native form, or after oxidative modification by the addition of Cu^{2+} ions, by incubation with macrophages, or after minimal modification by prolonged storage, as described in Chapter 8.

10.2 Methods
All reagents used in this chapter were obtained from Sigma Chemical Co., Poole, UK unless otherwise stated.

10.2.1 Monocyte-endothelial cell adhesion assay
This assay was used to quantify the adhesion of radiolabeled peripheral blood monocytes to coronary artery endothelial cell monolayers.

10.2.1.1 Monocyte isolation and labeling
Monocyte isolation was performed with care to avoid activation of the isolated cells, using a modification of the method described by Böyum in 1976. 25ml heparinised blood from a healthy volunteer was mixed with an equal volume of PBS/0.05% glucose. A discontinuous density gradient was created by layering the blood onto
Histopaque ρ1.077 in polycarbonate tubes, followed by centrifugation at 400G for 30 minutes at 4°C. Subsequent steps were also performed at 4°C. Mononuclear cells were removed from the interface between the Histopaque and PBS/glucose, washed twice in HBSS, and counted.

Monocytes were isolated from the mononuclear cell population by negative selection, as follows. The mononuclear cell suspension was incubated for 10 minutes at 4°C with magnetic Dynabeads coated with monoclonal antibodies to CD19 and CD2 (Dynal, Oslo, Norway) to remove B and T lymphocytes, respectively. Isolated monocytes were labeled by incubation with 300μCi of 51Cr sodium chromate in 10% serum containing M199 for 90 minutes at 37°C with constant agitation, then washed twice in HBSS.

10.2.1.2 Adhesion assay

Endothelial cells on a 96 well plate were pre-stimulated for 4-6 hours by incubation with native or modified LDL at 1-200μg/ml, in M199 (plus 10% FCS). The oxidation status of the LDL preparations was determined by the TBA test, described in Chapter 8. The malonaldehyde content of minimally modified LDL was 3-fold, and copper-oxidised LDL 9-fold, higher than that for native LDL. IL-1, TNFα, or lipopolysaccharide (LPS), were used as positive controls. At the end of the incubation time, cells were washed to remove the stimulating agent. Endothelial cells were then incubated with 5-6 x10⁴ monocytes per well for 30 minutes at 37°C, and unattached monocytes removed. The endothelial cells and attached monocytes were lysed overnight at 4°C in 0.1% Triton.

Counts were determined using a Beckman 5500 Gamma counter, and statistical analysis performed by one way ANOVA. The percentage of bound monocytes was calculated as follows:
% monocytes bound = \[
\frac{\text{Counts / minute in 100\mu l lysate}}{\text{Counts / minute in 100\mu l original monocyte suspension}} \times 100\%
\]

\[10.2.2 \text{ Endothelial cell adhesion molecule ELISA}\]

Induction of endothelial cell adhesion molecule expression, either directly by modified LDL or via interactions with other cell types, was investigated using a modification of the ELISA described by Wellicome et al (1990). Statistical analysis of data was performed by one way ANOVA.

\[10.2.2.1 \text{ Cell stimulation and fixation}\]

Endothelial cells were passaged onto 96 well plates and allowed to reach confluence. Plates were fibronectin coated at 2\mu g/cm\(^2\) in order to help prevent cell detachment during the ELISA. Cells were incubated for 4-8 hours at 37\degree C in M199 (supplemented with 10% FCS) and test substances, in triplicate. This time scale was chosen since, as discussed in Chapter 4, for E-selectin, the maximum response time to stimulation is seen at 4-6 hours, for VCAM-1 it is 4-10 hours, and for ICAM-1, 12-24 hours. Some experiments investigated adhesion molecule expression over periods of up to 24 hours.

At the end of the incubation time, the cells were fixed at 4\degree C as follows. Test media were removed and 200\mu l/well fresh PLP fixative (see Appendix II) immediately added for 10 minutes. Fixative was tapped out and the cells washed twice in blocking solution (see Appendix II). Fixed cells were stored at 4\degree C in blocking solution.

\[10.2.2.2 \text{ Endothelial cell adhesion molecule ELISA}\]

The assay was performed with care to prevent detachment of cells from the ELISA plate. Each stage was followed by three washes in 0.1% BSA in PBS (Unipath Ltd, Basingstoke, UK).
Cells were incubated for 2 hours in 10μg/ml mouse monoclonal anti VCAM-1, E-selectin or ICAM-1 antibody (6.5B5, 1.2B6, 6.5B5, respectively; kindly provided by Dr DO Haskard, Royal Postgraduate Medical School), or 0.1% BSA (negative control), followed by 1:200 biotinylated goat antimouse immunoglobulin, then avidin/biotin complex (both Dakopatts). The plate was developed in OPD (1,2 ortho phenylenediamine dihydrochloride) in citrate phosphate buffer ph5 (see Appendix II) with the addition of hydrogen peroxide. The reaction was stopped by the addition of 1M sulphuric acid, and the plate read at 492nm.

10.2.2.3 Adhesion molecule expression and lipopolysaccharide

In order to avoid false positive results from endotoxin contamination from the LDL preparations, dose response curves to lipopolysaccharide were constructed. Thus the lowest concentration of lipopolysaccharide to stimulate adhesion molecule expression was determined, both in the presence and absence of polymyxin B sulphate 5μg/ml. The antimicrobial actions of polymyxin include the binding to and disruption of lipopolysaccharide within the bacterial cell wall; it may therefore be used as a lipopolysaccharide neutralising agent (Storm et al 1977). However, the resulting increase in cell membrane permeability also renders polymyxin cytotoxic to endothelial cells, and may therefore alter cellular metabolism of LDL (Liao Wet al 1991). The intention was therefore to include polymyxin in assays only if endotoxin levels in the LDL preparations proved to be above those shown to stimulate adhesion molecule expression.

As shown in Figure 10.1, LPS induction of adhesion molecule expression in HUVEC followed a dose response pattern. No expression occurred at concentrations of 0.0001μg/ml (100pg/ml) LPS or less.
Figure 10.7. The effect of lipopolysaccharide on endothelial cell adhesion molecule expression in HUVEC

Significant (p<0.05) VCAM-1, ICAM-1 and E-selectin expression was induced in HUVEC by 0.001 μg/ml LPS, and expression followed a dose response pattern. Adhesion molecule expression was not induced by concentrations of 0.0001 μg/ml (100pg/ml) LPS or less. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.

The addition of 5 μg/ml polymyxin B reduced the sensitivity of the endothelial cells to endotoxin: 0.01 μg/ml (10000 pg/ml) lipopolysaccharide was required to induce ICAM-1 expression, with no induction of either VCAM-1 or E-selectin, as illustrated by Figure 10.2.
Figure 10.2. The effects of polymyxin on lipopolysaccharide stimulation of adhesion molecule expression in HUVEC

Significant (p<0.05) ICAM-1 expression was induced by 0.01μg/ml (10000pg/ml) LPS. No induction of VCAM-1 or E-selectin occurred. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.

Therefore, LDL preparations containing less than 100pg/ml endotoxin would not require the addition of the endotoxin neutralising agent, polymyxin B. As discussed in Chapter 8, the endotoxin content of LDL preparations was less than 30pg/ml in all cases. The addition of polymyxin B to ELISAs was therefore not required, and there could be no spurious results from contaminating endotoxin (Thorne et al 1993).
10.2.2.4 Adhesion molecule expression and CuSO₄, EDTA and fibronectin

Experiments were performed to ensure no stimulation of adhesion molecule expression was produced by contaminants or additions to the LDL preparations. The effects of copper sulphate, EDTA (both BDH Ltd, Dagenham, UK) and fibronectin coating were therefore studied. HUVECs were incubated with the maximum concentrations of CuSO₄ and EDTA to which they would be exposed. No induction of adhesion molecule expression occurred with up to 0.25mM EDTA or 1.66μM CuSO₄. In addition, pre-coating 96-well plates with 2μg/cm² of bovine fibronectin did not influence endothelial cell adhesion molecule expression in response to the cytokines IL-1 or TNFα.

10.2.2.5 Adhesion molecule expression and cytokines

Adhesion molecule expression dose response curves were constructed for the cytokines tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1). Both cytokines were International Standards obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). Since they were all human recombinant DNA types, they were tested for contaminating endotoxin using the Limulus amoebocyte lysate assay, described in Chapter 8. At 100IU/ml, the cytokines were found to contain <6pg/ml (equivalent to <0.06EU/ml) of endotoxin; insufficient to induce adhesion molecule expression.

Figure 10.3 illustrates the typical pattern seen for endothelial cell adhesion molecule expression induced by TNFα. No differences in response to cytokine stimulation were found between venous and arterial endothelial cells.
Expression of VCAM-1, ICAM-1 and E-selectin were significantly induced by TNF\(\alpha\), following a dose response pattern. Experiments (n=6) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.

### 10.2.2.6 Adhesion molecule expression and LDL

The effects of native and modified LDL on adhesion molecule expression in coronary artery and umbilical vein endothelial cells were investigated. Native, minimally modified, copper and macrophage oxidised (see Chapter 8 for methods) LDL were applied to endothelial cells in a range of concentrations from 0.0001-400 \(\mu\)g/ml. Incubation times ranged from 4-24 hours. In addition, experiments were performed to identify any synergistic effects on adhesion molecule expression between TNF\(\alpha\) and native or modified LDL. Cells were incubated with sub-maximal concentrations of TNF\(\alpha\), and the effects of co-incubation with LDL quantified.
10.3 Results

10.3.1 Monocyte-endothelial cell adhesion assay

Both copper-oxidised and minimally modified LDL significantly enhanced monocyte adhesion to coronary artery endothelial cell monolayers in concentrations as low as 10\(\mu\)g/ml. In contrast, native LDL had no effect in concentrations of up to 200\(\mu\)g/ml. The positive controls, IL-1 and TNF\(\alpha\), and LPS all significantly enhanced monocyte adhesion.

The results are illustrated in the following figures. Figure 10.4 and Table 10.i demonstrate the adhesion of monocytes to resting and stimulated coronary artery endothelial cells.

![Graph showing monocyte adhesion to stimulated coronary artery endothelial cells](image)

**Figure 10.4. Monocyte adhesion to stimulated coronary artery endothelial cells**

Monocyte adhesion to endothelial cells stimulated with oLDL and mmLDL 10\(\mu\)g/ml, and with IL-1 10 IU/ml was significantly enhanced (*\(p<0.05\), and **\(p<0.01\), respectively). Experiments (n=4) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.
### Table 10.i. Adhesion of monocytes to stimulated coronary artery endothelial cells

<table>
<thead>
<tr>
<th></th>
<th>Monocyte adhesion (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>TNF 100 IU/ml</td>
<td>18.0</td>
<td>0.003</td>
</tr>
<tr>
<td>LPS 1 ng/ml</td>
<td>17.7</td>
<td>0.002</td>
</tr>
<tr>
<td>oLDL 200 μg/ml</td>
<td>16.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>100</td>
<td>18.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>15.9</td>
<td>0.031</td>
</tr>
<tr>
<td>mmLDL 200</td>
<td>16.3</td>
<td>0.006</td>
</tr>
<tr>
<td>100</td>
<td>15.7</td>
<td>0.007</td>
</tr>
<tr>
<td>10</td>
<td>15.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL 200</td>
<td>9.7</td>
<td>NS</td>
</tr>
<tr>
<td>100</td>
<td>10.1</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>9.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

The percentage of monocytes adherent to endothelial cells stimulated with oLDL or mmLDL 10-200 μg/ml, TNFα, or LPS, was significantly greater than to unstimulated endothelial cells. Native LDL did not significantly increase adhesion above basal levels. Experiments (n=3) were performed in triplicate and statistical analysis was by one way ANOVA.

### 10.3.2 Endothelial cell adhesion molecule ELISA

No concentration of native, minimally modified, copper or macrophage oxidised LDL induced expression of the adhesion molecules VCAM-1, ICAM-1 or E-selectin in coronary artery or HUV endothelial cells, for any of the time courses studied (Thorne et al 1993). In addition there was no synergy between TNFα and native or modified LDL.

Figure 10.5 illustrates typical results.
Figure 10.5. Native and modified LDL do not induce VCAM-1 expression in coronary artery endothelial cells.

In parallel experiments, TNFα 100 IU/ml induced significant VCAM-1 expression (p<0.0001). Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean and statistical analysis was by one way ANOVA.

The modified LDL preparations used in this ELISA had all been shown to enhance monocyte adhesion to endothelial cells (section 10.3.1). The mechanism by which modified LDL enhanced monocyte-endothelial cell adhesion therefore did not involve induction of the adhesion molecules VCAM-1, ICAM-1 or E-selectin.
SECTION II: EXPERIMENTAL

CHAPTER 11
SMOOTH MUSCLE CELL ADHESION STUDIES

SUMMARY

Elevated plasma levels of low density lipoprotein (LDL) are associated with an increased risk of atherosclerotic disease, and oxidative modification of LDL is believed to enhance its atherogenic potential. Modified LDL is thought to be involved in intimal fatty streak formation: the earliest detectable stage of atherosclerosis. The characteristic cells within the fatty streak are lipid laden macrophage foam cells. Foam cells are derived from circulating monocytes, which adhere to the vessel wall before migrating into the intima. This thesis is an in vitro investigation of the interactions between modified LDL and the cells of the human coronary artery wall: endothelial and smooth muscle cells, and of the role of modified LDL in the adhesion of monocytes to these cells. The isolation, culture and characterisation of human endothelial cells derived from coronary artery, aorta, endocardium, saphenous and umbilical vein, and the cardiac microvasculature is described. In addition, the isolation, culture and characterisation of human coronary artery and aortic smooth muscle cells is reported. Techniques in the isolation and characterisation of low pyrogenicity human LDL are presented, and comparisons are made between the diverse methods of oxidatively modifying LDL. An investigation of the cytotoxic effects of native and modified LDL on endothelial and smooth muscle cells is reported, as are the effects of LDL on proliferation and migration in these cells.

The induction of monocyte adhesion to endothelial and smooth muscle cells which have been stimulated by native or modified LDL is investigated, and studies of the role of adhesion molecules in this process are presented.
CHAPTER 11
SMOOTH MUSCLE CELL ADHESION STUDIES

11.1 Introduction
As discussed in Chapter 5, there is histological evidence of smooth muscle ICAM-1 expression in human atherosclerotic plaques (Poston et al 1992), and VCAM-1 expression is present in the plaques of atherosclerotic rabbits (Cybulsky et al 1991b). Furthermore, cytokines induce ICAM-1 and VCAM-1 respectively, in human uterine artery smooth muscle cells in vitro, and baboon dermal smooth muscle cells in vivo (Stemme et al 1992, Briscoe et al 1992).

Smooth muscle cells are usually regarded as effector cells in the pathogenesis of atherosclerosis, responding to atherogenic stimuli by migration and proliferation. However, the above observations lead to speculation that intimal smooth muscle cells may facilitate monocyte adhesion by the expression of adhesion molecules, and thus play an active role in monocyte recruitment and foam cell formation.

An investigation of the effects of LDL and cytokines on monocyte adhesion to coronary artery and aortic smooth muscle cells, and on smooth muscle cell adhesion molecule expression is therefore the subject of this chapter.

11.2 Methods
11.2.1 Monocyte-smooth muscle cell adhesion assay
This assay quantified the adhesion of \(^{51}\)Cr labeled peripheral blood monocytes to coronary artery and aortic smooth muscle cells stimulated by native, minimally modified, or copper-oxidised LDL, or the cytokines TNF\(\alpha\) or IL-1. Statistical analysis of data was performed by one way ANOVA.
Monocyte isolation and labeling was performed as described in Chapter 11, as was the adhesion assay, except that smooth muscle cells were incubated in DMEM with 10% FCS.

11.2.2 Smooth muscle cell adhesion molecule ELISA
The adhesion molecule ELISA described in Chapter 10 was used to investigate the effects of native and modified LDL, and of IL-1 and TNFα, on the expression of VCAM-1 and ICAM-1 by coronary artery and aortic smooth muscle cells. E-selectin was not investigated, being expressed solely by activated endothelium. Statistical analysis of data was performed by one way analysis of variance. The technique used was the same as that described in Chapter 10, except that the incubation medium used was DMEM with 10% FCS.

11.3 Results
11.3.1 Monocyte-smooth muscle cell adhesion assay
Copper-oxidised and minimally modified LDL enhanced the adhesion of monocytes to coronary artery and aortic smooth muscle cells, whereas native LDL had either a lesser, or no effect. IL-1 and TNFα both enhanced monocyte adhesion to smooth muscle cells.

Typical results are illustrated by Figures 11.1 and 11.2, and Table 11.i. Table 11.i and Figure 11.1 illustrate the adhesion of unstimulated monocytes to resting and LDL-stimulated coronary artery smooth muscle cells. Figure 11.2 demonstrates unstimulated monocyte adhesion to TNFα-stimulated aortic smooth muscle cells.
<table>
<thead>
<tr>
<th></th>
<th>Monocyte adhesion (%)</th>
<th>p value</th>
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<tbody>
<tr>
<td>Basal</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>TNF 100 IU/ml</td>
<td>17.8</td>
<td>0.008</td>
</tr>
<tr>
<td>oLDL 200 μg/ml</td>
<td>11.8</td>
<td>0.016</td>
</tr>
<tr>
<td>100</td>
<td>10.4</td>
<td>0.011</td>
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<tr>
<td>10</td>
<td>8.6</td>
<td>NS</td>
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<tr>
<td>mmLDL 200</td>
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<tr>
<td>100</td>
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<td>6.9</td>
<td>NS</td>
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<td>10</td>
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Table 11.i. Adhesion of monocytes to stimulated coronary artery smooth muscle cells
The percentage of monocytes adherent to smooth muscle cells stimulated with oLDL or mmLDL 100-200μg/ml, or TNFα, was significantly greater than to unstimulated smooth muscle cells. Native LDL did not significantly increase adhesion above basal levels. Statistical analysis was by one way ANOVA.
Figure 11.7 Monocyte adhesion to LDL-stimulated coronary artery smooth muscle cells

Monocyte adhesion to smooth muscle cells stimulated with oLDL and mmLDL 1μg/ml was significantly enhanced (*p<0.01 and **p<0.001, respectively) compared to unstimulated cells. Significantly increased monocyte adhesion was observed for all concentrations of oLDL and mmLDL tested, and was comparable to that induced by IL-1. Native LDL caused a lesser increase in adhesion at a concentration of 100μg/ml and above (*p<0.01). Experiments (n=4) were performed in triplicate. Error bars are standard deviation from the mean.

Statistical analysis was by one way ANOVA.
Figure 11.2. Monocyte adhesion to TNFα stimulated aortic smooth muscle cells
Monocyte adhesion was significantly enhanced by TNFα, following a dose response pattern. Experiments (n=3) were performed in triplicate. Error bars are standard deviation from the mean and statistical analysis was by one way ANOVA.
11.3.2 Smooth muscle cell adhesion molecule ELISA

The cytokines TNFα and IL-1 induced VCAM-1 and ICAM-1 expression in smooth muscle cells derived from both normal and atherosclerotic coronary artery and aorta, as shown in Figures 11.3 and 11.4.

Neither native or modified LDL induced adhesion molecule expression in smooth muscle cells, in concentrations of up to 200 μg/ml. The modified LDL preparations used in this ELISA had been shown previously to induce monocyte-smooth muscle cell adhesion. Therefore, the mechanism by which this occurred did not involve induction of the adhesion molecules VCAM-1 or ICAM-1.

![Graph showing absorbance at 492nm vs TNFα (IU/ml)]

**Figure 11.3.** TNFα-induced adhesion molecule expression in aortic smooth cells

The expression of VCAM-1 and ICAM-1 were significantly induced by TNFα, following a dose response pattern. Experiments (n=4) were performed in triplicate. Error bars are standard deviation from the mean. Statistical analysis was by one way ANOVA.
Figure 11. IL-1-induced adhesion molecule expression in coronary artery smooth muscle cells

The expression of VCAM-1 and ICAM-1 were significantly induced by IL-1, following a dose response pattern. Experiments (n=4) were performed in triplicate. Error bars are standard deviation from the mean and statistical analysis was by one way ANOVA.
12.1 Human endothelial cell culture

12.1.1 Endothelial cell isolation and culture

Endothelial cells derived from the human coronary artery and aorta are involved in the atherogenic process; it was therefore of value to study them in the investigation of atherosclerosis, rather than endothelial cells from other tissues or species. There are, however, difficulties inherent in human cell culture compared to that of animals. Such difficulties included the limited, and often unpredictable availability of human tissue, and the fact that, at least for cells of cardiac origin, the tissues obtained were adult and frequently abnormal.

Nonetheless, sufficient pure endothelial cell cultures from normal coronary artery and aorta were achieved to allow their characterisation and use in adhesion studies.

Further evidence supporting the use of coronary artery endothelial cells in the study of coronary atherosclerosis was the observation of heterogeneity between coronary artery and other large vessel endothelial cells in culture. Coronary artery endothelial cells were more fastidious in their culture requirements than other large vessel endothelial cells. Although they proliferated at comparable rates in the same growth media, coronary cells were not able to withstand short periods under serum free conditions, unlike aortic, umbilical and saphenous vein endothelial cells. Furthermore, unlike aortic and umbilical vein endothelial cells, which demonstrated a cobblestone morphology when confluent, coronary artery endothelial cells formed a less regular pattern. In addition, although all endothelial cells could be induced to undergo \textit{in vitro} differentiation on Matrigel, coronary artery endothelial cells also
spontaneously differentiated on uncoated dishes if left at confluence for >24 hours, a phenomenon not observed with other large vessel endothelial cells.

HUVEC were used routinely in establishing assay techniques since they were readily available and pure in primary culture. Endothelial cells from saphenous vein showed no heterogeneity of response to cytokine induction of adhesion molecule expression (as described in Chapter 10), compared with the HUVEC, and were therefore not routinely used in adhesion studies. In addition, although endocardial endothelial cells were successfully cultured, the limited availability of specimens precluded their use in adhesion studies.

The endothelium is readily removed, and surgical handling of sections of aorta and saphenous vein may have accounted for the failure to isolate endothelial cells from some of the 10-20% of specimens where culture was unsuccessful. Such intra-operative disturbance of saphenous vein endothelium may have a bearing on vein graft survival. The inability to isolate and culture endothelial cells from LIMA segments may also have been partly due to handling of the artery during its dissection from the chest wall. However, other contributing factors are likely to have been the small vessel calibre, and the short lengths (1-1.5cm) of the segments available.

Capillary endothelial cells from human heart were isolated, cultured and characterised, as discussed in Chapter 6. This has not been described by other workers (Thorne et al 1991). However, insufficient cells were obtained to perform experiments. Possible reasons for this include the low initial cell yield, such that each cell had to undergo many mitoses to produce a 35mm diameter confluent monolayer. As a result, some cells might have lost differentiated characteristics or become senescent in early passage cultures. Furthermore, low initial cell seeding densities might have permitted the selection of a subpopulation of cells capable of repeated division and therefore not representative of the whole capillary bed.
In addition, specimens of papillary muscle and ventricle were not normal tissues. The papillary muscles were often fibrotic, being chiefly from hearts with rheumatic valve disease, and the ventricular tissue was from cardiac explants with cardiomyopathy or ischaemic ventricular disease. In contrast, atrial appendages obtained during coronary artery bypass surgery were not usually affected by ischaemic disease, and this may explain the relatively greater success in culturing atrial appendage capillary endothelial cells.

Nevertheless, there was evidence that the cells established in culture were not only endothelial, but also microvascular in origin. Firstly, they demonstrated vWF immunoreactivity. Secondly, as described in Chapter 6, macroscopic vessels were excised, and endocardium either excised or rendered non-viable with absolute alcohol, making contamination by large vessel endothelium unlikely. Thirdly, the isolated endothelial cells had more fastidious growth requirements than those derived from large vessels or endocardium, requiring 40% foetal calf serum and a three fold higher concentration of endothelial cell growth factor to support proliferation.

Primary cultures of coronary artery and aortic endothelial cells were frequently contaminated with smooth muscle cells, even when there was no visible atherosclerosis in the vessel of origin, perhaps reflecting the intimal smooth muscle cell population present in normal human arteries (Stary 1989).

Manual weeding of non-endothelial cells from primary cultures produced cultures with endothelial morphology. However, the endothelial morphology may be taken by other cell types, and therefore endothelial cell specific magnetic cell sorting methods were also used.

The magnetic beads could not be reproducibly removed from the endothelial cells, raising the question that the continued presence of the beads might activate the cells,
despite their normal growth pattern in culture. E-selectin is expressed exclusively by activated endothelial cells. Therefore, the demonstration by ELISA of the absence of E-selectin upregulation in endothelial cells attached to beads indicated that the beads did not activate the cells.

12.1.2 Endothelial cell characterisation

The demonstration of a cobblestone morphology, binding to magnetic beads recognising the CD31 antigen and the lectin UEA-1, and the uptake of fluorescently labeled acetylated LDL allowed cells in culture to be identified as endothelial. The formation of capillary-like tubules is considered to be the most reliable means of identifying endothelial cells (Folkman et al 1980). As discussed in Chapter 6, although all endothelial cells differentiated on a Matrigel substrate, in vitro differentiation on uncoated plates enabled the demonstration of heterogeneity between coronary artery and other large vessel endothelial cells.

Only a subset of endothelial cells expressed CD34, as assessed by QB-END/10 immunocytochemistry, despite having been characterised as endothelial by vWF immunoreactivity. CD34 expression could therefore not be used to characterise endothelial cells in culture. However, the pattern of CD34 expression was of interest. As discussed in Chapter 6, CD34 positive endothelial cells had the appearance of migrating cells, overlying the monolayer, and with bidirectional processes. Expression was proportional to the degree of confluence, but not increased by stimulation with LDL or TNFα, a finding supported by Schlingemann et al 1990. The morphological appearances of CD34 positive cells lead to speculation that CD34 might represent an adhesion molecule, and be involved in cell migration. The observations that CD34 expression is localised to the luminal cell surface, to interdigitating surfaces between endothelial cells, and to cellular microprocesses (Fina et al 1990), and is expressed by the endothelia of vascular tumours (Anthony et
al 1991, Schlingemann et al 1990) provide indirect evidence in support of this hypothesis.

The demonstration of vWF immunoreactivity provided a rapid and convenient method of characterising endothelial cells. Detection of vWF secretion and E-selectin expression allowed the functional assessment of endothelial cells in culture. The detection of von Willebrand factor secretion by ELISA demonstrated the constitutive vWF release characteristic of endothelial cells (Jaffe et al 1974), and their rapid increase in release of vWF after stimulation by PMA to cause Weibel Palade body exocytosis.

There are several possible reasons as to why secretion of vWF by endothelial cells on PDVF membrane (cell blot assay) was not demonstrated. vWF released by the cells may not have absorbed onto the PVDF membrane, or vWF may have absorbed onto the membrane in a conformation masking the antigenic epitope. However, neither of these explanations is likely in view of the positive staining achieved with the dot blots. It is more likely that the cells adherent to the PVDF membrane did not actually release vWF, either basally, or in response to thrombin. After 22 hours incubation on the membrane, the cells, though adherent, remained rounded and did not spread or divide. Thus the cytoskeleton would be condensed and the cells at a low basal metabolic rate, rendering Weibel Palade body exocytosis and vWF release impossible. In conclusion, this method of cell blot analysis, using PVDF membrane is unsuitable for the detection of peptide secretion by endothelial cells.

No basal expression of E-selectin was detected in cultured endothelial cells by ELISA, indicating that the cells were not activated by their culture conditions. Cultured endothelial cells showed the characteristic response to activation by cytokines or LPS, expressing E-selectin within 4 hours of stimulation.
12.1.3 Endothelial cell culture summary

The cell culture methods described herein provided pure endothelial cell cultures to establish techniques in the investigation of atherogenesis. As a result of the isolation and culture of human coronary artery and aortic endothelial cells, the studies of atherogenesis described in this thesis were performed on cells derived from vessels subject to atherosclerosis.

12.2 Human arterial smooth muscle cell culture

12.2.1 Culture and characterisation of smooth muscle cells

As was the case for endothelial cell culture, there were difficulties inherent in the culture of smooth muscle cells from adult human vessels subject to atherosclerosis. Not only was the availability of relevant tissues (coronary artery and aorta) limited, but also normal and atherosclerotic segments of vessel had to be separated prior to culture.

Furthermore, the slow initial growth pattern observed in these cells was typical of adult human vascular smooth muscle cells in culture (Smirnov et al 1990), with primary cultures reaching confluence in 3-4 weeks. Thus cell culture studies using adult human smooth muscle cells take longer to prepare than those using animal cells, primary rabbit aortic smooth muscle cell cultures reaching confluence in approximately 10 days (Fallier-Becker et al 1990).

The explant technique proved a reliable means of obtaining smooth muscle cells in culture from small tissue specimens. However, inherent in this technique is the possibility that a subpopulation of cells capable of migrating from the explant may be selected, and this should be considered when interpreting results.

The smooth muscle cell population which becomes established in culture is largely of the synthetic phenotype, and secretes type I collagen. This compares to a
predominance of type III collagen in the normal human aorta, and type I collagen in atherosclerotic lesions (Chamley-Campbell et al 1979). Furthermore, the synthetic phenotype of smooth muscle cell predominates in atherosclerotic lesions (Campbell et al 1990). Cultured human arterial smooth muscle cells therefore provide an appropriate model for the study of atherogenesis.

The finding of early senescence in cells from atheromatous areas is in accordance with Ross et al (1984), who noted that smooth muscle cells from atherosclerotic lesions became senescent by the 2nd passage, compared with about the 7th passage for cells from non-atheromatous areas of the same vessel.

Smooth muscle α-actin and desmin immunoreactivity demonstrated that primary smooth muscle cell cultures were consistently pure, with no significant contamination by other cell types. Despite their higher proliferation rates in culture, endothelial cells were not significant contaminants; this was due in part to their removal by collagenase digestion prior to processing the tissue of origin for smooth muscle cell culture. The growth of any remaining endothelial cells was slowed by the use of a selective growth medium (DMEM), containing only 10% foetal calf serum, no endothelial cell growth factor, and no smooth muscle cell-inhibiting heparin.

12.2.2 Smooth muscle cell culture summary
The tissue culture methods described in this thesis provided pure smooth muscle cell cultures derived from human coronary artery and aorta, for the study of atherogenesis.

12.3 Low density lipoprotein
12.3.1 Isolation and characterisation of endotoxin free LDL
The isolation procedure described in Chapter 8 was performed rapidly at 4°C in the presence of EDTA; all factors which minimised the possibility of spontaneous LDL
oxidation. The total centrifuge time was 10 hours at 55 000 rpm, or 5 hours at 65 000 rpm, and compares favourably with that of other investigators, for example, 36 hours described by Morel et al (1984).

The isolated LDL was shown not to be contaminated with the lipoprotein fractions VLDL and HDL. These lipoproteins would have interfered with subsequent biological and biochemical assays, as discussed in Chapter 3.

Contamination of LDL preparations by bacterial lipopolysaccharide (LPS, or endotoxin), may invalidate cell culture experiments, since LPS is a potent activator of cells, and is cytotoxic in high concentrations. This is illustrated by the induction of endothelial tissue factor by LPS (Drake et al 1991), by enhanced leucocyte-endothelial adhesion (Bevilacqua et al 1987), and as has been shown in this thesis, the induction of both monocyte adhesion to endothelial cells and endothelial adhesion molecule expression in concentrations as low as 1ng/ml (Chapter 10).

Chapter 8 described the techniques used to achieve endotoxin levels (<30pg/ml) in LDL preparations insufficient to stimulate cells. It is noteworthy that merely applying an aseptic technique did not prevent significant LPS contamination. This was due to the fact that cellulose dialysis tubing contains large amounts of endotoxin which is neither inactivated by standard sterilisation procedures such as boiling, nor removed by filter sterilisation using a 0.2µm mesh. The use of low endotoxin sterile cellulose ester dialysis membrane (produced to order by Spectrum Medical Industries, Inc, LA, USA), and its minimal handling by starch and pyrogen free sterile gloves, eliminated significant LPS contamination.

A review of the literature suggests that some workers, when investigating the biological effects of LDL, have not considered the possibility of endotoxin contamination of their LDL preparations; their results should therefore be interpreted
with caution. Berliner's group achieved low endotoxin levels in their studies of mmLDL and monocyte transmigration (Berliner et al. 1990, Cushing et al. 1990, Drake et al. 1991, Liao F et al. 1991, Navab et al. 1991, Navab et al. 1993), and Murugesan et al. (1993) assessed LDL endotoxin levels in their investigation of aortic endothelial cell migration. Other groups (Boulanger et al. 1992) comment that they used sterile techniques in the isolation of LDL, but as discussed above, this does not preclude significant LPS contamination.

Other investigators make no mention of LPS contamination and the possible introduction of this confounding factor limits interpretation of the results. For example, Quinn et al. (1987), and Frostegård et al. (1991) demonstrated that modified LDL enhanced monocyte recruitment and adhesion to cultured endothelial cells, phenomena which could be influenced by contaminating endotoxin. Other workers who apparently did not consider or mention LPS contamination of LDL when studying its effects on cells in culture include Steinbrecher (1988), Parthasarathy et al. (1989), Autio et al. (1990), Jessup et al. (1991) and Sparrow et al. (1992a).

12.3.2 Oxidative modification of LDL

LDL is a heterogeneous molecule, and the oxidative modification of its different components was monitored by a variety of techniques.

Copper initiated oxidation of LDL was monitored by a fall in β-carotene content as its intrinsic antioxidants became exhausted. This was accompanied by a rise in conjugated dienes, representing lipid peroxidation, and an increase in MDA (malonaldehyde), an end product of lipid peroxidation. Modification of the apo B moiety of LDL, rendering it recognisable by the scavenger receptor, was reflected by its increased electrophoretic mobility.
To perform the above biochemical techniques in assessing the oxidation status of macrophage modified LDL would have required its re-isolation from small volumes of cell culture media. It was therefore assessed biologically rather than by these biochemical methods. Lipid peroxidation precedes protein modification in the oxidation of LDL. Therefore, the demonstration that the protein moiety of the LDL was modified to a form recognised by macrophage scavenger receptors, confirmed that the LDL had been oxidised.

MmLDL was assessed by an increase in MDA, and the absence of a rise in conjugated dienes or increased electrophoretic mobility, the latter reflecting the unaltered nature of its protein moiety. In addition, the rise in MDA was less than half that seen for copper oxidised LDL, confirming that its modification was indeed minimal.

The 3 techniques used to oxidatively modify LDL thus resulted in modifications with potentially different biological activities. Both copper and macrophage oxidised LDL were sufficiently modified to be recognised by the scavenger receptor; however the latter may have been a more physiological modification and thus more likely to occur in vivo. The biological effects of mmLDL, recognised by the native LDL receptor, would be due to minor modifications of its lipid constituents.

### 12.3.3 LDL Summary

The techniques described in this thesis to isolate and modify LDL resulted in preparations which were uncontaminated by endotoxin or other lipoprotein fractions, and which had potentially diverse biological activities with which to study the process of atherogenesis.
12.4 LDL-cell interactions

Techniques to determine the cytotoxic, proliferative and migratory effects of native and modified LDL on both endothelial and smooth muscle cells were the subject of Chapter 9, which confirmed that modification of LDL conferred upon it marked alterations in biological activity.

12.4.1 Cytotoxicity

The \(^{51}\text{Cr}\) release assay demonstrated significant cytotoxic effects by 400\(\mu\text{g/ml}\) oLDL on both endothelial and smooth muscle cells. This concentration compares with normal plasma LDL levels of 600-700\(\mu\text{g/ml}\) (Chapter 3.4). Circulating antioxidants would protect the cells of the vessel wall from exposure to such high levels of oxidised LDL. However, within the microenvironment of the early atherosclerotic plaque it is possible to speculate that antioxidants could be consumed by the actions of activated macrophages and endothelial cells, and significant concentrations of oxidatively modified LDL accumulate.

It is of interest that some \(^{51}\text{Cr}\) release experiments also showed that 400\(\mu\text{g/ml}\) native LDL caused significant smooth muscle cell cytotoxicity, albeit significantly less than that caused by oLDL. There was no evidence that the preparation had become oxidised, and the serum present in the culture medium would have prevented smooth muscle cell mediated oxidation of the LDL. Such a finding may therefore either represent increased sensitivity of smooth muscle cells to LDL, or heterogeneity between LDL preparations.

Visual observations made during the \(^{51}\text{Cr}\) release assay showed that changes in cell morphology (rounding up of cells) allowed the detection of cytotoxicity at lower LDL concentrations than did the \(^{51}\text{Cr}\) release assay. Thus although only semi-quantitative, morphological change was a more sensitive method of detecting LDL
cytotoxicity, occurring in endothelial cells incubated in 200\,\mu g/ml of oxidised, and to a lesser extent, minimally modified, LDL.

12.4.2 Proliferation

The studies described in this thesis investigated the effects of native and oLDL (0.1-100\,\mu g/ml) on endothelial and smooth muscle proliferation using the BrDU DNA synthesis assay. Cellular DNA synthesis was inhibited by 100\,\mu g/ml oLDL and to a lesser extent, by mmLDL, with no effect produced by native LDL. Thus proliferation assays might be considered the most sensitive form of cytotoxicity assay.

This contrasts with results reported by Stiko-Rahm et al (1992), who described enhanced DNA synthesis and PDGF gene expression in smooth muscle cells exposed to both native and o-LDL. Several methodological differences may explain this apparent disparity. Firstly, the cells used by Stiko-Rahm et al had been subject to prolonged periods of culture and were used at the 8-16th passages. This may have resulted in changes in the growth characteristics of the cells. In contrast, all the cells used in the experiments described in this thesis were before their 3rd passage. Secondly, the pro-proliferative effects seen by Stiko-Rahm et al were transitory, disappearing after 4 hours of LDL stimulation, whereas the experiments describe in Chapter 9 of this thesis exposed the cells to LDL for up to 17 hours. The latter time scale may be more applicable to effects seen \textit{in vivo}. Lastly, the cells used by Stiko-Rahm et al were serum starved, in comparison to those described herein. As a result of serum starvation, the cells were also cholesterol-starved, since the culture medium (Ham's F12, Gibco) is devoid of cholesterol. Thus the return of cholesterol, in the form of LDL, to the growth medium, might itself be expected to enhance indices of proliferation.

In addition, Harris-Hooker \textit{et al} 1992 reported that native LDL had diverse effects on smooth muscle cell proliferation, dependent upon the nature of the extracellular...
matrix. Although the extracellular matrix may influence cellular morphology and hence cellular responses to factors such as LDL, other factors may have influenced the results obtained by Harris-Hooker et al. Firstly, their LDL preparations may have been contaminated with endotoxin, thus causing cell activation or cytotoxicity. Secondly, as was the case in the study described by Stiko-Rahm et al above, the cells were serum starved in a growth medium (DMEM, Gibco) which did not contain cholesterol.

Although smooth muscle cell proliferation was inhibited by oLDL in the experiments described herein, a monoculture system was used, and it is possible to speculate that within the atherosclerotic plaque, oLDL may activate other cell types (macrophages and endothelial cells) to release smooth muscle mitogens such as PDGF.

12.4.3 Migration

Experiments to investigate the effects of LDL on cell migration were performed on endothelial, but not smooth muscle cells. Smooth muscle cells proved unsuitable for use in the migration assay described in Chapter 9; because of their large size, and their strong intercellular connections, it was not possible to remove a strip of cells and leave "wound" edges which could be monitored by a photomicrographic field. Alternative methods, such as that described by Murugesan et al (1993), in which a razor was used to wound the monolayer were also unsuccessful. Not only did this result in injured cells at the wound edge, but it was also difficult to avoid scoring the tissue culture plastic surface and creating an obstacle over which the cells had to migrate.

Oxidised LDL significantly inhibited endothelial cell migration, native and mm- LDL had no effect. It is therefore possible to speculate that oLDL may inhibit endothelial cell migration in vivo, thus preventing the re-endothelialisation of areas of coronary artery denuded of endothelium during the atherogenic process.
LDL was found to have no effect on CD34 expression by endothelial cells, although the LDL preparations and incubation times used were sufficient to enhance monocyte adhesion in parallel experiments. Therefore, CD34 does not act as an o-LDL-induced monocyte-endothelial cell adhesion molecule.

15.4 Summary of LDL-cell interactions
The inhibition by oLDL of endothelial proliferation and migration described in this thesis might further contribute to the denudation of endothelium overlying atheromatous plaques, thus exposing the intima to procoagulant and smooth muscle growth factors. Such events may enhance the progression of the atherosclerotic process.

The studies described in this part of the thesis assessed the effects of LDL on basic cellular functions and cytotoxicity, and provided a background on which adhesion studies were performed and interpreted.

12.5 Endothelial cell adhesion studies
Since oxidatively modified LDL is implicated in the recruitment of monocytes to the intima in atherosclerosis, the studies described in Chapter 10 were performed. They investigated the role of LDL in the adhesion of monocytes to the human coronary artery endothelium.

12.5.1 Monocyte-endothelial cell adhesion studies
The monocyte isolation procedure was designed to avoid monocyte activation. It was rapid, performed at 4°C, and minimised manipulation and centrifugation of the cells by the use of negative selection of monocytes from the mononuclear cell population by magnetic cell sorting. The low basal adhesion of the monocytes to endothelial cells confirmed that they had not been activated by the isolation or labeling procedures. CD25 is expressed by activated, but not quiescent monocytes, future
studies may use monoclonal antibodies directed against CD25 as a further method to remove any activated cells.

Oxidised and minimally modified LDL enhanced monocyte adhesion to human coronary artery endothelial cells, whereas native LDL had no effect. Although this phenomenon has been reported in human endothelial cells (Navab et al 1991), its demonstration in human coronary artery endothelial cells is a new observation. Adhesion occurred at concentrations of modified LDL (10μg/ml), considerably lower than normal plasma LDL concentrations (600-700μg LDL protein/ml plasma). It is therefore biochemically possible that the adhesion induced by modified LDL between monocytes and endothelial cells in vitro, may also occur in vivo.

Observations which led to the investigation of the effect of LDL on endothelial cell adhesion molecule expression were as follows. Firstly, TNFα, IL-1, LPS and modified LDL all enhanced monocyte-endothelial cell adhesion; the first 3 agents are also known inducers of endothelial cell adhesion molecule expression. Secondly, there is evidence of adhesion molecule expression in atherogenesis, detailed in Chapter 5. The adhesion molecules VCAM-1, E-selectin and ICAM-1 were investigated. The expression of VCAM-1 was of greatest interest, since this molecule mediates the adhesion of monocytes and lymphocytes, whereas E-selectin and ICAM-1 also mediate neutrophil adhesion. Since monocytes, some T lymphocytes, but not neutrophils are found in fatty streaks (Tsukada et al 1990), it was hypothesised that the main adhesion molecule involved might be VCAM-1. No form of LDL induced VCAM-1, ICAM-1 or E-selectin expression in any of the endothelial cell types studied (in agreement with the findings of JA Berliner, personal communication). Cytokines and LPS did induce adhesion molecule expression, confirming the validity of the assay.
Therefore, the mechanism by which modified LDL enhanced monocyte adhesion to the human coronary endothelium did not involve the direct induction of VCAM-1, ICAM-1 or E-selectin. The consideration of future studies to elucidate the mechanism by which modified LDL enhances monocyte adhesion to the endothelium is the subject of Chapter 13.

12.5.2 Summary of monocyte-endothelial cell adhesion studies

The studies described in this thesis support a role for modified LDL in atherogenesis. They have demonstrated that modified LDL enhances monocyte adhesion to the human coronary endothelium. The mechanism does not involve the direct induction of the adhesion molecules VCAM-1, ICAM-1 or E-selectin.

12.6 Smooth muscle cell adhesion studies

Poston et al (1992) observed that adhesion molecule expression within the human atherosclerotic plaque is not confined to the endothelium and infiltrating leucocytes, raising the possibility that other cells may also be involved in the recruitment of monocytes to the intima. Studies investigating whether human coronary artery smooth muscle cells could support monocyte adhesion and express adhesion molecules, and the role of LDL in these processes, were therefore the subject of Chapter 11.

12.6.1 Monocyte-smooth muscle cell adhesion studies

Human coronary artery smooth muscle cells could be stimulated to support the adhesion of unstimulated monocytes; this has not been reported previously. Monocyte adhesion was induced by cytokines, by both oxidatively and minimally modified LDL, and to a significantly lesser degree, by native LDL. This phenomenon was observed at modified LDL concentrations as low as 1μg/ml, implying, as discussed in 12.5.1, that it could occur in vivo.
At sites where the endothelium overlying areas of early atherosclerotic change is breached (Davies et al 1988), intimal smooth muscle cells are exposed to circulating monocytes. They are also exposed to modified LDL within the intima, and to cytokines released from activated mononuclear cells and endothelial cells. As a result of the studies described in this thesis it is therefore hypothesised that smooth muscle cells play an active role in monocyte recruitment to the early atherosclerotic plaque, by mediating monocyte-smooth muscle cell adhesion.

The mechanism by which modified LDL and cytokines induced smooth muscle cells to support the adhesion of unstimulated monocytes was subsequently investigated. It was hypothesised that modified LDL and cytokines might enhance monocyte-smooth muscle cell adhesion by inducing smooth muscle VCAM-1 and ICAM-1; this was investigated by ELISA.

The cytokines TNFα and IL-1 both induced VCAM-1 and ICAM-1 expression in human coronary artery and aortic smooth muscle cells; a finding not reported before. Therefore the mechanism by which cytokines stimulate smooth muscle cells to support monocyte adhesion may involve the induction of ICAM-1 and VCAM-1 expression. However, modified and native LDL had no effect on adhesion molecule expression in smooth muscle cells. The mechanism by which modified LDL induces monocyte-smooth muscle cell adhesion does therefore not involve the direct induction of ICAM-1 or VCAM-1.

12.6.2 Summary of smooth muscle cell adhesion studies

This thesis presents evidence that human coronary artery smooth muscle cells are capable of playing an active role in monocyte adhesion; thus they are not solely passive effector cells in the atherogenic process.
Smooth muscle cell-monocyte adhesion was induced by cytokines and modified LDL, lending further support to the notion that modified LDL is atherogenic. Although VCAM-1 and ICAM-1 were inducible by cytokines, the mechanism by which modified LDL enhanced smooth muscle cell-monocyte adhesion did not involve the direct induction of VCAM-1 or ICAM-1. Further possible mechanisms for LDL-induced monocyte-smooth muscle cell adhesion are explored in Chapter 13.
13.1 Implications of results

This thesis examined the hypothesis that oxidatively modified LDL interacts with human coronary artery endothelial and smooth muscle cells to promote monocyte adhesion and the atherosclerotic process.

Oxidised, and to a lesser extent, minimally modified LDL have been shown to induce cytotoxicity in human coronary artery endothelial and smooth muscle cells, at concentrations to which the cells may be exposed in vivo. The antioxidant activity of plasma may prevent significant concentrations of circulating oLDL. However, the microenvironment of the early atherosclerotic plaque is thought to be pro oxidant; activated macrophages, endothelial and smooth muscle cells are capable of oxidatively modifying LDL, resulting in its avid scavenger receptor-mediated uptake, foam cell formation, attraction of further monocytes, and the further oxidation of LDL. In addition, as macrophage derived foam cells necrose, they release their lipid contents, including oLDL, into the extracellular milieu of the plaque. Thus oLDL may accumulate in the intimal matrix in sufficient concentrations to be cytotoxic and play a role in the focal endothelial denudation observed overlying areas of atheromatous change. This proposed sequence of events is summarised in Figure 13.1.
**Figure 13.1 Oxidatively modified LDL and foam cell formation: a summary.**

LDL is minimally modified and subsequently oxidised to a form recognised by the scavenger receptor by reactive oxygen species (ROS) released from activated cells in an intimal microenvironment protected from circulating antioxidants. The avid uptake of oLDL by macrophage scavenger receptors results in the formation of lipid laden foam cells. The actions of modified LDL include: (i) The induction of MCP-1 release from endothelial cells (EC) and smooth muscle cells (SMC), thus attracting circulating monocytes. (ii) Cytotoxic effects which may contribute to focal endothelial denudation and exposure of intimal smooth muscle cells to the circulation. (iii) The induction of undefined monocyte adhesion mechanisms on the endothelium and exposed smooth muscle cells.
This thesis has presented evidence that modified LDL may enhance monocyte recruitment and subsequent foam cell formation in the human coronary artery intima, by enhancing the adhesive properties not only of the endothelium, but also of intimal smooth muscle. That modified LDL can induce monocyte adhesion to smooth muscle cells is a new finding, implying that coronary artery smooth muscle cells may be more than effector cells in the atherogenic process, responding to stimuli by migration, proliferation and secretion of extracellular matrix. Thus, in areas of endothelial denudation, where intimal smooth muscle cells are exposed to the circulation, smooth muscle cells may be actively involved in the recruitment of monocytes to the intima.

The mechanism by which modified LDL enhances monocyte adhesion to the coronary endothelium and smooth muscle has not been identified; studies presented herein indicate that the adhesion molecules VCAM-1, ICAM-1 and E-selectin are not directly involved. However, both VCAM-1 and ICAM-1 have been demonstrated in atherosclerotic lesions (Cybulsky et al 1991b, Poston et al 1992); these adhesion molecules may be induced by other stimuli (TNFα, IL1-1) to participate in monocyte recruitment to the atherosclerotic plaque.

Since monocyte adhesion can be induced by modified LDL in a single cell-type culture system, the mechanism does not require interactions between several different cell types. Speculations as to possible candidates are considered below.

MCP-1 appears to play a crucial role in monocyte transmigration, and is synthesised by endothelial and smooth muscle cells in response to modified LDL. Although monocyte transmigration across co-cultures of aortic wall cells is inhibited by antibody to MCP-1 (Navab et al 1991), whether MCP-1 influences monocyte adhesion has not yet been defined. MCP-1 does modify monocyte adhesion molecule expression, upregulating the CD11b and CD11c integrin subunits, and it enhances
cytokine release from monocytes (Jiang et al 1992). Whether it also modulates endothelial and smooth muscle cell adhesion molecule expression is not known.

The role of monocyte integrins should also be considered. It is possible that oLDL stimulates changes in the extracellular matrix secreted by smooth muscle cells, and it may be to this, rather than to their immunoglobulin adhesion molecule ligands, that the monocyte integrins bind. Furthermore, if integrin pathways are activated by oLDL, they may not only influence monocyte adhesion, but also monocyte activation, proliferation and differentiation within the plaque via interactions with both extracellular matrix and immunoglobulin ligands (Pardi et al 1992).

Other monocyte adhesion molecules may be involved, such as L-selectin. Its oligosaccharide ligands, the vascular addressins, might be upregulated by oLDL on endothelial cells, thus enhancing adhesion.

Further possibilities exist; the oLDL-induced binding of monocytes to the arterial wall may rely on a sequence of combined adhesion mechanisms, rather than on a single binding event. In addition, adhesion may be mediated by new, as yet undiscovered, pathways.

**13.2 Future work**

The cell culture models described in this thesis may be extended to include other factors which might influence monocyte adhesion in atherosclerosis. Co-cultures of endothelial and smooth muscle cells may be established as a closer approximation to the intimal microenvironment in which monocyte adhesion occurs, allowing not only the formation of an extracellular matrix similar to that of the arterial intima, but also interactions between cytokines, growth and chemotactic factors released by the different cells. The effects of shear stress on adhesion may also be determined using modifications of the static systems described in Chapters 10 and 11. Such
modifications may enable the continued investigation of adhesion pathways induced by oLDL and cytokines.

Studies of the effects of LDL on cell proliferation may be extended to investigate the effects of co-culture and different extracellular matrices. The effects of LDL on smooth muscle cell migration along an LDL concentration gradient may also be investigated.

The mechanism by which oLDL induces monocyte adhesion to endothelial and smooth muscle cells may be further studied at several levels, from the control of gene expression and mRNA transcription, to the cell surface expression of adhesion molecules and the adhesion event itself. The ensuing discussion considers each in turn.

The induction of cellular adhesion mechanisms by modified LDL may be investigated at the level of gene expression. It is the control of gene expression that allows the specific response of a particular cell in producing a protein in response to a stimulus; in recent years it has become apparent that such control occurs largely at the level of transcription of DNA to produce RNA (Latchman 1991). Transcription factors are proteins which bind to DNA sequences and control the expression of the gene to which they bind. Examples include the protein products of the oncogenes c-jun, c-fos and c-myb. Jun and Fos are transiently expressed to induce the activation of growth regulatory genes, whereas Myb activates genes to allow continued cellular proliferation (Latchman 1991). Of note, c-myb expression has been implicated in the smooth muscle hyperplasia associated with restenosis following balloon angioplasty (Simons et al 1992).

Of particular interest however, is the effect of modified LDL on the activation of the transcription factor, NF-κB (nuclear factor κB). NF-κB is a DNA-binding protein.
which controls the expression of certain genes. It is present in many cell types, including endothelial cells, lymphocytes and monocytes. NFκB may play an important role in inflammatory disease: it is activated by inflammatory mediators including IL-1, TNFα, LPS and by radical species (H₂O₂) (Schreck et al 1991), as illustrated in Figure 13.2. Furthermore, its activation induces the transcription of genes involved in inflammatory processes, including those coding for the immunoglobulin κ light chain, IL-2, IL-6, TNFα, and some acute phase and viral proteins (Baeuerle et al 1991, Blank et al 1992). In addition, NF-κB has been implicated in controlling the expression of the genes for the adhesion molecules VCAM-1, ICAM-1 and E-selectin in HUVECs (Montgomery et al 1991, Iadecarlo et al 1992, Mackay et al 1993).

NF-κB is a heterodimer, comprising a 50kDA DNA-binding subunit, and a 65kDA IκB-binding subunit. It exists in an inactive form in the cytosol of endothelial cells, bound to its inhibitor, the protein IκB (Baeuerle et al 1991, Blank et al 1992). On stimulation of the cell, NF-κB is activated by separating from IκB. The active NF-κB is translocated to the nucleus, where it binds to κB motifs present in DNA enhancer sequences, thus inducing gene transcription, resulting in mRNA synthesis. The mRNA may subsequently be translated to allow protein synthesis (Baeuerle et al 1991, Blank et al 1992), as shown in Figure 13.2.

Since reactive oxygen species have been shown to activate NF-κB, and NF-κB activation is involved in the expression of genes encoding for some known adhesion molecules, it may be speculated that the lipid peroxide radical species of modified LDL might activate NF-κB and initiate the transcription of genes encoding new adhesion molecules. This hypothesis is illustrated in Figure 13.2.
Figure 13.2. NF-κB activation and gene transcription in endothelial cells

Inactive cytosolic NF-κB -IκB is activated by cytokines, reactive oxygen species, and, as indicated by studies in this thesis, by oLDL. The activated NF-κB is translocated to the nucleus where it binds to κB motifs on DNA enhancer sequences and signals RNA polymerase and hence transcription. The mRNA is subsequently translated and may result in adhesion molecule expression.
Preliminary experiments have been undertaken to investigate this hypothesis. Results from DNA electrophoretic mobility shift assays performed on HUVEC stimulated for 2 hours by LDL, indicated that 10μg/ml oLDL activates NF-κB to an extent comparable with that of the positive control (TNFα 100μ/ml). Activation of NF-κB by native LDL occurred to a lesser extent and required higher concentrations (100 μg/ml). NF-κB activation may therefore be the initiating step in the activation of endothelial adhesion mechanisms by oxidatively modified LDL.

That the enhanced adhesion induced by modified LDL involves de novo protein synthesis may be confirmed by experiments using inhibitors of protein synthesis to block adhesion. Techniques used to identify currently known adhesion molecules may be applied in the further identification of an adhesion molecule induced by modified LDL. The identity of endothelial and smooth muscle cell surface proteins induced by modified LDL may be determined by 2 dimensional electrophoresis. This technique involves the biotinylation of cell membrane proteins and their subsequent separation and visualisation by 2 dimensional electrophoresis using an avidin-biotin complex. Monoclonal antibodies raised against the adhesion molecule may be used to immunoprecipitate the protein and define its molecular weight and biochemical properties (de Fougerolles et al 1992), and the protein may be purified by monoclonal antibody immunoaffinity chromatography (Marling et al 1987). Monoclonal antibodies may also be used to quantify cell membrane expression of such a protein by ELISA or immunofluorescence flow cytometry (de Fougerolles et al 1992). Such studies may be facilitated by polymerase chain reaction techniques, used to clone the gene encoding an adhesion protein (Polte et al 1991). The gene may subsequently be used to transfect cell lines, thus providing relatively large amounts of the protein under investigation.

The possible roles of known adhesion molecules such as the integrins and L-selectin, and of MCP-1, in mediating oLDL-induced monocyte adhesion to endothelial and smooth muscle cells may also be further investigated.
Integrin expression in endothelial monolayers has been localised to sites of cell-cell contact, using monoclonal antibodies (Lampugnani et al 1991). The same investigators used monoclonal antibodies to correlate integrin expression with potential integrin ligands: components of the extracellular matrix secreted by the cells. Such techniques may be used to not only to demonstrate sites of integrin expression in monocytes, endothelial and smooth muscle cells, but also to identify the constituents of the extracellular matrix secreted by the endothelial and smooth muscle cells. The effects of modified LDL on integrin distribution and extracellular matrix composition may thus be determined. In addition, monoclonal antibodies directed against integrins and matrix components may be used to block specific adhesion paths, thus allowing their contribution to modified LDL-induced monocyte adhesion to be determined. Similar monoclonal antibody techniques may be applied to the study of a possible role for L-selectin in mediating modified LDL-induced monocyte adhesion to vessel wall cells.

Jiang et al (1992) demonstrated that MCP-1-induced monocyte chemotaxis is inhibited by monoclonal antibodies against β2 and α4 integrin subunits, suggesting that integrins participate in monocyte adhesion. Further studies using monoclonal antibodies may determine whether LDL-induced MCP-1-mediated chemotaxis is associated with the upregulation of other adhesion molecules.

The development of transgenic mice harbouring human genes may enable the development of animal models of "humanised" atherosclerosis; "knockout" mice lacking the gene encoding apolipoprotein E have already been established (Zhang et al 1992). In the future, transgenic mice may provide further useful models for the in vivo investigation of monocyte adhesion mechanisms, using techniques discussed above, such as the selective blockade of adhesion pathway with monoclonal antibodies.
13.3 Future therapies for atherosclerosis

Therapy is aimed at preventing the initiation of atherosclerosis, preventing its progression, and bringing about its regression.

The studies described in this thesis have addressed mechanisms involved in the early, asymptomatic stages of atherosclerosis. Improved understanding of the cellular and molecular mechanisms of atherosclerosis will enable strategies aimed at primary prevention to be devised. Reductions in plasma LDL concentrations may reduce the risk of the initiation and progression of atherosclerosis. Whether antioxidant therapy, aimed at preventing the modification of LDL to its more atherogenic oxidised form, will reduce the incidence of clinically important atherosclerotic disease remains to be seen. Current studies suggest that vitamin E supplementation might reduce the incidence of coronary artery disease in both men and women (Rimm \textit{et al} 1993, Stamper \textit{et al} 1993). However long term, blinded trials confirming this observation are awaited, as are such trials investigating other antioxidants such as ascorbate, β-carotene and probucol.

Since in the early stages of the atherosclerotic process, clinically important disease cannot be localised, any attempt to prevent monocyte adhesion and foam cell formation would have to use a systemic approach. It would be unlikely that targeting adhesion mechanisms to prevent monocyte adhesion to the vessel wall would be without important immunological side effects. It is in secondary prevention, during the later stages of the disease, that new molecular techniques might be applied. The \textit{focal} lesion at risk of restenosis following angioplasty lends itself to molecular interference at the levels of lipid accumulation and oxidation, smooth muscle migration, proliferation and connective tissue production, and monocyte proliferation. Recent developments have focused on the prevention of smooth muscle cell hyperplasia; the techniques described below might also be applied to prevent monocyte proliferation and lipid accumulation.
Restenosis has been prevented in animal models by the use of monoclonal antibodies to smooth muscle mitogens such as PDGF and FGF (Ross 1993). The development of balloon delivery systems (Nabel et al 1993) will allow the precise delivery of molecules to the exact site of the coronary lesion. Thus the effects of antibodies against other smooth muscle mitogens such as TGFβ, inhibitors of smooth muscle cell proliferation such as heparin and IFNγ, and antibodies against MCP-1 and monocyte/macrophage colony stimulating factor may also be studied.

Balloon delivery techniques may also allow the delivery of antisense oligonucleotides to genes involved in smooth muscle cell growth regulation. Antisense c-myb oligonucleotides have been shown to inhibit smooth muscle cell accumulation following angioplasty in the rat (Simons et al 1992). Such methods may be applied to other growth regulatory genes to inhibit smooth muscle cell proliferation and restenosis.

In addition, smooth muscle hyperplasia in porcine arteries has been induced by a gene transfer technique in which the gene encoding PDGF-B was introduced into smooth muscle cells in vitro. In future, the transfer of genes encoding defective growth factors, or inhibitors of proliferation, such as NO synthase or IFN-γ, might reduce smooth muscle cell hyperplasia and restenosis.

There is however, a caveat. Before attempting to inhibit smooth muscle cell hyperplasia following intervention in atherosclerotic vessels, it should be remembered that smooth muscle cell proliferation is the response of the artery to injury, and that plaques with a low smooth muscle cell to lipid ratio are at increased risk of ulcerating, resulting in unstable angina or myocardial infarction (Richardson et al 1989).
13.4 Conclusion

This thesis has aimed to investigate the atherogenic effects of oxidatively modified LDL on the cells of the human coronary artery wall. To this end it has described techniques to achieve pure cultures of human coronary artery endothelial and smooth muscle cells, and to isolate and modify pure human LDL of low pyrogenicity.

Modified LDL has been demonstrated to exert atherogenic effects on these cells, and the role of modified LDL in monocyte adhesion investigated. Furthermore, the new observation that monocyte adherence to coronary artery smooth muscle cells is enhanced by stimulation of the smooth muscle cells by modified LDL has been described.

Modified LDL-induced monocyte adhesion may involve the induction of gene expression by the activation of the transcription factor, NF-κB. It has been shown not to involve the direct induction of the adhesion molecules VCAM-1, ICAM-1 or E-selectin. The mechanism by which modified LDL induces monocyte adhesion to both endothelial and smooth muscle cells therefore remains to be elucidated.
PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS THESIS

Publications


Presentations

In preparation:


Kus M, Thorne SA, Winyard P, Blake DR. The isolation of plasma and synovial low density lipoprotein by near vertical rotor ultracentrifugation. Targetted journal: FEBS LETTERS.

176
APPENDIX I

Cell culture media and reagents

Media were obtained from Gibco (Life Technology), Paisley, UK, foetal calf serum from Advanced Protein Products, Brierly Hill, UK, and heparin from CP Pharmaceuticals Ltd, Wrexham, UK. All other reagents were from Sigma Chemical Co, Poole, UK.

Crude collagenase solution in HBSS

<table>
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<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagenase</td>
<td>0.5%</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.1%</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>100mM</td>
</tr>
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</table>

20% serum containing Medium 199

Medium 199 with additions of:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal calf serum</td>
<td>20%</td>
</tr>
<tr>
<td>Heparin sodium (mucous)</td>
<td>1000u/ml</td>
</tr>
<tr>
<td>Endothelial cell growth supplement</td>
<td>0.03mg/ml</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 000u/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Amphotericin (primary cultures only)</td>
<td>5µg/ml</td>
</tr>
</tbody>
</table>

40% serum containing Medium 199

Medium 199 with additions of:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>Foetal calf serum</td>
<td>40%</td>
</tr>
<tr>
<td>Heparin sodium (mucous)</td>
<td>1000u/ml</td>
</tr>
<tr>
<td>Endothelial cell growth supplement</td>
<td>0.1mg/ml</td>
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<tr>
<td>Penicillin</td>
<td>10 000u/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Amphotericin (primary cultures only)</td>
<td>5µg/ml</td>
</tr>
</tbody>
</table>
10% serum containing Dulbecco's modified Eagle medium (DMEM)

Dulbecco's modified Eagle medium with additions of:

- Foetal calf serum 10%
- Penicillin 10 000u/ml
- Streptomycin 100μg/ml
- Amphotericin (primary cultures only) 5μg/ml
- HEPES buffer 20mM

(N-2- hydroxyethylpiperazine-N'-2-ethanesulphonic acid)
APPENDIX II

Buffers and reagents

All chemicals were obtained from BDH Ltd, Dagenham, UK, unless otherwise stated.

Buffers

All figures for buffer solutions are per litre distilled deionised water.

0.1M acetate buffer pH 6
Sodium acetate 8.2g
Acetic acid to acidify

Barbital buffer pH 8.6
Sodium barbital 6.49g
Diethylbarbituric acid 1.05g
Glycine 28.13g
Sodium azide 0.325g
Tris hydroxymethylamine 22.63g

0.02M Borate buffered saline pH7.2 / tween
Boric acid 1.236g
Sodium chloride 8.774g
Polyoxyethylenesorbitan monolaurate (Tween 20) 0.5ml Sigma Chemical Co, Poole, UK

0.5M Borate buffered saline pH 9.5
Citric acid 210.2g
Boric acid 61.8g
Sodium dihydrogenphosphate dihydrate 138g
Sodium hydroxide to adjust pH
0.05M Carbonate buffer pH 9.6
Sodium carbonate anhydrous 1.59g
Sodium hydrogen carbonate 2.93g
Sodium azide 0.2g

0.1M Citrate phosphate buffer pH 5
Di-sodium hydrogen orthophosphate dodecahydrate 23.87g
Citric acid anhydrous 7.3g

Tris HCl buffer
0.2M Tris hydroxy methylamine 240ml
0.1M HCl 380ml

Tris buffered saline
Sodium chloride 8.0g
Trihydroxy methylamine 0.605g
1M hydrochloric acid 3.8ml

Reagents
Blocking solution
Bovine serum albumin 1% Sigma Chemical Co, Poole, UK
Glycine 100mM Sigma Chemical Co, Poole, UK
Sodium azide 0.05% Sigma Chemical Co, Poole, UK
Hank's balanced salt solution 100ml Gibco (Life Technology), Paisley, UK

DAB peroxidase substrate solution
"DAB stock" buffer 10ml Sigma Chemical Co, Poole, UK
Di-aminobenzidine 5mg Sigma Chemical Co, Poole, UK
1% hydrogen peroxide 100μl Sigma Chemical Co, Poole, UK
**Lipoprotein electrophoresis fixative**

- Ethanol 180ml
- Glacial acetic acid 30ml
- Deionised water 60ml

**Nickel-DAB peroxidase substrate solution**

- Ammonium nickel sulphate 0.625g
- 0.1M acetate buffer 25ml
- Di-aminobenzidine 12.5mg Sigma Chemical Co, Poole, UK
- 30% hydrogen peroxide 17.5μl Sigma Chemical Co, Poole, UK

**PLP fixative**

8% filtered paraformaldehyde

- /distilled water 5ml
- Solution A* 15ml
- Sodium periodate 42.8mg Sigma Chemical Co, Poole UK.

*Solution A:*

- 0.2M disodiumhydrogen phosphate 9.5ml
- 0.2M sodiumdihydrogen phosphate 40.5ml
- L-lysine hydrochloride 1.827g Sigma Chemical Co, Poole UK.
- distilled water 100ml

**Triple stain**

- Lissamine green 0.5g
- Thiazine red R 0.5g
- Amido black 0.5g
- Mercuric chloride 5.0g
- 2% acetic acid 100ml
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BBS</td>
<td>Borate buffered saline</td>
</tr>
<tr>
<td>BrDU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster designation</td>
</tr>
<tr>
<td>DAB</td>
<td>Di-amino benzidine</td>
</tr>
<tr>
<td>Dil-AC-LDL</td>
<td>1,1-dioctadecyl 1-3,3,3,3-tetramethyl- indocarbocyanine- perchlorate low density lipoprotein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>ECGF</td>
<td>Endothelial cell growth factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELAM-1</td>
<td>Endothelial leucocyte adhesion molecule-1</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GMP-140</td>
<td>Granule membrane protein-140</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2- hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
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</table>
KBr  Potassium bromide

LDL  Low density lipoprotein

LPS  Lipopolysaccharide

LECAM-1  Leucocyte endothelial cell adhesion molecule-1

LFA-1  Lymphocyte function associated antigen

LIMA  Left internal mammary artery

M199  Medium 199

MCP-1  Monocyte chemotactic protein-1

M-CSF  Macrophage colony stimulating factor

MDA  Malonaldehyde

mmLDL  Minimally modified low density lipoprotein

NO  Nitric oxide

NFκB  Nuclear factor κB

oLDL  Oxidised low density lipoprotein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLG</td>
<td>Plasminogen</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>UEA-1</td>
<td><em>Ulex europaeus</em> agglutinin type-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule</td>
</tr>
</tbody>
</table>
VLA-4  Very late activation antigen

VLDL  Very low density lipoprotein

vWF  von Willebrand factor
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Sparrow CP, Doebber TW, Olszewski J, Wu MS, Ventre J, Stevens KA, Cjao Y. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol fed rabbits by the antioxidant N,N'-dephenyl-phenylenediamine. *J Clin Invest* 1992;89:1885-91.


Low density lipoprotein and endothelial adhesion molecule expression in the human coronary artery

SA Thome, SE Abbot, PG Mills, DR Blake.

Monocytes adherence to the endothelium is a prerequisite for foam cell formation. Modified low density lipoprotein (LDL) enhances this process by a mechanism not yet fully elucidated. The aim of this study was to investigate the effect of native or modified LDL on the expression of the leucocyte adhesion molecules (AM) VCAM-1, ICAM-1 (vascular cell and intercellular adhesion molecule, respectively) and E-selectin in cultured human coronary artery (HCA) endothelial cells (EC).

Methods. LDL was isolated from healthy volunteers by ultracentrifugation in the presence of 1mM EDTA. The endotoxin content was less than 30pg/ml (LAL test, Sigma), thus ensuring no lipopolysaccharide stimulation of AM expression. LDL was either used in its native state, copper oxidised, oxidised by incubation with macrophages, or minimally modified by prolonged storage. LDL modification was assessed by electrophoresis, formation of conjugated dienes and malonaldehyde content. HCA and umbilical vein EC were incubated for 4-22 hours in 10% serum containing Medium 199 alone, or with additions of the different forms of LDL 0.001-100μg/ml, or the positive control, tumour necrosis factor-α 100U/ml. Some experiments investigated possible synergy between LDL and TNFα. AM expression was quantified by an enzyme linked immunosorbent assay (monoclonal antibodies provided by D Haskard, Royal Postgraduate Medical School, UK).

Results. No induction of VCAM-1, ICAM-1 or E-selectin expression was observed in either HCA or umbilical vein EC following stimulation with native or modified LDL, either alone, or in the presence of TNFα.

Conclusion. Neither copper-oxidised, macrophage-modified, nor minimally-modified LDL enhances monocyte adhesion to the endothelium by the direct induction of the adhesion molecules VCAM-1, ICAM-1 or E-selectin.
Endothelial adhesion molecule expression and low density lipoprotein


Objective. Modified low density lipoprotein (LDL) has been implicated in atherogenesis. It enhances monocyte adhesion to the endothelium and subsequent foam cell formation by mechanisms not yet fully elucidated. This study investigated the effect of native, minimally modified (mm-) and oxidised (o-) LDL on endothelial expression of the leucocyte adhesion molecules VCAM-1, ICAM-1 (vascular cell and intercellular adhesion molecule, respectively) and E-selectin. Interactions between different cell types of the vessel wall were also investigated by incubation of LDL with human coronary artery smooth muscle cells (SMC) prior to its application to endothelial cells (EC).

Methods. LDL was isolated from healthy volunteers by ultracentrifugation (NVT 65, Beckman) in the presence of 1mM EDTA. Purity was confirmed by electrophoresis and electron microscopy. Endotoxin content was less than 30pg/ml (LAL test, Sigma), thus ensuring no lipopolysaccharide stimulation of adhesion molecule (AM) expression. LDL was either copper oxidised, minimally modified by prolonged storage, or used in its native state. Modification of LDL preparations was assessed by electrophoresis, formation of conjugated dienes and malonaldehyde content. Primary cultures of human coronary artery and umbilical vein EC, and human coronary artery SMC were assessed for purity by morphological appearance and by von Willebrand factor and α-actin expression, respectively. No cytotoxicity was observed by chromium⁵¹ release or morphological change with any form of LDL for the concentrations and time courses described below. EC were transferred to 96 well plates and allowed to reach confluence. They were incubated for 4 hours in Medium 199 (plus 10% foetal calf serum) alone, or with additions of LDL 0.001-100μg/ml, or the positive control, interleukin-1 100U/ml. In some experiments, test media underwent 18 hours pre-incubation with the SMC, before being applied to the EC. AM expression was quantified by an enzyme linked immunosorbent assay (monoclonal antibodies provided by Dr D Haskard, Royal Postgraduate Medical School).

Results. No induction of VCAM-1, ICAM-1 or E-selectin or expression was observed in either coronary artery or umbilical vein EC following stimulation with native, mm-, or o-LDL. In addition, pre-incubation of native or modified LDL with SMC did not induce endothelial AM expression.

Conclusions. The mechanism by which o-LDL and mm-LDL enhance monocyte adhesion to the endothelium does not involve the direct induction of VCAM-1, ICAM-1 or E-selectin. In addition, modified LDL does not induce expression of these endothelial AMs via an indirect action on vascular SMC.
Stress proteins, self defence, and the myocardium

Despite advances in the treatment of coronary artery disease, the search for ways to preserve myocardium threatened by ischaemia or reperfusion injury continues. A group of proteins known as the heat shock or stress proteins represent the cell’s innate ability to protect itself. Do these proteins have a role in protecting the heart in coronary artery disease and can this self preservation mechanism be exploited therapeutically?

Heat shock proteins were originally described in the 1960s and over the next two decades the concept of thermotolerance developed. It was discovered that cells and organisms exposed to sublethal hyperthermia synthesised stress proteins and were rendered tolerant or resistant to subsequent hyperthermic stress. This group of inducible stress proteins is phylogenetically well preserved, being present in various organisms from bacteria to humans. It is unlikely that their expression in mammals is merely an evolutionary remnant of little relevance to homoeothermic animals, since their induction is not confined to the stimulus of heat. Other stimuli include various metabolic stressors such as cellular hypoglycaemia, reactive oxygen species (hydrogen peroxide and superoxide), and ischaemia—all factors that suppress the synthesis of other cellular proteins.

Animal studies have shown that after preconditioning with a brief period of ischaemia the heart is temporarily protected from further ischaemic episodes, and recent evidence suggests that this phenomenon also exists in humans. In addition, a constitutively (basally) expressed stress protein of the 70 kDa heat shock protein (HSP70) family is selectively inducible in rabbit heart by ischaemia. It is well recognised that heat shock and the subsequent synthesis of stress proteins is protective, whether stress proteins contribute to myocardial preconditioning remains controversial. Attention to date has focused on the 70 kDa stress protein, which has been well reviewed.

A role for stress proteins in myocardial preconditioning was supported by work that showed that rendering animals hyperthermic (thus inducing the heat shock response) before an episode of cardiac ischaemia had a protective effect both during the ischaemic period and on restoration of perfusion. However, a few minutes of myocardial ischaemia has been shown to exert a protective effect, pre-conditioning the heart against subsequent ischaemic episodes. Such a time course suggests that this preconditioning effect is unlikely to be due to stress protein induction: increased expression of HSP70 mRNA is not seen in rabbits until an hour after a short ischaemic period, and an increase in the stress protein itself is only detected after two hours, peaking at 24 hours. In addition, it has been shown that protein synthesis is not a prerequisite for preconditioning.

A further group of stress proteins may contribute to the myocyte’s defences. The glucose-regulated stress proteins are induced by cellular hypoglycaemia, a state that occurs during ischaemia when the synthesis of other proteins is suppressed. Though little is known of their functions, they may modulate the response of the myocyte to hypoxia, perhaps by regulating the folding and assembly of proteins disrupted by hypoxia.

Stress proteins also seem to have a role in myocardial adaptation (by hypertrophy), to haemodynamic overload. Cardiomyocytes adapt rapidly to pressure overload with increased protein synthesis. Though the bulk of such proteins comprise contractile units, increased expression of the HSP70 gene is detected as early as an hour after the onset of pressure overload, followed by the appearance of the HSP70 protein. This induction is a short-lived phenomenon; concentrations of HSP70 mRNA return to baseline after 24 hours despite continuous pressure overload. Although the transience of their induction suggests that stress proteins are not the sole cause of compensatory hypertrophy, their perinuclear distribution implies that they may have a permissive role in mediating overload-induced hypertrophy. By the mechanisms discussed below they may permit the rapid recovery of protein synthesis interrupted by the onset of overload, thus enabling the cell to survive the initial stress and to adapt by hypertrophy to continued overload.

What are the mechanisms by which stress proteins provide protection? It has been hypothesised that they recognise and repair hypoxia-induced damage to cellular proteins; rescue protein synthesis and mRNA splicing halted by the onset of stress; and are involved in protein folding and unfolding, assembly and disassembly, and translocation. A further possibility is that stress proteins exert their effect by affording protection against the oxidative stress of reactive oxygen species liberated during post-ischaemic reperfusion: heat shock is accompanied by the induction of the antioxidant catalase during myocardial reperfusion injury. SP32 has been identified as haem oxygenase-1, the rate limiting enzyme in the degradation of haem to bilirubin. Bilirubin is a potent scavenger of reactive oxygen species, its efficacy increasing with the decrease in pH associated with ischaemia. Haem oxygenase-1 (SP32) is active only in the presence of oxygen. It is therefore possible that during reperfusion (for example after thrombolysis for myocardial infarction) the induction of haem oxygenase-1 mediated by reactive oxygen species has a protective antioxidant effect. It may therefore be hypothesised that the heart is
protected by the innate ability of the cardiomyocyte to defend itself against hypoxic, oxidant, and haemodynamic stress by the induced expression of a stress protein response. The heart is not unique in this respect: the inflamed joint shows striking similarities. It too is subject to pressure-induced ischaemia and reperfusion events that facilitate the generation of reactive oxygen species and the induction of protective stress proteins. Additionally, the 60 kDa stress protein family has been implicated in inflammatory arthritis, inviting speculation that because of the highly conserved nature of stress proteins, molecular mimicry may lead to the development of autoimmunity.

Some fundamental questions remain. Do the stress proteins provide the heart with purely a self defence mechanism or can they also attack (for example in autoimmune conditions such as Dressler’s syndrome and lupus carditis)? While research to elucidate the molecular biology of the stress proteins continues, questions about their therapeutic exploitation should be considered. Has this already been achieved during surgery by the hypothermic induction of protective stress proteins during cold cardioplegia and organ transport? Finally, will pharmacological intervention prolong or stimulate the induction of stress proteins, thereby providing a means to protect the myocardium in coronary artery disease?

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Adhesion molecule expression in human coronary artery smooth muscle cells

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Intimal smooth muscle cells (SMCs) are present in both normal and atherosclerotic human coronary arteries (HCA); they are exposed to the circulation when the endothelium is breached. The aim of this study was to demonstrate whether leucocyte adhesion molecules (AMs) are inducible on HCA SMCs, providing a potential role for these cells in the recruitment of monocytes and T lymphocytes to the atherosclerotic plaque.

Methods: SMCs were cultured by a primary explant technique from normal HCAs and characterised by α-smooth muscle actin expression. They were incubated for 4 hours in 10% serum containing DMEM alone, or with interleukin-1 (IL-1) 0.001-500U/ml, or tumour necrosis factor-α (TNFα) 0.1-100U/ml. Expression of ICAM-1 and VCAM-1 (intercellular and vascular cell adhesion molecule, respectively) was quantified by an enzyme linked immunosorbent assay (monoclonal antibodies provided by D Haskard, Royal Postgraduate Medical School, UK).

Results: There were low levels of basal ICAM, and no basal VCAM expression. The expression of both AMs was significantly induced by IL-1 and TNFα in a dose dependent manner. Maximal responses were: for VCAM p=0.004 and 0.015, and for ICAM p=0.007 and <0.001 for TNFα and IL-1, respectively.

Conclusions: SMCs within the atherosclerotic plaque may be exposed to AM stimulating cytokines from already resident macrophages. We have shown ICAM and VCAM to be inducible in human coronary artery SMCs; this has not been demonstrated previously. VCAM, unlike ICAM, is specific for the mononuclear cells which typify the leucocyte plaque population. VCAM may thus provide a specific mechanism whereby mononuclear cells are recruited to the atherosclerotic plaque.
Human recombinant tissue-type plasminogen activator enhances wound healing in human coronary artery endothelial cells

SA Thome, SE Abbot, DR Blake, PG Mills.

Endothelial cell (EC) migration is essential to angiogenesis and the repair of breached endothelium. Tissue plasminogen activator (tPA), synthesised by ECs, converts plasminogen to plasmin which disrupts the basement membrane, facilitating EC migration. tPA alone promotes migration in umbilical vein ECs, however ECs of different origins exhibit functional heterogeneity. We have therefore investigated the effect of tPA on migration in a novel human coronary artery EC system. The system is serum-free, permitting investigation of tPA in the absence of plasminogen.

Normal coronary artery and aortic ECs were isolated by collagenase digestion of surgical specimens, cultured, and passaged onto fibronectin coated Thermanox® coverslips. Cells were characterised as endothelial by their cobblestone morphology, and granular pattern of von Willebrand factor immunoreactivity. The monolayer was wounded and washed in serum free medium (SFM, with 0.5% bovine serum albumin, 0.2% gelatin). During 30 hours incubation in SFM containing human recombinant tPA (rtPA, Boehringer Ingelheim) 10-6000 iu/ml, migration was measured by sequential photography. Proliferating cells were quantified after detection by immunocytochemistry (Ki 67 antibody, Dako; specific for non-G0 cells). Wound healing occurred by movement of the whole cell sheet, migration of individual cells, and cell proliferation at the wound edge. rtPA enhanced cell migration and proliferation, following a dose dependent trend, but was less effective (p<0.001) than foetal calf serum (FCS). A combination of rtPA and FCS (supplying plasminogen) had a synergistic effect on cell migration (p<0.001).

We have demonstrated that rtPA enhances coronary artery and aortic EC migration in vitro. This effect is due to the action of tPA alone as well as to migration enhancement by plasmin. In the presence of fibrin, tPA binds rapidly to the endothelium with resultant protection from circulating tPA inhibitors. Our results suggest that the role of tPA in coronary artery disease is not only fibrinolytic, but that it may also promote endothelial repair in the coronary artery.
The isolation, culture and characterisation of human cardiac microvascular endothelial cells.


The cardiac microvascular endothelium has been implicated in mediating myocardial hypoxic reperfusion injury. Endothelial cell heterogeneity may preclude the application of data from non-cardiac or animal endothelial cell *in vitro* studies to the situation in the human heart. We have therefore performed the isolation, culture and characterisation of human cardiac microvascular endothelial cells; this has not been previously described perhaps in part due to limited tissue availability and the fastidious nature of adult microvascular endothelial cells in culture.

Fresh tissues were obtained from operative specimens (mitral valve papillary muscles, right atrial appendages (RAA) and whole cardiac explants). Endocardial and epicardial surfaces, fibrous tissue and macroscopic blood vessels were excised. RAA endocardium was rendered non-viable by immersion in absolute alcohol, followed by washing in Hank's balanced salt solution. Endothelial cells were isolated by mincing the tissue into 2mm\(^3\) pieces and incubating with a 0.5% crude collagenase solution containing 100mM CaCl\(_2\) (to ensure non-viability of contaminating myocytes). Capillary segments were expressed from the digest using a flat spatula. Material was disaggregated and filtered through a 250μm mesh to remove large tissue pieces. The filtrate was centrifuged and the pellet resuspended in culture medium and incubated at 37\(^\circ\)C. After 24 hours, the cells were washed and the medium replaced, thus removing debris and leaving the adherent endothelial cells. Confluent cultures were passaged every 10-14 days. The endothelial nature of the cultured cells was confirmed by electron microscopy, by characteristic granular staining for von Willebrand factor and by staining with the endothelial cell specific monoclonal antibody QB-END/10.

To ascertain the optimal site for endothelial cell extraction, source tissues were also immunostained with the above endothelial cell markers. Morphometric studies demonstrated that all tissues contained high endothelial cell densities. However, sections from left ventricle (LV) were found to contain a significantly greater density of microvascular endothelial cells (mean 486/mm\(^2\)) than either RAA (mean 288/mm\(^2\)) or papillary muscle (mean 336/mm\(^2\), \(p<0.05\) using a Student's t test. Thus when available, LV myocardium will be used to gain the maximum endothelial cell yield.