EFFECT OF HIGH DENSITY LIPOPROTEINS ON THE EXPRESSION OF ADHESION MOLECULES ON ENDOTHELIAL CELLS

A Thesis submitted by

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CHAPTER SIX

EFFECT OF FENOFIBRATE ON PLASMA LEVELS OF LIPIDS AND SOLUBLE ADHESION MOLECULES IN DIABETIC SUBJECTS

ABSTRACT

High density lipoproteins (HDLs) protect against the development of coronary heart disease. There are several potential mechanisms which may account for the cardioprotective function of HDLs. These include their proposed role in the reverse cholesterol transport pathway, the capacity of HDLs to reduce the atherogenicity of low density lipoproteins by inhibiting their oxidative modification and, based on recent observations, they may be anti-atherogenic by virtue of their ability to inhibit the expression of adhesion molecules on endothelial cells. The investigations in this thesis centre on the effects of HDLs on the expression of endothelial cellular adhesion molecules with emphasis on the effects of HDL subpopulations, HDL composition, acute-phase HDLs and the effects of altering plasma HDLs on adhesion molecule expression in endothelial cells.

Chapter 3, shows that the HDLs from different subjects vary in their ability to inhibit vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells. This difference is attributable to differing proportions of HDL_2 and HDL_3. The HDL_3 subfraction is shown to be superior to HDL_2 as an inhibitor of the cytokine-induced expression of VCAM-1 in human umbilical vein endothelial cells (HUVECs).

The experiments in chapter 4 further investigate the HDLs mediated inhibition of VCAM-1 expression in endothelial cells by addressing the influence of apolipoprotein (apo) composition. Experiments show that altering the apolipoprotein composition on HDL_3 by replacing the apoA-I with apoA-II did not affect its ability to inhibit VCAM-1 expression. Experiments designed to show if the differences in

the inhibition of VCAM-1 expression by HDL_2 and HDL_3 may be due to differences in their protein or lipid components are presented. Neither the isolated HDL proteins nor the HDL lipids inhibit VCAM-1 expression when added separately to the endothelial cells. This is consistent with the results from other studies presented in chapter 4 in which lipid-free apoA-I and di-myristoyl-phosphatidylcholine (DMPC) vesicles alone have no inhibitory effect on VCAM-1 expression. However, when lipid-free apoA-I and DMPC are combined to make discoidal reconstituted HDLs (rHDLs), these particles are able to inhibit the cytokine-induced VCAM-1 expression on HUVECs .

The acute-phase protein serum amyloid-A (SAA) is predominantly carried on HDLs in the plasma and SAA has been shown to have potential proatherogenic properties. We originally hypothesised that HDLs that were associated with SAA would be less effective inhibitors of VCAM-1 expression in endothelial cells than unmodified HDLs. Experiments presented in chapter 5 show that this is not the case, with HDL_3 that is associated with SAA (SAA-HDL_3) being just as effective as unmodified HDL_3 in the inhibition of cytokine-induced VCAM-1 expression in HUVECs. Lipid-free SAA was also incubated with HUVECs and as is the case with lipid-free apoA-I, there is no inhibitory effect on cytokine-induced VCAM-1 expression.

Chapter 6 describes a preliminary study designed to determine whether raising the plasma HDL-C with the drug fenofibrate has any effect on the levels of serum soluble adhesion molecules in 20 subjects with type II diabetes mellitus. After 6 weeks of therapy with fenofibrate there is a significant reduction in soluble E-selectin

but no significant effect on soluble intracellular adhesion molecule-1 and soluble VCAM-1. These results are of sufficient interest to warrant a larger, randomised, double-blind, placebo controlled trial which is currently underway in our department.

This thesis expands upon the original discovery that HDLs inhibit the cytokine-induced expression of endothelial cell adhesion molecules and extends our understanding of this potential antiatherogenic function of HDLs.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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PUBLICATIONS

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ABSTRACTS

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PRIZES

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PRESENTATIONS

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ABBREVIATIONS

A-I HDLs	HDLs containing ApoA-I only
A-I/A-II HDLs	HDLs containing ApoA-I and ApoA-II
A-II HDLs	HDLs containing ApoA-II only
ApoA, B, C….	ApolipoproteinA, B, C….
bFGF	basic Fibroblast Growth Factor
CABG	Coronary Artery Bypass Graft
CHD	Coronary Heart Disease
DMPC	di-myristoyl-phosphatidylcholine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
E-selectin	Endothelial-Selectin
FACS	Fluorescent Activated Cell Sorting
FGF	Fibroblast Growth Factor
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
FPLC	Fast Protein Liquid Chromatography
g/ml	grams per milli-litre
G-CSF	Granulocyte - colony stimulating factor
h	Hour
HB-EGF	Heparin Binding- Epidermal Growth Factor
HDL	High Density Lipoprotein
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1 and 2	Intercellular Adhesion Molecule-1 and 2
IDL	Intermediate Density Lipoprotein
IGF-1	Insulin like Growth Factor-1
IL-1 and -2	Interleukin-1 and -2
INF-γ	Interferon γ
LCAT	Lecithin:Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide

L-selectin	Leukocyte-Selectin
MAdCAM-1	Mucosal Addressin Cellular Adhesion Molecule-1
MCP-1	Monocyte Chemotactic Protein-1
M-CSF	Monocyte - colony stimulating factor
μmol/L	micro-moles per litre
mg/ml	milli-grams per milli-litre
mmol/L	milli-moles per litre
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PECAM	Platelet Endothelial Cell Adhesion Molecule
PGI_2	Prostacyclin
Post-Op	Post-Operative
Pre-Op	Pre-Operative
P-selectin	Platelet-Selectin
PSF	PGI_2 stabilizing factor
SAA	Serum Amyloid-A
SAA-HDL_3	HDL_3 containing Serum Amyloid-A
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SMC	Smooth Muscle Cells
TBS	Tris Buffered Saline
TGF-β	Transforming Growth Factor β
TNF-α	Tumour Necrosis Factor α
U	Units
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA	Very Late Appearing
VLDL	Very Low Density Lipoprotein

CHAPTER ONE

INTRODUCTION

1.1 EPIDEMIOLOGY OF CORONARY HEART DISEASE

Coronary heart disease (CHD) accounts for more Australian deaths than any other disease. CHD mortality in Australia reached a peak in the mid 1960's and since then there has been a subsequent decline (National Heart Foundation of Australia 1989). Reductions in age adjusted death rates for CHD between 1967 and 1989 in the 30-64 year age group were 61% for men and 64% for women. The total mortality fell by 41% and 40% respectively in the same time period (Lloyd, 1994). Other Westernised countries have had similar decreases in mortality from CHD, but despite this atherosclerosis and its clinical syndromes (CHD and stroke) remain the biggest killers in Westernised countries.

The cause of the reduction in age adjusted mortality rates from coronary artery disease over the past twenty to thirty years is multifactorial. Goldman and Cook estimated that 54% of the decline in CHD mortality rate was due to decreased disease incidence and occured mainly as a result of changes in lifestyle and diet and reduced cigarette smoking (Goldman and Cook 1984). They attributed up to 24 % of the CHD reduction to smoking cessation alone. Other factors that have had a beneficial effect on CHD mortality include healthier diet (less saturated fats, less red meat and fewer dairy products), increased aerobic exercise and better management of hypertension, diabetes and dyslipidemia. Improvements in medical care and treatments have also led to a reduction in age adjusted CHD mortality. This includes improvements in pre-hospital care, widespread introduction of coronary care units, use of thrombolytic therapy and other drug treatments for acute myocardial infarction and the increased availability of revascularization procedures for CHD.

1.2 ATHEROSCLEROSIS

Atherosclerosis is the major cause of death in the Western world with the mortality rates from CHD and stroke combined causing more than 50% of the total number of deaths (Davies and Woolf 1993). In addition it causes significant morbidity with clinical syndromes such as angina, myocardial infarction, heart failure, cerebrovascular accidents and peripheral vascular disease.

Atherosclerosis is a chronic, progressive disease that usually starts in childhood but doesn't present with clinical manifestations until middle to late adult life. Atherosclerosis is a disease entity which has a specific pathogenesis and pathological picture and yet there are many factors which predispose to it. Risk factors for atherosclerosis include a Western diet, smoking, increased plasma cholesterol (or a low HDL cholesterol), diabetes, hypertension, obesity, age, male sex and a genetic predisposition (National Heart Foundation of Australia 1989).

1.2.1 PATHOLOGY OF THE ATHEROSCLEROTIC PLAQUE

The atherosclerotic lesion or plaque consists of lipid deposited in the arterial intima, recruitment of inflammatory cells into the intima, proliferation of smooth muscle cells in the arterial intima and the increased production of collagen and matrix proteins by smooth muscle cells (Raines and Ross, 1993). The more advanced atherosclerotic lesions may have areas of necrosis in the central areas of the plaques (Ross, 1986). There are three major phases in the development of atherosclerotic

plaques: the fatty streak, intermediate lesions and finally the formation of the advanced, complex, occlusive lesions called fibrous plaques (Ross, 1993).

The earliest macroscopically detectable lesion in atherogenesis is the "fatty streak". These initial lesions of atherosclerosis can be found in children and young adults (Stary, 1983). Macroscopically the fatty streak appears as an area of yellow discolouration occurring in patches surrounded by areas of normal endothelium. The yellow colour is due to cholesterol and cholesteryl esters deposited in foam cells. These foam cells are lipid-laden macrophages and they co-exist in the fatty streak with T-lymphocytes and varying numbers of lipid filled smooth muscle cells. As the lesions increase in size and complexity they develop to intermediate atherosclerotic plaques (Faggiotto and Ross, 1984; Masuda and Ross, 1990). Intermediate atherosclerotic plaques are composed of layers of macrophages and smooth muscle cells. These lesions may progress to develop into the more advanced, complex, occlusive lesions called fibrous plaques.

The mature atherosclerotic lesions are called fibrous plaques. They may be associated with plaque rupture with resulting thrombosis and haemorrhage and may become calcified in which case they are known as a complicated lesion (Ross, 1992). Fibrous plaques are usually elevated and they protrude to varying degrees into the lumen of the artery. The flow down the artery may be compromised if the plaque is of sufficient size. Macroscopically the fibrous plaques are white in appearance and may have signs of calcification. Microscopically they lie beneath the endothelium and consist of a smooth cellular fibrous cap with an underlying lipid rich core. The

portion of the fibrous cap closest to the endothelium consists of flattened smooth muscle cells that are surrounded by dense connective tissue made up of collagen and proteoglycans. Beneath this lies a mixture of smooth muscle cells, macrophages, and numerous lymphocytes (mainly CD-8+ and some CD-4+ T cells) and in this area there is also a large amount of connective tissue (Ross, 1992). The inner portion of the fibrous plaque consists of the soft, lipid rich core which is devoid of supporting collagen and is avascular and hypocellular (except at the periphery of the core) (Lundberg, 1985).

Coronary occlusion and myocardial infarction occur frequently from plaques causing only mild to moderate degrees of stenosis (Ambrose et al, 1988; Little et al, 1988). These smaller plaques tend to be more unstable than larger more occlusive plaques (Fuster et al, 1992). The smaller plaques are lipid-rich and their fibrous caps are thinner than the larger plaques that have a thick fibrous tissue cap with only a small lipid core. The unstable plaque contains a soft atheromatous core and if the fibrous cap ruptures, then the highly thrombogenic material from the core is suddenly exposed to the flowing blood. This may result in an acute coronary syndrome and such disrupted plaques are found beneath about 75% of the thrombi responsible for acute coronary syndromes (Richardson et al, 1989; Falk, 1988). The risk of plaque disruption is related to both the intrinsic properties of individual plaques and to the extrinsic forces acting on the plaques. Plaque rupture most commonly occurs at the unstable shoulder region of the plaque (Ross, 1992). In this area the fibrous cap is thinnest and there is a high concentration of foam cells which can trigger plaque

rupture by the release of proteases such as MCP-1 and matrix metalloproteinases which chemically digest the plaque cap (Topel and Van de Werf, 1998).

1.2.2 PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is caused by the interaction of various factors. Our current knowledge on the pathogenesis of atherosclerosis is based on the original hypothesis by Ross and Glomset from 1973 which was titled "the response to injury hypothesis of atherosclerosis" (Ross and Gloset, 1973). This hypothesis states that some form of structural or functional injury to the endothelium occurs which results in increased permeability of the endothelial barrier to cells, hormones and lipoproteins in the bloodstream. The "injury" may be caused by a number of factors such as mechanical forces, lipoproteins, homocysteine, viruses, bacteria (such as chlamydia), toxins or immunological factors. Manifestations of the dysfunction of the endothelium caused by the injury include increased trapping of lipoprotein in the arterial wall and the appearance of glycoproteins called adhesion molecules on the endothelial cell surface (Ross, 1993). Monocytes and T-lymphocytes attach themselves to the endothelial cells and then migrate between the endothelial cells to the intima under the influence of growth factors and chemoattractants released by nearby cells. Once in the intima, the monocytes differentiate to become macrophages which then accumulate LDLs to become foam cells. These foam cells and T-lymphocytes make up the fatty streak (Adams and Shaw, 1994). If the injury to the endothelium continues the fatty streaks will eventually become mature fibrous plaques which may go on to form complicated plaques and cause the clinical syndromes of atherosclerosis.

Three factors of major importance in the pathogenesis of atherosclerosis are lipids and lipoproteins, growth factors and cells. Each will be discussed in turn.

1.2.2.1 LIPIDS AND LIPOPROTEINS IN ATHEROSCLEROSIS

Dyslipidemia is a major risk factor for atherosclerosis and lipoproteins are an integral part of the formation of the atherosclerotic plaque. Cholesterol and triglycerides are important in many physiological processes such as membrane and hormonal synthesis, intercellular and extracellular signaling and energy metabolism. Cholesterol and triglycerides are hydrophobic and are carried in the plasma in water soluble structures called lipoproteins. Lipoprotein particles consist of an outer monolayer that contains phospholipids, unesterified cholesterol and apolipoproteins. The inner core contains the hydrophobic cholesteryl esters and triglycerides. There are five major classes of plasma lipoproteins and they are grouped according to their density (Table 1.1). Two of them, the chylomicrons and the very low density lipoproteins (VLDLs), are triglyceride rich and function as transporters of the triglyceride which originates in the intestine and liver respectively. Increased concentrations of either or both of these lipoproteins lead to hypertriglyceridemia. The low density lipoproteins (LDLs) are breakdown products of VLDLs. The LDLs are formed by the hydrolysis of VLDLs by lipoprotein lipase and the transfer of cholesteryl esters from high density lipoproteins (HDL) by the action of cholesteryl ester transfer protein (CETP). HDLs mediate removal of cholesterol from the circulation.

Table 1.1 Classification of human plasma lipoproteins[a]

Lipoprotein Class	Diameter (nm)	Hydrated density (g/ml)	Electrophoretic Mobility[b]	Main Apolipoproteins
Chylomicrons	75 - 1,200	$d < 0.94$	origin	B_{48}, C
VLDL	30 - 80	$0.94 < d < 1.006$	pre-β	B_{100}, C
IDL	25 - 35	$1.006 < d < 1.019$	slow pre-β	B_{100}, E
LDL	18 - 25	$1.019 < d < 1.063$	β	B_{100}
HDL	7 - 12	$1.063 < d < 1.21$	α	A-I, A-II

[a] Source - Skipski, 1972; Scanu, 1979; Blanche et al, 1981

[b] On agarose gels.

Raised plasma LDL levels and low plasma HDL levels are both independent risk factors for atherosclerosis (Gofman et al, 1966, Kannel et al, 1971, Gordon et al, 1977). The mechanisms of how LDLs cause atherosclerosis are now largely understood and are discussed below. The mechanisms of HDLs' protection against atherosclerosis are less clear and will be discussed later.

One of the first steps in the formation of the fatty streak is the transport of LDLs into the arterial wall. This is a concentration-dependent process that does not require receptor-mediated endocytosis (Steinberg et al, 1989). There is strong evidence from work by Brown and Goldstein examining mutations in the LDL receptor that elevations in LDL levels are sufficient to induce all the components of atherosclerosis (Brown and Goldstein, 1986). Schwenke and Carew showed in a rabbit model of atherosclerosis that for any given LDL plasma concentration, LDL retention in the arterial wall was more important for the development of atherosclerosis than the rate of transport of the LDL into the arterial wall (Schwenke and Carew, 1989a, 1989b).

Once they have crossed the endothelium, LDLs become trapped in the extracellular matrix (Nievelstein et al, 1991; Nievelstein et al, 1994) and then cells of the artery wall release oxidative products that initiate oxidation of the trapped LDL particles (Witztum, 1994). Unmodified LDL particles do not cause appreciable lipid accumulation in cultured arterial cells and do not promote atherosclerosis (O'Brien

and Chait, 1994). LDLs that have been oxidatively modified do accumulate in arterial cells and are atherogenic.

Trapped LDL particles in the arterial intima are subject to oxidative modification by the cells of the arterial wall (endothelial cells, monocyte-macrophages and arterial smooth muscle cells) (Chisolm, 1991). It has been shown that even minimally oxidised LDLs can initiate inflammatory cell recruitment into the arterial intima hence initiating or continuing the development of an atherosclerotic plaque. Minimally oxidised LDLs do this by a number of mechanisms including: stimulating the expression of a monocyte-specific chemoattractant, monocyte chemotactic protein-1 (MCP-1) (Cushing et al, 1990), upregulating the expression of the cellular adhesion molecules P-selectin (Berliner et al, 1990) and VCAM-1 (Khan et al, 1995), and the increased expression of monocyte and granulocyte colony stimulating factors (M-CSF and G-CSF) (Rajavashisth et al, 1990). Hence, minimally oxidised LDLs have a role in monocyte binding to the endothelial surface and the transmigration of the monocyte through the endothelial layer under the influence of MCP-1 into the arterial intima.

Once in the subendothelial space, the monocytes differentiate into macrophages under the influence of M-CSF (also influenced by minimally oxidised LDLs) (Rajavashisth et al, 1990). This in turn enriches the microenvironment with reactive oxygen species which converts the trapped minimally oxidised LDLs into highly oxidised LDLs. Minimally oxidised LDLs are recognised by the LDL receptor and are taken up into cells predominantly via the LDL receptor (Berliner, 1990). Highly

oxidised LDLs can enter the macrophage by either binding to the LDL receptor (which is subject to downregulation when intracellular cholesterol content becomes elevated) or via scavenger receptors (whose expression is not affected by cellular cholesterol content) present on macrophages and endothelial cells (Brown and Goldstein, 1983). The lack of downregulation of these scavenger receptors leads to a massive accumulation of cholesterol in the macrophage and due to the foamy appearance of their cholesterol laden cytoplasm, these macrophages are called foam cells. These foam cells are the basis of the fatty streak.

1.2.2.2 Molecular Regulators of Atherosclerosis

Numerous cell functions are under the regulation of growth factors, cytokines and other small molecules such as nitric oxide. These factors may act in cell recruitment and migration, cell proliferation and the control of lipid and protein synthesis.

Growth regulatory molecules can cause either stimulation or inhibition of cell proliferation and many of the proliferative agents can act as chemoattractants. The molecules important in stimulating smooth muscle proliferation are platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), insulin-like growth factor (IGF-1), interleukin-1 (IL-1), tumour necrosis factor α (TNF-α) and transforming growth factor β (TGF-β) (Ross, 1993). These factors are generally not expressed in the normal artery but are upregulated in the atherosclerotic plaques. There are several molecules that, as well as stimulating cellular proliferation, can in certain circumstances cause inhibition of cellular proliferation. These include TGF-β, TNF-

α, IL-1 and interferon-γ (IFN-γ), which all exert a negative effect on cell proliferation when they occur in excess. In addition, they cause a downregulation of PDGF which itself has a powerful proliferative effect on smooth muscle cells (Battegay et al, 1990).

Chemotaxis is a critical event in the development of the lesions of atherosclerosis. It is important in the transfer of leukocytes into the arterial wall and bringing smooth muscle cells from the media into the intima of the artery. Factors important in monocyte chemotaxis and endothelial transmigration are colony-stimulating factors (CSFs), monocyte chemotactic protein-1 (MCP-1), oxidised LDL and TGF-β (Ross, 1990). PDGF and IGF-1 are important in inducing smooth muscle chemotaxis (Ross, 1990).

The cytokines IL-1, TNF-α, interferon-γ and IL-2 together with the colony stimulating factors are the modulators of the inflammatory response that occurs once the endothelium has been exposed to injurious agents (Ross, 1990).

The process of atherosclerosis is complex and the cellular regulators mentioned above have varying roles to play. The cells important in the pathogenesis of atherosclerosis and their role will be discussed next.

1.2.2.3 Cellular Mechanisms in Atherosclerosis

The major cell types involved in the formation of the atherosclerotic plaque are: endothelial cells, monocytes/macrophges, T-lymphocytes and smooth muscle cells.

1.2.2.3.1 THE ENDOTHELIUM AND ATHEROSCLEROSIS

Until relatively recently, the endothelium was thought of as an inert structure which functioned only as a barrier between the blood elements and the artery wall. It is now established that the endothelium has a number of physiological roles including: acting as a permeable barrier to transport substances from the blood to the artery wall, provision of a non-thrombogenic surface, maintaining vascular tone by the release of substances such as nitric oxide (NO), prostacyclin (PGI_2) and endothelin, formation and secretion of cytokines and growth factors, oxidising lipoproteins as they are transported into the arterial wall and helping to maintain the basement membrane collagen and proteoglycans upon which they rest.

Atherosclerosis can occur in situations of endothelial injury caused by substances such as oxidised LDL or by conditions such as smoking or hypertension. The initial step is the increased expression of cell surface glycoproteins called adhesion molecules which cause adherence and migration of monocytes and T-lymphocytes into the subendothelial space. Then oxidised LDL and other substances cause the monocytes to become macrophages which themselves take up oxidised LDL to become foam cells (Ylä-Herttuala et al, 1989). The other important role the endothelium has in atherosclerosis is its ability to maintain a strict cellular monolayer such that when an area of endothelium is denuded or damaged, cells on the margin of the wound either stretch to maintain the monolayer or replicate in order to close the wound. Theoretically, repeated injury (such as may occur in atherosclerosis) could

result in the endothelial cells losing their ability to replicate and could lead to alterations of the surface of the artery at this site.

Lastly the endothelium plays a critical role in thrombotic and coagulant activities by producing antithrombotic substances such as heparin sulphate, NO, PGI_2, plasminogen activator and urokinase. The endothelium can also increase the thrombotic state by producing plasminogen activator inhibitor and von Willebrand factor (Braunwald 4th Ed, p1109).

1.2.2.3.2 MONOCYTES AND MACROPHAGES AND ATHEROSCLEROSIS

The macrophage is probably the key cell responsible for the promotion of connective tissue proliferation in the atherosclerotic plaque due to it's scavenging capacity and its ability to produce and secrete growth factors (Ross, 1992). Macrophages initially exist as monocytes in the plasma. Their major role in the development of the atherosclerotic plaque is as follows: monocytes initially adhere to the endothelium via adhesion molecules expressed on endothelial cells in response to either minimally oxidised LDL or to inflammatory cytokines such as TNF-α, IL-1 or IFN-γ (O'Brien and Chait, 1994). MCP-1 then causes transmigration of the monocyte across the endothelium into the subendothelial space. MCP-1 is expressed by endothelial cells, monocytes and macrophages and smooth muscle cells in response to various cytokines including TNF-α, IL-1 and INF-γ and M-CSF can also induce monocytes and macrophages to express MCP-1 (Faruqi and DiCorleto, 1993). After entering the vessel wall, monocytes differentiate into macrophages (Fuster, 1994). Once in the

intima, macrophages internalise oxidised LDL through both scavenger receptors and LDL receptors and become lipid laden foam cells that are seen in the fatty streak.

Other roles of macrophages in the development of the atherosclerotic plaque is to release cytokines such as IL-1 and TNF-α which increase the expression of cellular adhesion molecules on the endothelium and to synthesize PDGF which is a potent chemoattractant and stimulator of proliferation for smooth muscle cells (Raines and Ross, 1993). Other growth factors that macrophages synthesize and secrete include fibroblast growth factor (FGF), epidermal growth factor (EGF) and TGF-β.

1.2.2.3.3 T-Lymphocytes and Smooth Muscle Cells and Atherosclerosis

Atherosclerotic plaques contain T-lymphocytes (both CD-8+ and CD-4+) and this supports the notion that these lesions develop, at least in part, as a result of an immune or possibly autoimmune response (Emeson and Robertson, 1988). Most of the T lymphocytes have cell surface markers which indicate that they have been antigen stimulated, but the nature of the antigens is unknown. Experimentally induced autoimmunity has been shown to produce proliferative lesions of atherosclerosis in rabbits (Minick and Murphy, 1973). It has been shown that when monocytes and T lymphocytes are co-cultured in vitro there is secretion of cytokines such as IFN-γ from the T lymphocytes (Frostegård et al,1992). This may affect the other cells of the plaque as IFN-γ is an inhibitor of cellular proliferation.

Smooth muscle cells (SMC) that proliferate in the arterial intima and cause the intermediate and advanced plaques of atherosclerosis are originally derived from the media. There are two major types of smooth muscle cell found in the arterial wall (Campbell and Campbell, 1985). The contractile phenotype contains large numbers of myofilaments in their cytoplasm and is mainly found in the media of the artery wall. The second major SMC phenotype in the arterial wall are SMC of the synthetic state (Campbell and Campbell, 1985). These SMC are most commonly found in the intima and have less myofilaments in their cytoplasm and a better developed Golgi apparatus and rough endoplasmic reticulum. These synthetic SMC are capable of expressing genes for a number of growth-regulatory molecules and cytokines and unlike the contractile phenotype SMC, these synthetic SMC respond to mitogens such as PDGF. Hence it is these synthetic SMC that proliferate and form the intermediate and advanced lesions in atherosclerosis.

1.2.3 Adhesion Molecules and Atherosclerosis

Adherence of monocytes and lymphocytes to the arterial endothelial lining is one of the earliest detectable events in atherosclerosis. The subsequent transmigration of these leukocytes, their accumulation in the intima and transformation into lipid laden foam cells are important events in the initiation and progression of atherosclerotic plaques.

The endothelium is normally quiescent and maintains blood fluidity by inhibiting coagulation and resisting the adhesion of blood leukocytes. This state changes following activation of the endothelium by cytokines (TNF-α, IL-1 and IFN-γ) and

chemoattractants (C5a, leukotriene B4, platelet activating factor and members of the chemokine family of polypeptides) to express adhesion molecules. This allows leukocytes to attach to the endothelial cells and migrate towards the source of the inflammatory stimulus (Faull, 1995). Adhesion and subsequent transendothelial migration takes place preferentially at specialised sites in the blood vessels: postcapillary venules in nonlymphoid tissue and high endothelial venules in lymph nodes (Anderson and Shaw, 1993).

The sequence of events that occurs during the extravasation of leukocytes is as follows (Figure 1.1). The leukocyte in the bloodstream makes random contact with the endothelial surface and the group of adhesion molecules called the selectins cause tethering and rolling of the leukocyte along the endothelial surface (Lawrence and Springer, 1991; Bevilacqua and Nelson, 1993). The next stage is the firmer adhesion of the leukocytes to the endothelium, followed by changes in their shape from a spherical to flattened configuration so they can slide between the endothelial cells (Shimizu et al, 1992; Zimmermann et al, 1992). These interactions are mediated by adhesion molecules from the integrin and immunoglobulin superfamily classes. The leukocytes then pass through the basement membrane and into the arterial intima (Faull, 1995). Adhesion molecules from the CADherin family are responsible for the stable adhesion between the endothelial cells and their basement membrane (Takeichi, 1991).

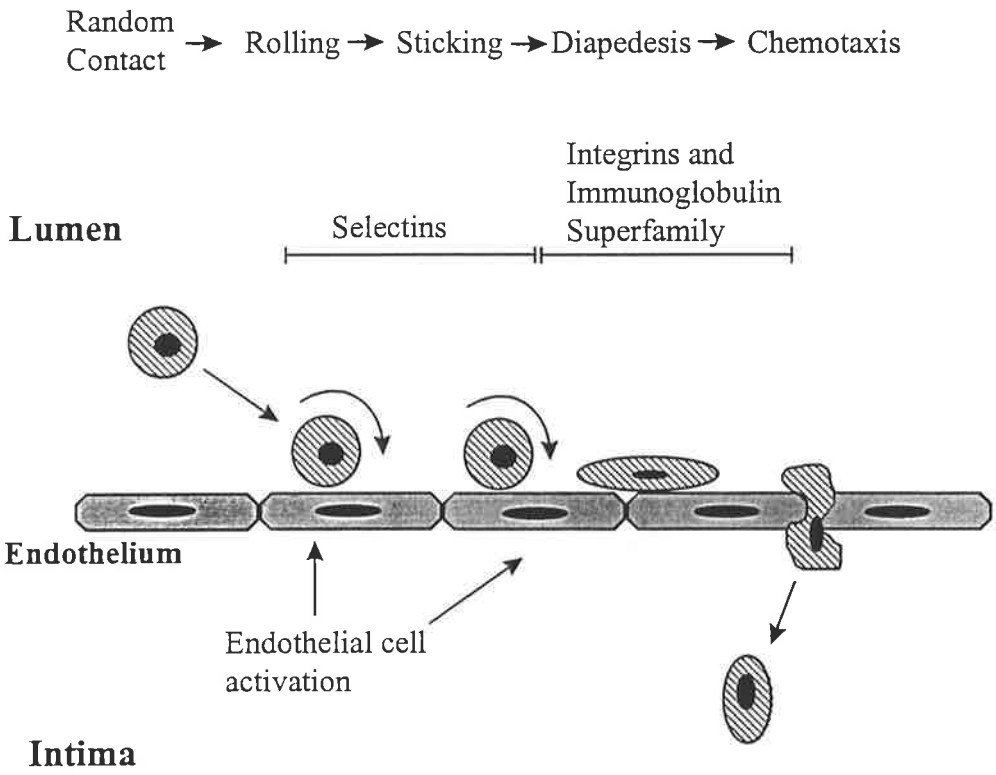

Figure 1.1. Leukocyte recruitment into cells. Circulating leukocytes come into random contact with the endothelium. The leukocytes are slowed by a series of transient attachments to selectin (eg P-selectin and E-selectin) and this causes rolling of the leukocyte along the endothelium. The rolling leukocytes then develop firm adhesion to the endothelium via integrins and immunoglobulin superfamily adhesion molecules. They then change shape and migrate in between the endothelial cells into the intima (Adapted from Faull, 1995; O'Brien and Chait, 1994).

Within each of the four families of adhesion molecules - the selectins, integrins, immunoglobulin superfamily and CADherins - molecules share highly significant structural and sequence homology and have many functional similarities.

1.2.3.1 The Selectins

Tethering and rolling of the leukocytes is mediated by the selectins which are a family of three lectin-like carbohydrate-binding molecules (Lawrence and Springer, 1991). They are designated by the prefixes E (endothelial), L (leukocyte) and P (platelet) according to the cells on which they were first described. L-selectin is constitutively expressed on most leukocytes, whereas E-selectin is synthesised and expressed on endothelial cells after cytokine activation (Bevilacqua and Nelson, 1993). P-selectin is expressed on activated endothelium and by platelets. P-selectin can be mobilised on activation from cytoplasmic granules to the endothelial cell surface (Lasky, 1992). E and P-selectins maybe important in atherosclerosis.

Selectins mediate a degree of adhesion strong enough to induce rolling along the vessel wall but not so strong as to halt the leukocyte completely (Lawrence and Springer, 1991). This is important as it allows the leukocytes to sample the local endothelium for the presence of specific trigger factors that can activate leukocyte integrins and allow the adhesion cascade to proceed. In the absence of these factors the leukocyte disengages and moves on (Adams and Shaw, 1994). The selectins function entirely within the vascular space and studies in knockout mice that are P-selectin deficient show they have markedly impaired leukocyte rolling and extravasation (Mayadas et al, 1993).

1.2.3.2 THE INTEGRINS

Strong adhesion of leukocytes to the endothelium is mediated by leukocyte integrins that bind to counter receptors on the endothelium (Springer, 1990). The integrins are the most widely distributed adhesion molecules and are all similar, being membrane-spanning heterodimeric glycoproteins with non-covalently associated α and β chains. The integrins are usually grouped into subfamilies according to the 8 β chains (table 1.2) which associate with a variable number of α chains.

The most important of these sub-families for leukocytes are: i) the β1 integrins (also known as the very late appearing antigen (VLA) integrins) which have a common β chain CD 29 paired with different α subunits CD 49a-CD49f, and ii) the β2 integrins which share the β chain CD18 paired with α-chains, CD11a (LFA-1), CD11b (Mac-1), or CD11c (p150, 95) (Adams and Shaw,1994). The integrins important in the binding of leukocytes to the endothelium are the β2 integrins LFA-1 (CD11a) on lymphocytes and Mac-1 (CD11b) on neutrophils and monocytes, and the β1 integrin VLA-4 (CD49d) on lymphocytes and monocytes (Jang et al, 1994).

The corresponding ligands on the endothelium for these leukocyte integrins are ICAM-1 and ICAM-2 for the β2 integrins and VCAM-1 for the β1 integrins. ICAM-1 and VCAM-1 are adhesion molecules from the immunoglobulin superfamily.

Table 1.2 Integrin molecules, their ligands and their distribution

Integrin	Alternative Name (s)	Ligand (s)	Distribution
α1β1	VLA-1, CD49a/CD29	collagen, laminin	broad
α2β1	VLA-2, CD49b/CD29	collagen, laminin	broad
α3β1	VLA-3, CD49c/CD29	epiligrin, fibronectin, collagen, laminin	broad
α4β1	VLA-4, CD49d/CD29	fibronectin, VCAM-1	lymphocytes, muscle, monocytes/macrophage fibroblasts, neural crest
α5β1	VLA-5, CD49e/CD29	fibronectin	broad
α6β1	VLA-6, CD49f/CD29	laminin	broad
α7β1		laminin	?
α8β1		?	?
α9β1		tenascin	?
αvβ1		fibronectin, vitronectin	epithelial cells
αLβ2	LFA-1, CD11a/CD18	ICAM-1, ICAM-2, ICAM-3	leukocytes
αMβ2	Mac-1, CD11b/CD18	fibrinogen, iC3b, ICAM-1, factor X	monocytes, granulocytes, NK cells, T-lymphocytes
αXβ2	p150, 95, CD11c/CD18	fibrinogen, iC3b	monocytes, granulocytes, activated B-lymphocytes
αIIbβ3	gpIIb/IIIa	fibrinogen, fibronectin, VWF, vitronectin	platelets, megakaryocytes
αvβ3	VnR	fibrinogen, fibronectin, VWF, vitronectin, collagen	endothelium, tumour cells
α6β4		laminin, BM protein (?)	epithelial cells
αvβ5		fibronectin, vitronectin	carcinoma cells
αvβ6		fibronectin	?
α4β7		VCAM-1, fibronectin, MadCAM-1	activated B and T lymphocytes, macrophages
αEβ7		E-cadherin	intraepithelial lymphocytes
αvβ8		vitronectin	?

Source : Faull, 1995, Jang et al, 1994.

1.2.3.3 THE IMMUNOGLOBULIN SUPERFAMILY

The immunoglobulin superfamily includes the cellular adhesion molecules ICAM-1, ICAM-2, VCAM-1 and MAdCAM-1 (Mucosal Addressin CAM-1). All members of this family are expressed or are inducible on vascular endothelium and all have one or more of the immunoglobulin homology units as a common structural feature. These units consists of 70-110 amino acids forming anti-parallel β-pleated sheets (Williams and Barclay, 1988).

ICAM-1 (CD 54) and ICAM-2 are closely related in structure and function. ICAM-1 is widely distributed being found on endothelial, epithelial, follicular dendritic and fibroblast cells and on leukocytes and it is a primary ligand for the integrin LFA-1 (Dustin et al, 1988). The expression of ICAM-1 is increased by the stimulation of the cytokines IL-1, IF-γ and TNF-α and the level of expression peaks at 18-24 hours after initial stimulation and remains persistent with an ongoing stimulus (Dustin et al, 1986). ICAM-2 is also a ligand for LFA-1 and is found on the same cell types as ICAM-1. The expression of ICAM-2 is constitutive and unlike ICAM-1, its expression is not increased by the presence of cytokines (de Fougerolles et al, 1991).

The third member of the immunoglobulin superfamily that serves as an endothelial adhesion molecule for leukocytes is VCAM-1. VCAM-1 was first identified in 1989 and is not constitutively expressed. Its expression is stimulated by lipopolysaccharide (LPS) and by the cytokines TNF-α, IL-1 and specifically by IL-4 and maximal expression is seen 4-10 hours after stimulation (Jang et al, 1994).

VCAM-1 is expressed on activated endothelial cells, follicular dendritic cells, bone marrow stromal cells and some macrophages. The counter receptor for VCAM-1 is the integrin $\alpha 4\beta 1$ (VLA-4) which is distributed on monocytes, macrophages, lymphocytes, neural crest cells and fibroblasts (Lobb and Hemler, 1994).

There is considerable evidence for the involvement of VCAM-1 in the development of early atherosclerotic lesions and in mature atherosclerotic plaques (Cybulsky and Gimbrone, 1991; Van der Wal et al, 1992). It has been shown in hyperlipidemic rabbits that endothelial adhesion of monocytes and lymphocytes is preceded by an increased expression of VCAM-1. In experiments with New Zealand White rabbits fed a 0.3% cholesterol diet, after 3 weeks on the hypercholesterolemic diet, numerous macrophages were found in the intima, but only under VCAM-1 expressing endothelial cells. After 6 weeks on the high cholesterol diet, the aorta contained areas of thickened intima with foam cell lesions. The endothelium covering most of these lesions expressed VCAM-1, whereas the endothelium of lesion-free areas usually lacked VCAM-1 (Li et al, 1993). Variable and low levels of E-selectin and VCAM-1 have been detected in the arterial endothelium overlying atherosclerotic plaques (Wood et al, 1993). VCAM-1 has been discovered at the base of atherosclerotic plaques in areas of inflammatory infiltrates and neovascularization which suggests that intimal neovascularization maybe an important site of inflammatory cell recruitment into advanced coronary lesions (O'Brien et al, 1993).

1.2.4 Factors Influencing Adhesion Molecule Expression and Leukocyte Binding to the Endothelium

Adhesion molecule expression is regulated by cytokines (TNF-α, IL-1 and IFN-γ) and chemoattractants (C5a, leukotriene B4, platelet activating factor and members of the chemokine family of polypeptides). Other factors can also affect the level of expression of cellular adhesion molecules.

Substances that attenuate the level of cytokine-induced expression of adhesion molecules include high density lipoproteins (Cockerill et al, 1995), omega-3 polyunsaturated fatty acids (De Caterina et al,1994), treatment with probucol in rabbits (Fruebis et al, 1997), TGF-β (Gamble et al, 1995), and L-arginine (Adams et al, 1997). Factors that increase the expression of cellular adhesion molecules include native LDL (Smalley et al, 1996; Lin et al,1996), oxidised LDL (Khan et al, 1995), lysophosphatidylcholine (Kume et al, 1992), Lp(a) (Allen et al, 1998) and cigarette smoke condensate (Shen et al, 1995).

Other factors can attenuate binding of leukocytes to the endothelium. Examples of these include: vitamin E inhibiting the LDL induced adhesion of monocytes to endothelial cells (Martin et al, 1997), vitamin C preventing smoke induced leukocyte aggregation and adhesion to the endothelium (Lehr et al, 1994) and fluvastatin decreasing the level of leukocyte-endothelial adhesion induced by feeding rats a hypercholesterolemic diet (Kimura et al, 1997).

1.2.5 SOLUBLE ADHESION MOLECULES AND ATHEROSCLEROSIS

Soluble forms of cellular adhesion molecules can be found in the circulation. The soluble adhesion molecules are thought to be shed from membrane bound versions of the adhesion molecule. The shedded soluble forms are probably generated by cleavage at a site close to the membrane insertion as they lack the membrane-spanning and cytoplasmic domains that are present in the membrane-bound forms (Pigott et al, 1992). The origins, metabolism and functional significance of the soluble adhesion molecules are not yet known, however it is apparent that their levels are altered in a range of diseases and that the treatment of these diseases can affect the levels.

ELISA kits are now commercially available to measure soluble adhesion molecules. The major soluble adhesion molecules measured are from the selectin family (soluble L-selectin (sL-selectin) soluble P-selectin (sP-selectin) and soluble E-selectin (sE-selectin)) and from the immunoglobulin super family (soluble ICAM-1(sICAM-1) and soluble VCAM-1 (sVCAM-1)). Disease states in which soluble adhesion molecule levels are elevated include sepsis, autoimmune diseases, malignancies, organ failure and chronic inflammatory diseases such as atherosclerosis (Gearing and Newman, 1993) (Table 1.3). The soluble forms of adhesion molecules are biologically active and their purpose may be to partially block the adhesion of

Table 1.3. Blood levels of soluble adhesion molecules in disease states

Adhesion Molecule	Fold elevation in disease
sL-selectin	x 0.5 Kawasaki disease x 2 Sepsis x 3 HIV infection
sP-selectin	x 2 Haemolytic uremic syndrome x 3 Thrombotic thrombocytopenic purpera
sE-selectin	x 2 Diabetes x 2 Breast/gastro intestinal (GI) cancer x 3 - x 23 Septic shock x 4 Systemic lupus erythematosis (SLE) x 2 Scleroderma x 2 Malaria
sVCAM-1	x 2 Ovarian, GI, renal, bladder cancers x 8 Septic shock x 2 Impaired renal function x 2 Haemodialysis x 4 SLE x 2 Diabetes x 3 Vasculitis x 2 Rheumatoid arthritis
sICAM-1	x 2 Diabetes x 2 Impaired renal function x 3 Chronic ambulatory peritoneal dialysis x 3 Septic shock x 1.5 Bacterial meningitis x 3 - x 5 Metastatic cancer x 2 Idiopathic pulmonary fibrosis x 2 Crohns disease x 2 Ulcerative colitis

Source : Gearing and Newman, 1993; Gearing et al, 1992.

leukocytes to surface bound adhesion molecules and compete with the surface bound adhesions for their ligands (Gearing et al, 1992).

sVCAM-1 has been shown to correlate with the degree of atherosclerosis in subjects with peripheral vascular disease (Peter et al, 1997) and elevated levels of sVCAM-1 have been reported in patients with advanced atherosclerosis of their aortas compared with asymtomatic controls (Nakai et al, 1995). In other studies patients with ischemic heart disease and peripheral arterial disease were compared to normal controls with respect to their serum levels of soluble adhesion molecules (Blann and McCollum, 1994; Blann et al, 1996). In these studies, sP-selectin and sICAM-1 were elevated in the patient groups compared with the control subjects. A prospective case-control study showed that the baseline plasma concentration of sICAM-1 was significantly higher in men who went on to have a myocardial infarction than in matched controls (Ridker et al, 1998).

There are also elevations in soluble adhesion molecules in conditions known to predispose to atherosclerosis such as dyslipidemia (Hackman et al, 1996), diabetes mellitus (Lampeter et al, 1992) and hypertension (Lip et al, 1995). Studies have shown that improved diabetic control and treatment of dyslipidemias can reduce soluble adhesion molecule levels back towards the normal range. Ceriello and colleagues (1996) showed a significant reduction in sICAM-1 levels after a three month period of improved diabetic control. Studies of hypercholesterolemic subjects showed a reduction in certain soluble adhesion molecules after cholesterol lowering therapy. In subjects with familial hypercholesterolemia treated with LDL

apheresis there was a significant reduction in sICAM-1 and sE-selectin after therapy (their removal by extracorporeal circulation components was excluded) (Sampietro et al, 1997). In a different study, 10 hypercholesterolemic subjects were treated aggressively with atorvastatin ± colestipol for a mean of 42 weeks. Comparison of soluble adhesion molecules before and after treatment showed a significant reduction in sE-selectin, but no significant change in sVCAM-1 and sICAM-1 (Hackman et al, 1996). The same group also assessed the effects of purified n-3 fatty acid (Omacor) 4 g/day on soluble adhesion molecule levels in subjects with hypertriglyceridemia (Abe et al, 1998). The baseline levels of sE-selectin, sVCAM-1 and sICAM-1 were elevated in the subjects with hypertriglyceridemia compared to controls. After > 7 months of purified n-3 fatty acid therapy, there was a significant reduction in sE-selectin and sICAM-1 but no change in sVCAM-1.

Soluble adhesion molecule levels may provide an in-vivo guide to the level of expression of cell membrane based adhesion molecules (Blann and McCollum, 1994), and as discussed above, their levels correlate with the degree of atherosclerosis and the level of control of the atherosclerotic risk factors diabetes and dyslipidemia.

1.3 High Density Lipoproteins

1.3.1. HDL Subpopulations

HDLs are the smallest and most dense of the plasma lipoproteins. The HDL fraction in human plasma is heterogeneous and can be separated into several subclasses by

preparative, rate zonal and analytical ultracentrifugation, selective polyanion precipitation, gradient gel electrophoresis, and immunoaffinity chromatography (Alaupovic, 1984). When isolated on the basis of density by ultracentrifugation, human HDLs separate into two major subfractions: HDL_2 and HDL_3. Table 1.4 summarizes some of the properties of these two major HDL subfractions with the major differences being HDL_2 are larger and less dense than HDL_3 and contain less cholesterol esters, triglycerides and proteins.

Non-denaturing polyacrylamide gradient gel electrophoresis has been used to further separate HDL_2 and HDL_3 into subpopulations according to their size. Using this technique, Blanche et al, (1981) separated HDLs into five distinct subpopulations. In order of decreasing particle size these are HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c}.

Subpopulations of HDLs which differ with respect to their apolipoprotein content have also been identified. The main HDL apolipoproteins are apoA-I and apoA-II, with apoA-I comprising 60-70% (by mass) and apoA-II 20-30% of the HDL protein content (Patsch and Gotto, 1987). Using immunoaffinity chromatography HDLs have been separated into three major apolipoprotein-specific subpopulations. One subpopulation consists of HDLs that contain apoA-I without apoA-II (A-I HDLs), another comprises particles that contain both apoA-I and apoA-II (A-I/A-II HDLs) and the third subpopulation contain apoA-II without apoA-I (A-II HDLs) (Cheung and Albers, 1984). The A-I HDLs are found in both the HDL_2 and HDL_3 density ranges, while most of the A-I/A-II and A-II HDLs are found in the HDL_3 density range. The other apolipoproteins that exist on HDLs are apoA-IV, C-I, C-II, C-III,

Table 1.4 - Properties of human HDL_2 and HDL_3[a].

A - Physical properties

	Radius (nm)	Hydrated density (g/ml)	Floatation coefficient[b]
HDL_2	4.4-6.0	$1.063 < d < 1.125$	$S_{f(1.21)}$ 3.5-9.0
HDL_3	3.8-4.4	$1.125 < d < 1.210$	$S_{f(1.21)}$ 0.0-3.5

B - Chemical composition (% composition by weight)

	TG[c]	CE	UC	PL	Protein
HDL_2	4.1	12.0	5.4	30.0	41.0
HDL_3	4.5	16.0	2.9	23.0	55.0

[a] Source - Skipski, 1972; Scanu, 1979; Blanche et al, 1981

[b]Flotation coefficient units - 10^{-13} cm sec^{-1} dyn^{-1} g^{-1}.

[C] TG, triglyceride; CE, cholesteryl esters; UC, unesterified cholesterol; PL, phospholipid.

D, E, G, H and J (McConathy and Alaupovic, 1986). The enzymes and transfer proteins associated with HDLs are cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), lecithin:cholesterol acyl transferase (LCAT) and paraoxonase. HDLs have also been separated on the basis of their surface charge by agarose gel electrophoresis (Neary et al, 1991). The majority of HDLs have alpha migration on the agarose gels, but small discoidal HDLs have pre-beta and pre-alpha migration (Castro and Fielding, 1988).

There is evidence that specific subpopulations of HDL have differing functions. For instance, the rate of LCAT-catalysed cholesterol esterification is influenced by both HDL particle size and apolipoprotein composition and the preferred initial acceptor of cholesterol in the first step of reverse cholesterol transport has been identified as a minor subpopulation of very small, prebeta migrating HDLs (Castro and Fielding, 1988). In-vitro studies have suggested that HDL particles containing apoA-I without apoA-II could be as effective (Johnson et al, 1991; Oikawa et al, 1993) or better (Barkia et al, 1991) acceptors of cholesterol than HDL particles containing both apoA-I and apoA-II.

1.3.2. HDL METABOLISM

Nascent HDLs are discoidal complexes of apoA-I and phospholipids. They are secreted from both the human intestine and liver (Hamilton et al, 1976; Green and Glickman, 1981). Discoidal HDLs can also be assembled in the plasma from lipid-poor apoA-I and phospholipids which are derived from cell membranes and the surface of VLDLs which are undergoing lipolysis (Forte et al, 1995). These discoidal

HDLs acquire unesterified cholesterol from tissues and the enzyme LCAT catalyses the esterification of the cholesterol to form cholesteryl esters. The discoidal complexes are then converted into small spherical particles due to the accumulation of cholesteryl esters in their core (Rye et al, In Press). These small spherical HDLs are in the HDL_3 density range and are subsequently modified to become larger, less dense HDL_2 particles by acquisition of apolipoproteins and lipids from the lipolysis of triglyceride rich chylomicrons and VLDLs and by further activity of LCAT (Nichols et al, 1985; Liang et al, 1995). HDL_2 can also be converted back to HDL_3 by the removal of cholesteryl esters into LDL and VLDL by cholesteryl ester transfer protein (CETP), PLTP and hepatic lipase (Rye et al, In Press). In this process HDLs are interconverted between HDL_2 and HDL_3 as cholesterol is transferred from peripheral tissues to the liver or to apoB containing lipoproteins.

1.3.3. HDLs and Coronary Heart Disease

There is evidence that HDL subpopulations may vary in terms of their ability to protect against CHD. A number of case-control studies have suggested that the HDL_2 subfraction may be more protective than the HDL_3 subfraction (Lamarche et al, 1997; Robinson et al, 1987; Salonen et al, 1991). Other studies have suggested that the HDL_3 subfraction may be superior to HDL_2 in its protection against CHD (Kempen et al, 1987; Sweetnam et al, 1994). There are still other studies showing that both HDL_2 and HDL_3 may be protective against CHD (Buring et al, 1992; Stampfer et al, 1991). A review by Barter and Rye (1996) presents convincing evidence that apolipoprotein composition may influence the capacity of HDLs to protect against atherosclerosis.

A-I HDLs appear to be superior to A-I/A-II HDLs in their ability to protect against atherosclerosis. Clinical support for this comes from a study of adolescents whose parents had suffered a myocardial infarction under the age of 55 years (Amouyel et al, 1993). When compared with matched controls with no family history of CHD, the adolescents with a positive family history of CHD had significantly lower concentrations of A-I HDL particles and no difference in the level of the A-I/A-II HDL particles. Another clinical study showed that A-I HDLs but not A-I/A-II HDLs were inversely correlated with angiographically established coronary artery disease (Puchois et al, 1987).

Finally, studies of transgenic mice provide direct evidence that while both A-I HDLs and A-I/A-II HDLs give protection against atherosclerosis it is the A-I HDLs that are superior. Transgenic mice that overexpress human apoA-I have superior protection against the development of atherosclerosis (Plump et al, 1994; Patszty et al 1994) than mice overexpressing the combination of human apoA-I and apoA-II (Schultz et al, 1993) or human apoA-II alone (Schultz and Rubin, 1994). Mice overexpressing murine apoA-II have actually been shown to develop atherosclerosis (Warden et al, 1993).

Hence, according to retrospective human studies and transgenic animal studies, A-I HDLs appear to give superior protection against atherosclerosis. To confirm these findings for humans, prospective studies are needed and the mechanism of A-I HDLs superiority needs to be investigated.

1.3.4 Mechanisms of The Anti-Atherogenic Effects of HDLs

The precise mechanism of how HDLs inhibit atherosclerosis is still not clear. One of the most widely documented theories is the involvement of HDLs in reverse cholesterol transport, the pathway in which HDLs transport cholesterol from extrahepatic tissues into the liver (Figure 1.2). There are four basic steps in reverse cholesterol transport (Barter, 1994): i) cholesterol is transferred from cell membranes in unesterified form to HDLs; ii) the cholesterol is esterified by LCAT. The movement of esterified cholesterol to the core of the HDLs leaves the surface depleted of cholesteryl esters which creates a concentration gradient. This allows more cholesterol to move from the cell membranes to the surface of the HDL particle; iii) a substantial proportion of the cholesteryl esters from the HDLs are transferred by CETP to the apoB containing lipoproteins VLDLs, IDLs and LDLs; iv) most of the cholesteryl esters are eventually delivered to the liver contained in LDLs, but a small amount are delivered to the liver directly by HDLs. In support of the reverse cholesterol transport pathway, it has been clearly shown that HDLs promote the efflux of cholesterol from cholesterol laden cells (Fielding and Fielding, 1995) and HDLs reduce the cholesterol content of foam cells (Miyazaki et al, 1992). There is also evidence which suggests that reverse cholesterol transport may not be the major pathway in which HDLs protect against CHD. Some subjects with atherogenic lipoprotein profiles, including low plasma levels of HDL, have increased rates of at least some steps in the reverse cholesterol transport pathway: cholesterol

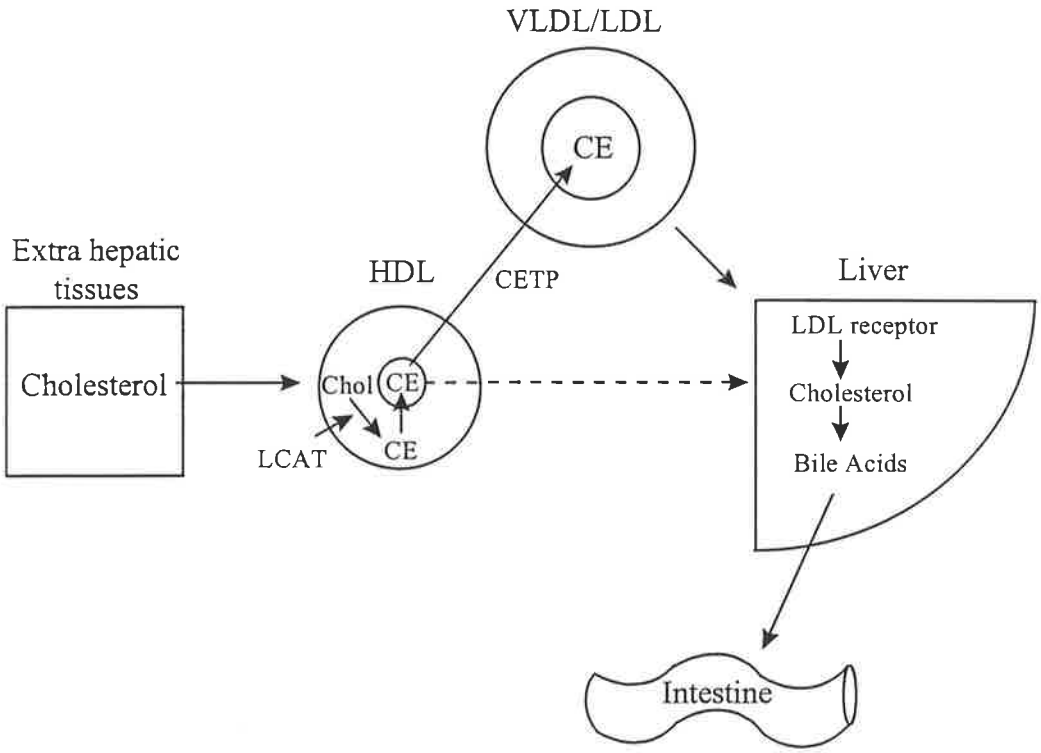

Figure 1.2. Schematic diagram of reverse cholesterol transport. Cholesterol is transferred from cell membranes in unesterified form to HDLs. Within the HDLs under the influence of LCAT the cholesterol is esterified. Esterified cholesterol is not water soluble and hence moves to the core of the HDLs leaving the surface depleted of cholesterol thus creating a concentration gradient which allows more cholesterol to move from the cell membranes to the surface of the HDL particle. A substantial proportion of the cholesteryl esters from the HDLs are transferred by CETP to the apo B containing lipoproteins VLDLs, IDLs and LDLs. Most of the cholesteryl esters are eventually delivered to the liver contained in LDLs, but a small amount are delivered to the liver directly by HDLs (Adapted from Barter, 1994).

esterification by LCAT is increased and there is an increase in the level of prebeta HDLs, the preferred acceptor of cellular cholesterol (Barter and Rye, 1996).

HDLs have a number of functions that may be unrelated to their role in plasma lipid transport but which may contribute to the ability of HDLs to prevent atherosclerosis. These functions include the antioxidant properties of HDLs, the fact that HDLs are mitogenic, HDLs can reduce cellular growth factors, HDLs have anti-inflammatory properties, HDLs have favourable effects on preventing blood coagulation and increasing vascular reactivity and HDLs inhibit the cytokine-induced expression of adhesion molecules in cultured endothelial cells.

HDLs may protect against atherosclerosis by their antioxidant properties. One of the early, important events in atherosclerosis is the oxidation of LDL in the arterial intima. HDLs have been shown to inhibit this oxidation of LDL both in vitro (Maier et al, 1994; Decossin et al, 1995) and in vivo (Klimov et al, 1993). The mechanism by which HDLs prevent the oxidation of LDLs is not known, but may be related to the enzyme paraoxonase which is associated with HDL. Mackness et al showed that HDL and paraoxonase inhibited the lipid peroxide generation in LDL by 32% and 25% respectively after 24 hours of incubation (Mackness et al, 1993).

Tauber et al have reported that HDLs are mitogenic as they caused proliferation of cultured bovine vascular endothelial cells (Tauber et al, 1980). LDLs were also studied and were mitogenic for the vascular endothelial cells only when added at low concentration. When the LDLs were added at physiological concentrations they were

toxic to the cells. It is possible that the mitogenic effect of HDLs for endothelial cells may result in the endothelium having improved capacity for repair after injury.

HDLs have also been shown to reduce epidermal growth factor (EGF)-induced DNA synthesis in vascular smooth muscle cells (Ko et al, 1993). Abnormal cell growth of vascular smooth muscle cells plays a pathophysiological role in the development of hypertension and atherosclerosis (Schwartz et al, 1986) and hence the ability of HDLs to reduce the levels of EGF may partly explain how HDLs protect against atherosclerosis.

HDLs also have anti-inflammatory properties. In vitro studies of mediator production by monocyte/macrophages show that binding of lipopolysaccharide (LPS) to HDL and other lipoproteins greatly reduces production and release of TNF-α, IL-1, and IL-6 (Ulevitch and Johnston, 1978; Ulevitch et al, 1981; Mathison et al, 1988). Levine et al (1993) showed that transgenic mice with 2-fold-elevated plasma HDL levels and overwhelming bacterial infections had more endotoxin bound to HDL, lower plasma cytokine levels, and improved survival rates compared with low HDL mice.

The antiatherogenic effects of HDLs may also relate to their effects on the blood coagulation system. Epand et al (1994) have shown that the generation of procoagulant activity in human erythrocytes by the calcium ionophore A23187 and Ca^{2+} is inhibited by apoA-I and HDLs. HDLs have also been shown to increase PGI_2 levels in cultured endothelial cells (Fleisher et al, 1982). PGI_2 has potent vasodilator

functions and inhibits platelet aggregation. Yui et al (1988) have shown that the PGI_2 stabilizing factor (PSF), a factor found in serum that binds to PGI_2 and prolongs its half-life, is actually apoA-I, the major apolipoprotein of HDLs. Hence these effects on preventing blood coagulation maybe important to the antiatherogenic action of HDL.

In vivo studies have shown that in coronary arteries with varying degrees of atherosclerotic thickening, subjects with elevated plasma HDL had a significantly blunted constrictor response to acetylcholine than subjects with lower plasma levels of HDL (Zeiher et al, 1994). This suggests that HDL cholesterol may exert a beneficial effect on abnormal vascular reactivity, a fundamental functional disturbance that is associated with atherosclerosis.

A recent theory on how HDLs may protect against atherosclerosis is by their ability to inhibit cytokine induced adhesion molecule expression on endothelial cells. As described above, the adhesion of monocytes to the vascular endothelium is an early event in atherosclerosis. It has been shown that HDLs inhibit the cytokine-induced (both TNF-α and IL-I) expression of the cellular adhesion molecules VCAM-1, ICAM-1 and E-selectin in cultured endothelial cells (Cockerill et al, 1995). This inhibition is concentration dependent and occurs over the physiological range of HDL concentrations. There are parallel reductions in the steady state mRNA levels of these adhesion molecules (Cockerill et al, 1995). This inhibition of adhesion molecules is seen both with native human HDLs and reconstituted discoidal HDLs which contain only apoA-I, phosphatidylcholine and unesterified cholesterol. The

mechanism of how HDLs cause the inhibition of adhesion molecule expression is yet to be elucidated.

1.4 ACUTE PHASE REACTANTS AND HDLS

The acute-phase response to injury and infection is characterised by fever, an increase in granulocyte production, the release of bone marrow-derived cells and other lymphokine-mediated responses. This includes a major rearrangement of plasma protein synthesis by the liver, whereby the production of many acute-phase proteins are increased at the expense of albumin synthesis (Malle et al, 1993). One such acute phase protein is serum amyloid-A (SAA) which is carried predominantly by HDLs. SAA is a low molecular weight protein of around 12,000 daltons. In the acute phase, SAA can reach plasma levels 500- to 1000- fold greater than in the non-inflammatory state (Kisilevsky, 1991). The level of SAA in the plasma peaks on average 67 hours after the onset of the acute phase response and levels fall to baseline 7-10 days after the stimulus to the acute phase has resolved (Maury et al, 1988). The definitive physiological function of SAA is yet to be determined.

1.4.1 SAA AND HDL

Once released into the circulation from the liver, SAA rapidly associates with HDLs (Benditt and Eriksen, 1977; Malmendier et al, 1979; Eriksen and Benditt, 1980). SAA most likely binds to HDL via its amphipathic α-helical structure which is located mainly in the N-terminal region (Turnell et al,1986; Malle et al, 1995). SAA accounts for 17-87% of total apolipoproteins present in acute phase HDL (Clifton et al, 1985) and it mainly circulates as a component of the HDL_3 subfraction (Benditt

and Eriksen, 1977; Hoffman and Benditt,1982). The SAA enriched HDL_3 are enriched in triglycerides but depleted of phospholipids (Clifton et al, 1985). They are larger than normal HDL_3 having a diameter of 9.0 - 10.6 nm that is typical of HDL_2 rather than HDL_3. (Cabana et al, 1989).

In the acute phase, SAA displaces apoA-I and to a lesser extent apoA-II from the HDLs. As a consequence, the apoA-I and A-II levels in the plasma fall, although the HDL cholesterol level remains unchanged. When SAA levels fall, the HDL cholesterol level also falls and only begins to rise when the HDL apoA-I and A-II are regenerated (Bausserman et al, 1988).

1.4.2 SAA And Its Role In Atherosclerosis

The process of inflammation has an important role in the pathogenesis of atherosclerosis (Berliner et al, 1995). Plasma SAA levels are elevated in inflammatory states and there are now several theories as to how SAA could predispose to atherosclerosis.

SAA may alter the binding of HDLs to cells. HDLs that are associated with SAA have altered the binding characteristics to neutrophils (Shephard et al, 1987). The presence of SAA on HDLs enhances the binding of these HDL particles to macrophages by up to fourfold. This contrasts with a decreased affinity of SAA enriched HDL for normal hepatocytes (Kisilevsky and Subrahmanyan, 1992). Studies of human monocytic THP-1 cells indicate that acute phase HDL particles are

also preferentially bound by these cells when compared with normal HDL (Banka et al, 1995).

SAA may also promote atherosclerosis by adversely affecting reverse cholesterol transport. It has been shown in vitro that phospholipid vesicles exposed to apoA-I and low levels of SAA increase LCAT activity whereas high levels of SAA in these vesicles decrease LCAT activity (Steinmetz et al, 1989).

Normal HDLs protect against the oxidative modification of LDL. Van Lenten and colleagues showed that SAA enriched HDLs have a decreased ability to protect LDL against oxidation (Van Lenten et al,1995). They showed that paraoxonase levels in HDLs declined by 71% in the SAA-HDLs. They also demonstrated an enhanced expression of MCP-1 in cells treated with acute phase SAA-HDL compared to those treated with normal HDL.

Recombinant human SAA has been shown in vitro to be a chemoattractant and to induce the migration, adhesion and tissue infiltration of monocytes, polymorphonuclear leukocytes and T lymphocytes (Badolato et al, 1994; Xu et al, 1995). Recombinant human SAA also increases the expression of the cellular adhesion molecules Mac-1 and leukocyte adhesion molecule-1 (Badolato et al, 1994). In addition to being associated with HDLs, SAA has been localised to human atherosclerotic lesions and to macrophage/monocyte cell lines (Urieli-Shoval et al, 1994). In one study of atherosclerotic lesions in human coronary and carotid arteries, SAA mRNA was found in most endothelial cells, some smooth muscle cells and in

macrophage-derived "foam cells", adventitial macrophages and adipocytes (Meek et al, 1993).

The above studies show there is now extensive circumstantial evidence that SAA may have a pro-atherogenic role, although whether this translates into accelerated atherosclerosis in vivo remains to be determined.

1.5 AIMS OF THIS THESIS

The studies described in this thesis are concerned with the ability of HDL to inhibit the cytokine-induced expression of adhesion molecules by endothelial cells. The specific aims are as follows:

- to determine whether HDLs from different subjects vary in their ability to inhibit the cytokine induced expression of the adhesion molecule VCAM-1.

- to determine whether the HDL subfractions, HDL_2 and HDL_3 vary in their ability to inhibit the cytokine induced expression of the adhesion molecule VCAM-1.

- to determine whether preincubation of HDL_2 and HDL_3 with endothelial cells is sufficient to inhibit cytokine-induced VCAM-1 expression or whether the HDLs must be present during the endothelial cell activation.

- to determine whether the inhibitory effects of HDL reside in the lipid or protein components of the particle.

- to determine the effects of apolipoprotein composition of HDL_3 on their ability inhibit VCAM-1 expression.

- to determine whether inflammatory HDLs that are enriched in SAA retain their ability to inhibit the cytokine induced expression of the adhesion molecule VCAM-1.

- to determine whether treatment of diabetic subjects with fenofibrate influences their serum levels of the soluble adhesion molecules sVCAM-1, sICAM-1 and sE-selectin and if so, how the changes correlate with changes in plasma lipids and lipoproteins.

1.6 Contribution By the Candidate to the Thesis

The development of this thesis represents interaction between my supervisors and myself. My input at all levels was substantial, and after discussions with my supervisors decisions about how to proceed were my own.

CHAPTER 2

METHODS AND MATERIALS

2.1 ISOLATION AND PREPARATION OF PLASMA HDLS

Blood samples were collected from subjects after a 12 hour fast by standard venipuncture into tubes containing disodium EDTA (final concentration 1 mg/ml) and placed immediately on ice. Plasma was separated by centrifugation at 4°C. HDLs were isolated by sequential ultracentrifugation at 4°C in a Beckman TLA 100.4 rotor using a Beckman TL-100 Ultracentrifuge. Density adjustments were made by the addition of solid KBr (Hatch and Lees, 1963). HDLs were isolated in their appropriate density range: total HDLs $1.07 < d < 1.21$ g/ml, HDL_2 $1.07 < d < 1.12$ g/ml and HDL_3 $1.13 < d < 1.21$ g/ml. The samples were centrifuged at both the lower and higher densities. Each was at 100,000 r.p.m. for 17 h. Supernatants and infranatants in each spin were recovered by tube slicing. The resulting HDLs were dialysed against either 3 x 1 litre of endotoxin-free phosphate buffered saline (pH 7.4) (PBS) or Tris-buffered saline (TBS) (pH 7.4). The PBS was prepared from 0.12% (w/v) Na_2HPO_4 and 0.02% (w/v) KH_2PO_4 and contained 0.9% (w/v) NaCl, 0.02% (w/v) KCl, 0.01% (w/v) EDTA-Na_2 and 0.02% (w/v) NaN_3. The TBS contained 0.01 M Tris, 0.15 M NaCl, 0.02% (w/v) NaN_3 and 0.01% (w/v) EDTA-Na_2.

2.2 ISOLATION OF APO A-I, APO A-II AND SAA.

Blood samples were collected as described in section 2.1. Plasma for the isolation of apo A-I and A-II was isolated from healthy subjects and was pooled. Plasma for the isolation of SAA was obtained from coronary artery bypass grafting (CABG) patients on the third post-operative day and was pooled. HDLs were isolated from the plasma by sequential ultracentrifugation at 4°C in a Beckman Type 55.2 Ti rotor using a

Beckman L8-70M Ultracentrifuge . Density adjustments were made by the addition of solid KBr. Apo A-I and apo A-II were isolated from the total HDL fraction of 1.07 < d < 1.21 g/ml and SAA was isolated from the HDL_3 fraction of 1.13 < d < 1.21 g/ml. The sample was centrifuged at 55,000 r.p.m. for 17 h. The samples were then centrifuged for 26 h at the higher density at 55,000 r.p.m. The resulting HDLs were dialysed against 3 x 5 L of 20 mM NH_4HCO_3 before delipidation (Osborne, 1986). During the delipidation, lyophilization was used to concentrate the HDLs to 50% of their original volume. Methanol was then added to the HDLs and 3 hours later chloroform and chilled diethyl ether were added (methanol : chloroform : ether = 1:1:3 (v/v)). The mixture was chilled at - 20°C for 30 minutes, then subjected to centrifugation at 1,500 rpm at 4°C for 10 minutes. The infranatant was subjected to further delipidation using ethanol and chilled ether (1:4 v/v). The latter procedure was repeated 3 times, followed by drying the apo HDL pellet under nitrogen. The apo HDL was subsequently dissolved in 20 mM Tris, pH 8.2, lyophilised and stored at -20°C before being subjected to anion exchange chromatography for further separations of apolipoproteins.

The anion-exchange chromatography was performed on a column of Q-sepharose Fast Flow (Pharmacia LKB Biotechnology, Uppsala, Sweden) attached to a Fast Protein Liquid Chromatography (FPLC) system. The apo A-I, apo A-II and SAA were resolved by a modification of the method of Weisweiler (1987). The modifications involved using a salt gradient of 0.5M NaCl from 0-50% over 3 hours, using a Superose 6 Pharmacia column containing the Q-sepharose and loading 100mg of total protein (apo A-I, A-II and SAA) onto the column. The apo A-I, apo

A-II and SAA each appeared as a single band after electrophoresis on a 20% SDS-polyacrylamide gradient gel stained with Coomassie R 350. The purified apolipoproteins were dialysed against 3 x 5 L of 20 mM NH_4HCO_3, lyophilised and stored at -20°C. Prior to use they were reconstituted in 0.01 M Tris-HCl, 3.0 M guanidine HCl, 0.01% (w/v) EDTA-Na_2 (pH 8.2) for 1-2 hours and then exhaustively dialysed against TBS (5 x 1 L).

2.3 Preparation of Reconstituted HDLs

Discoidal rHDLs containing apo A-I and di-myristoyl-phosphatidylcholine (DMPC) were prepared by the cholate dialysis method described by Matz and Jonas (1982). The molar ratio for apo A-I : DMPC was 1 : 100. The cholate solution (30 mg/ml) was prepared by dissolving sodium cholate in TBS, pH 7.4. The mixing of the lipids and apo A-I was performed in glass test tubes. The first step involved the DMPC being dried as a thin film on to the wall of the test-tube with nitrogen. The cholate (6.32 μmol) was then added. TBS, pH 7.4 was also added to bring the volume to 0.5 ml. The test-tubes were kept on ice and vortexed every 10-15 minutes until the mixture became clear (1-2 hours). Apo A-I (2 mg), was added into each test-tube which was left on ice for another 2-3 hours. The DMPC-cholate-apo A-I mixtures from each test-tubes were pooled and dialysed against 5 x 1 L of TBS (pH 7.4) over 5 days to remove the cholate.

2.4 Chemical Analyses

All chemical assays were performed on a Cobas Fara Centrifugal Analyzer (Roche Diagnostics, Zurich, Switzerland). Protein concentrations were measured using the

method of Lowry et al. (1951) adapted for use on the Cobas Fara (Clifton et al, 1988) and using bovine serum albumin as a standard. Concentrations of apo A-I and apo A-II were measured immunoturbidometrically using sheep anti-human apo A-I and anti-human apo A-II anti-serum (Boehringer Mannheim, Germany). The reagent for the apolipoprotein assays was a solution of saline (0.9% (w/v) NaCl) containing 40% (v/v) polyethylene glycol 600 (Boehringer Mannheim), 0.1% Tween-20 (BDH Chemicals) and 0.025% (v/v) buffer concentrate (Behring). Concentrations of total cholesterol, free cholesterol, triglyceride and phospholipid were measured using enzymatic kits (Boehringer Mannheim). The concentration of esterified cholesterol was calculated as the difference between the concentration of total cholesterol (esterified plus free) and free cholesterol.

2.5 ELECTROPHORESIS

2.5.1 NON-DENATURING GRADIENT GEL ELECTROPHORESIS

The particle sizes of the HDL and rHDL were determined by electrophoresis on 3-40% non-denaturing polyacrylamide gradient gels (Gradipore, Australia). Samples (5 μg of protein) were pre-mixed with 20 μl of a solution containing 40% (w/v) sucrose and 0.01% (w/v) bromo-phenol blue before being applied onto the gel. The electrophoresis was carried out in Tris (0.09M)-Borate (0.08M)-EDTA-Na_2 (0.003M) buffer, pH 8.4, at 160-175 volts for a total of 3,000 volt hours. A calibration standard (High Molecular Weight Electrophoresis Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden) containing thyroglobulin (Stokes' diameter 17.0 nm), ferritin (12.2 nm), lactate dehydrogenase (8.16 nm) and bovine serum albumin (7.1 nm) was also subjected to electrophoresis for calculation of particle size.

At the completion of the electrophoresis, the gels were fixed with 10 % (w/v) sulphosalicylic acid for 60 minutes. The gels were then stained for 2 to 4 hours in 0.04% (w/v) Coomassie G-250 in 3.5% (v/v) perchloric acid and then de-stained in 5 % (v/v) acetic acid for 16-24 hours. Scanning of gels were performed with an Ultrascan XL laser densitometer (LKB, Bromma, Sweden) attached to a computer installed with the Gelscan XL Software Package (Pharmacia LKB Biotechnology). The HDL populations were resolved into peaks and the relative migration of each peak was determined. Assuming an inverse logarithmic relationship between particle size and migration distance on the gel, the particle size of each population was determined by comparing the relative migration of the peak with that of the known protein standards.

2.5.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on a Pharmacia PhastSystem (Pharmacia Biotechnology AB, Uppsala, Sweden) using 20% homogenous polyacrylamide gels (Pharmacia) and SDS buffer strips (Pharmacia). For molecular weight references, a calibration kit for low molecular weight proteins (Pharmacia Biotechnology AB, Uppsala, Sweden), including phosphorylase b (subunit MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), soybean trypsin inhibitor (MW 20,100) and α-lactalbumin (14,400), was used. The electrophoresis was for 95 volt hours. The gel was stained with 0.1% (w/v) PhastGel Blue R (350) (Pharmacia) containing 10 % (v/v) acetic

acid, destained with methanol: acetic acid: H_2O (30:10:60 v/v/v) and preserved with glycerol: acetic acid: H_2O (10:10:80 v/v/v).

2.6 Isolation and Culture of Human Umbilical Vein Endothelial Cells.

Human umbilical vein endothelial cells (HUVECs) were isolated as described by Wall et al (1978). Cells were cultured on gelatin coated culture flasks in medium M199 with Earles Salts (Trace Biosciences, Australia) supplemented with 20 % foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 20 mmol/L HEPES, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, non essential amino acids, penicillin, streptomycin, 20 mg/ml endothelial growth supplement (Collaborative Research, Australia) and 20 mg/ml heparin (Sigma). The HUVECs were passaged every two or three days. Cell viability was tested with the standard dye exclusion method and by assessing plating efficiency of the cultures. In addition the cells were continually observed with light microscopy and they maintained confluent cultures with a healthy "cobblestone" morphology. HDLs were not toxic to the cells as proven by the fact that in initial studies PECAM-1 was not inhibited in the pressence of HDLs (Cockerill et al, 1995) and cell viability (as discussed above) was not altered in the presense of HDLs.

2.7 Incubation conditions

Confluent preparations of passage 3, 4 or 5 HUVECs were incubated for 1 or 17 hours in the presence of the various concentrations of HDLs, rHDLs, lipid-free apolipoproteins or phospholipid vesicles. TNF-α (100 U/ml) (R & D Systems, USA)

was then added to the culture medium and 5 hours later the cell surface expression of VCAM-1 was determined.

2.8 Fluorescent Antibody Cell Sorting Analysis

The cell surface expression was quantified as described by Cockerill et al, 1995. In brief, HUVECs were first washed with FACS wash (RPMI 1640, containing 10 mmol/L HEPES, 0.02 % azide and 2.5 % foetal calf serum). They were then incubated with mouse monoclonal antibody to VCAM-1 (51-10C9) for 30 minutes at 4°C, then washed again with fluorescent antibody cell sorting (FACS) wash. Binding of the primary antibody was detected by incubating with FITC conjugated secondary antibody (Immunotech FITC conjugated F(ab)2 fragment goat (mouse IgG)) for 30 minutes at 4°C. After washing with PBS, the cells were harvested by trypsinization. FACS wash was added to neutralise the trypsin. The cells were centrifuged and the cell pellet resuspended in FACS fixative (PBS containing 2 % glucose, 0.02 % azide and 2.5 % formaldehyde). The expression of VCAM-1 was measured as fluorescence intensity using a Coulter Epics Profile II flow cytometer (Coulter, U.S.A.). Each sample counted 10,000 cells. Controls included absence of primary antibody and using an isotype-matched, nonrelevant antibody.

2.9 Determination of Serum Soluble Adhesion Molecule Levels.

Levels of sE-selectin, sICAM-1 and sVCAM-1 were determined by monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) using kits from R & D Systems (U.S.A.). Assays of all samples, controls and standards were performed in

duplicate. Concentrations of samples were determined by analysing standards with known concentrations of recombinant adhesion molecules coincident with samples and plotting of signal versus concentration.

2.10 Contribution By the Candidate to the Thesis

I was personally responsible for all the techniques involved in this thesis with the exception of harvesting and the initial culturing of the HUVECs and the experiments discussed in figure 4.4 which are acknowledged as the work of my colleagues in this section. Most methods used were established techniques with the exception of the SAA experiments in which there were modifications to the established techniques as discussed in section 5.2.

2.11 Other Methods

Methods specific to the work in individual studies are presented in the methods sections of their relevant chapters.

2.12 Chemicals and Reagents

Acetic acid glacial	BDH Chemicals 10001
Ammonium Hydrogen Carbonate	BDH Chemicals 10302
Ammonium Sulphate	BDH Chemicals 10033
Anti-human-apo A-I antiserum	Boehringer Mannheim 726478
Anti-human-apo A-II antiserum	Boehringer Mannheim 726486
Bovine serum albumin	Sigma chemicals 87H7603

Bromophenol Blue	BDH Chemicals 10058
Buffer concentrate for apolipoprotein determination	Behring OUEC 40/41
Calibration serum for apolipoprotein	Boehringer Mannheim 837237
Calibrator for automated systems	Boehringer Mannheim 759350
Chloroform	BDH Chemicals 10077
Cholesterol Reagent (CHOD-PAP)	Boehringer Mannheim 1442341
Cholesterol Standards	Boehringer Mannheim 125512
Coomassie brilliant blue G-250	Bio-Rad 161-0406
Coomassie brilliant blue R-250	Bio-Rad 161-0400
Coomassie brilliant blue R-350 (PhastGel Blue R-350)	Pharmacia 17-0518-01
Diethyl ether	BDH Chemicals 10094
Dimethylformamide	BDH Chemicals 10322
Di-myristoyl-phosphatidylcholine	Sigma Chemicals
di-Sodium hydrogen orthophosphate anhydrous	BDH Chemicals 30158
Earles Salts	Trace Biosciences
Endothelial growth supplement	Collaborative Research
EDTA-Na_2 (Ethylenediaminetetra-acetic acid disodium salt)	BDH Chemicals 10093
Ethanol	BDH Chemicals 10107
Foetal calf serum	Commonwealth Serum Laboratories
Folin & Ciocalteu's phenol reagent	Sigma Chemicals F-9252
Formaldehyde sodium	BDH Chemicals 10113
Free Cholesterol Reagent	Boehringer Mannheim 310328

Gelatin	Sigma G9391
Glutamine (L)	Trace Biosciences 21-125-0100V
Glutaraldehyde	Ajax Chemicals 698
Glycerol	BDH Chemicals 10118
Guanidine (aminomethanamide) hydrochloride	Sigma Chemicals G-3272\
Heparin	Sigma Chemicals H-9399
HEPES	Gibco BRL 11344-041
Methanol	BDH Chemicals 10158
Non-essential amino acids	Trace Biosciences
Penicillin-Streptomycin	Sigma Chemicals P 4458
Perchloric acid	BDH Chemicals 101764
Phospholipid reagent	Boehringer Mannheim 691844
Polyethylene glyco 6000	Boehringer Mannheim 240907
Potassium Bromide	BDH Chemicals 10195
Q Sepharose Fast Flow	Pharmacia Biotech 17-0510-01
Sodium Acetate	BDH Chemicals 10236
Sodium Azide	Sigma Chemicals S-2002
Sodium Bromide	BDH Chemicals 30116
Sodium Carbonate	BDH Chemicals 10240
Sodium chloride	BDH Chemicals10241
Sodium dihydrogen orthophosphate monohydrate	BDH Chemicals 10245
Sodium dodecyl sulphate (SDS)	BDH Chemicals 44244
Sodium hydrogen carbonate	BDH Chemicals 10247

Sodium pyruvate	Trace Biosciences 21-154-0100V
Sucrose	BDH Chemicals10274
5-Sulphosalicylic acid	BDH Chemicals 103464
Triglyceride Reagent	Boehringer Mannheim 877557
Tris (Tris[hydroxymethyl]amion-methane)	Sigma Chemicals T-1378
Trypsin	Gibco BRL 01500
Tween-20	BDH Chemicals 66368

CHAPTER 3

THE EFFECTS OF TOTAL HDL AND ITS SUBFRACTIONS ON THE CYTOKINE -INDUCED EXPRESSION OF VCAM-1 IN HUVECS

3.1 INTRODUCTION

Adhesion of monocytes to the vascular endothelium is one of the earliest events in atherosclerosis. In section 1.2.3, the role of adhesion molecules in the atherogenic process is described. The adhesion of monocytes to the endothelium is mediated by adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, all of which are rapidly synthesised by endothelial cells in response to stimulation by cytokines. VCAM-1, ICAM-1 and E-selectin have all been detected in the arterial endothelium over existing atheromatous plaques (Van der Wal et al, 1992) and the concentration of sVCAM-1 is elevated in the plasma of patients with atherosclerosis (Karlheinz et al, 1997).

It has been previously reported from this laboratory that HDLs inhibit the cytokine-induced expression of adhesion molecules in endothelial cells. These earlier studies assessed the effects of the total HDL fraction ($1.07 < d < 1.21$ g/ml) obtained from healthy volunteers and rHDLs on the expression of VCAM-1, ICAM-1, E-selectin and PECAM (platelet endothelial cell adhesion molecule). Both the native HDLs and rHDLs inhibited the cytokine-induced expression of VCAM-1, ICAM-1 and E-selectin in a concentration dependent manner. In contrast, neither type of HDLs had any effect on the constitutively expressed PECAM.

The experiments described in this chapter were designed to investigate the following specific questions: i) would the HDLs isolated from the same subject on separate occasions give reproducible levels of inhibition of cytokine-induced of VCAM-1

expression in HUVECs? ii) does freezing HDLs and then thawing them cause any change in their ability to inhibit VCAM-1 expression? iii) is there any difference in the ability of total HDL isolated from different subjects to inhibit the cytokine-induced expression of VCAM-1? iv) do the major HDL subfractions, HDL_2 and HDL_3, have the same ability to inhibit VCAM-1 expression? and v) are HDLs able to cause inhibition of VCAM-1 expression if they are removed from the cells prior to stimulation of the endothelial cells with cytokine?

3.2 METHODS

3.2.1 ISOLATION AND PREPARATION OF HDLS

The total HDL fraction, HDL_2 and HDL_3 were isolated from fasting, healthy volunteers as described in chapter 2. The HDLs used in the experiments were stored at 4°C and were not frozen, unless otherwise stated. HDL particle size distribution and concentrations of apolipoproteins and lipid constituents in the HDL were determined as described in chapter 2.

3.2.2 INCUBATION CONDITIONS

Confluent preparations of passage 3, 4 or 5 HUVECs were incubated for one hour in the presence of the various concentrations of total HDL, HDL_2 and HDL_3. TNF-α (100 U/ml) was then added to the culture medium and 5 hours later the cell surface expression of VCAM-1 was measured by FACS analysis as described in chapter 2.

3.2.3 Statistical Analysis

The data obtained in this chapter are expressed as mean ± sem. Students' t test for paired samples was used to determine whether differences between values were significant. Statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 Comparison of The Inhibition of VCAM-1 Expression by HDLs Isolated from the Same Subject on Different Occasions.

To test whether HDLs isolated from the same subject on different occasions would give similar levels of inhibition of VCAM-1 expression when added to the same batch of cultured HUVECs, the total HDL fraction ($1.07 < d < 1.21$ g/ml) was isolated from plasma obtained from the same subject on two separate occasions three days apart. Four separate subjects were studied. The HDLs from each subject inhibited the cytokine-induced expression of VCAM-1 in a concentration dependent manner when added to the cells at apoA-I concentrations of 2, 4 and 8 μmol/L (Figure 3.1). Preparations of HDLs isolated from the same subject on different occasions and added to the same batch of endothelial cells did not vary in their ability to inhibit VCAM-1 expression.

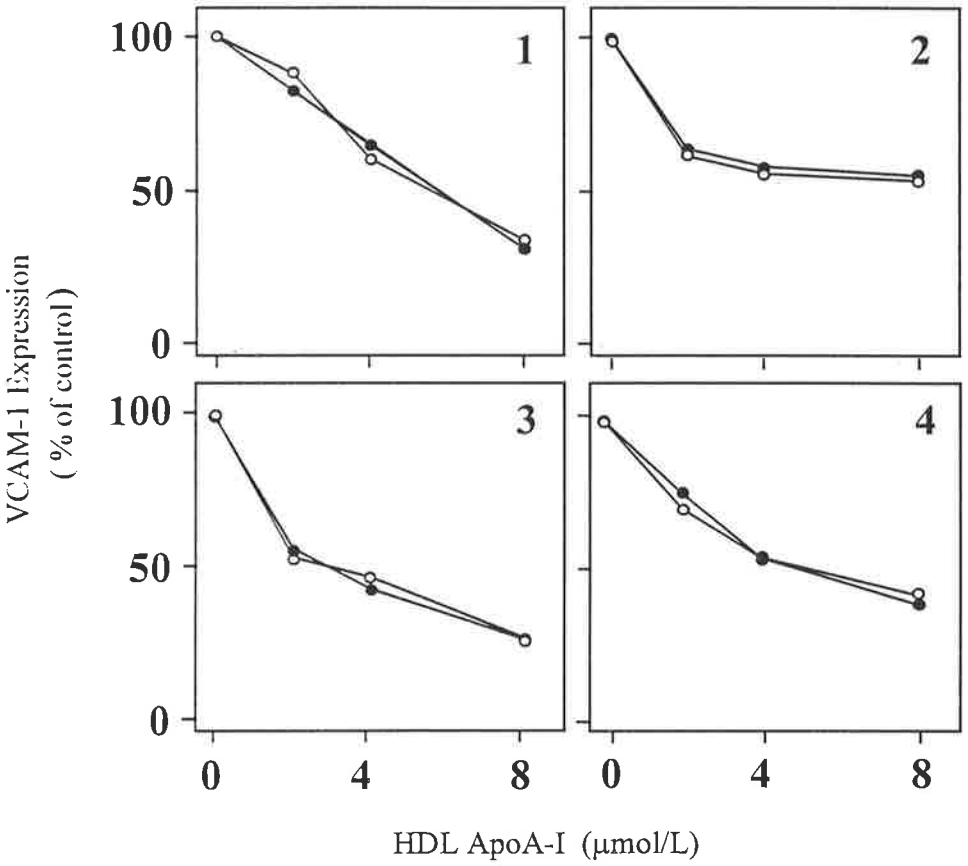

Figure 3.1 Inhibition of cytokine-induced endothelial cell VCAM-1 expression by HDLs isolated on different occasions from individual subjects.. HDLs from each of 4 subjects were isolated on two occasions three days apart. HUVECs were pre-incubated for one hour with HDLs before being activated with TNF-α (100U/ml) and incubated for a further 5 hours. Expression of VCAM-1 was quantified by flow cytometry. The values represent the mean fluorescence intensity detected with antibodies directed against VCAM-1. Values are expressed as a percentage of that in a sample without HDLs. The inhibitory activities of the two preparations from each subject (1-4) are shown. The initial HDL sample (•) and later HDL sample (○) are shown for each subject.

3.3.2 Effect of Freezing on the Ability of HDLs to Inhibit the Cytokine-Induced Expression of VCAM-1

The effects of freezing and thawing on the ability of HDLs to inhibit adhesion molecule expression were determined. Total HDL ($1.07<d<1.21$ g/ml) and HDL_3 ($1.13<d<1.21$ g/ml) were isolated from fasting, healthy volunteers. Each preparation of HDLs was then divided, half was kept at 4°C and the other half frozen to -70°C over-night before thawing back to 4°C the next day. The total HDLs and the HDL_3 from each preparation were then added to HUVECs for one hour before the HUVECs were stimulated with cytokine and the expression of VCAM-1 determined by FACS analysis 5 hours later (Figure 3.2). Both the fresh and frozen (then thawed) HDLs from each subject inhibited the cytokine-induced expression of VCAM-1 in a concentration dependent manner when added to the cells at apoA-I concentrations of 2, 4 and 8 μmol/L; there was no significant effect of freezing and thawing on their ability to inhibit VCAM-1 expression.

3.3.3 Inhibition of VCAM-1 Expression by the Total HDL Fractions Isolated from Different Subjects

The total HDL fractions were isolated from each of six subjects and compared in terms of their abilities to inhibit cytokine-induced VCAM-1 expression in HUVECs (Figure 3.3). When added at apoA-I concentrations of 4, 8, 12 and 16 μmol/L, the HDLs isolated from subjects 1, 2 and 3 achieved much greater inhibition of VCAM-1 expression than was the case with the HDLs isolated from subjects 4, 5 and 6. The HDLs from subject 4 were intermediate in their inhibitory activities. At an apoA-I

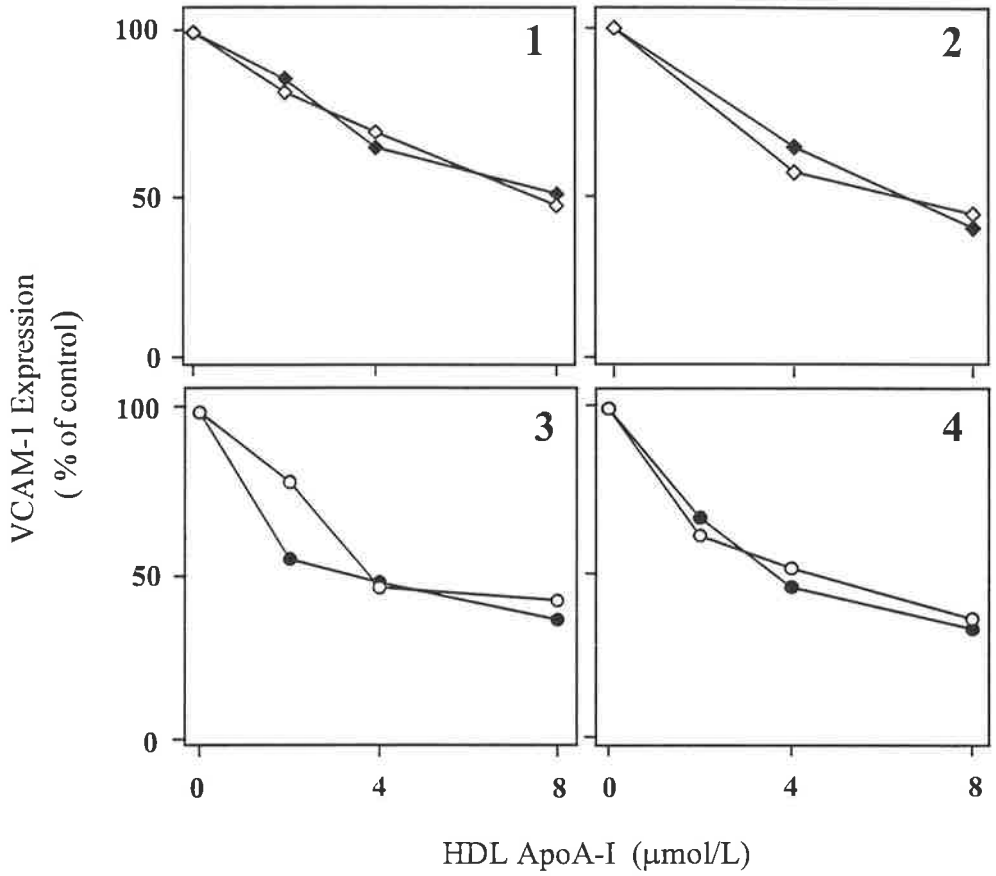

Figure 3.2 Inhibition of cytokine-induced endothelial cell VCAM-1 expression by HDLs that were either kept at 4°C or frozen to -70°C and then thawed. Experiments were conducted as described in the legend to figure 3.1. Total HDL was isolated from two subjects (1 and 2) and was added to the cells as fresh (◆) or frozen and thawed (◇). The HDL_3 subfraction was isolated from two other subjects (3 and 4) and was added to the cells as fresh (●) or frozen and thawed (○). Values are expressed as a percentage of that in a sample without HDLs.

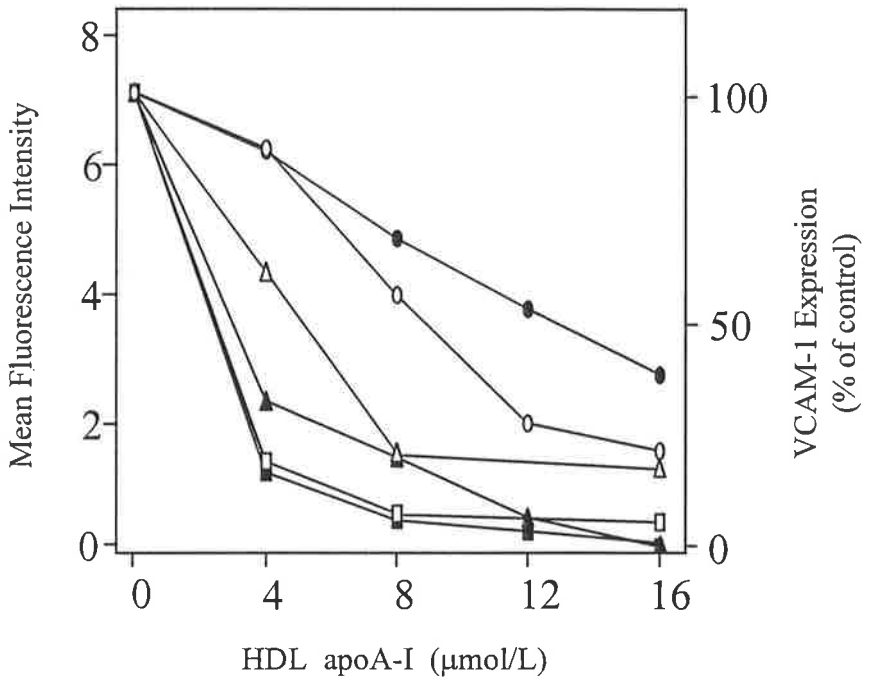

Figure 3.3 Inhibition of cytokine-induced endothelial cell VCAM-1 expression by the total HDL fraction from different subjects. Experiments were conducted as described in the legend to figure 3.1. Values are expressed as a percentage of that in a sample without HDLs. The donors of HDLs are individually identified: subject 1 (■), subject 2 (□) and subject 3 (▲), subject 4 (Δ), subject 5 (●) and subject 6 (○).

concentration of 4 μmol/L the HDLs from subjects 1, 2 and 3 inhibited VCAM-1 expression by more than 60%, while the HDLs from subjects 4, 5 and 6 inhibited by 38%, 16% and 14%, respectively. These differences were apparent up to apoA-I concentrations of 16 μmol/L. Comparable inter-subject variations were seen in other experiments with HDLs isolated from other subjects in studies using other preparations of HUVECs. In contrast, preparations of HDLs isolated on different occasions from the same subject did not vary in their ability to inhibit VCAM-1 expression as shown earlier (Figure 3.1).

3.3.4 Comparison of the Inhibition of VCAM-1 Expression by Total HDL and the HDL_3 Subfraction.

In order to determine whether there was a relationship between the ability of the HDLs to inhibit VCAM-1 expression and HDL particle size, the total HDL fraction from each subject was subjected to non-denaturing polyacrylamide gradient gel electrophoresis (Figure 3.4). The HDLs which had the greatest inhibitory activity (subjects 1, 2 and 3) consisted mainly of HDL_3 with very little HDL_2. In contrast, the HDLs which contained more HDL_2 (subjects 4, 5 and 6) were less effective at inhibiting VCAM-1 expression. To determine whether HDL_3 might therefore be responsible for most of the VCAM-1 inhibition in the total HDL fraction, the inhibitory activities of the total HDL fraction and the HDL_3 subfraction were compared.

Total HDL ($1.07 < d < 1.21$ g/ml) and HDL_3 ($1.13 < d < 1.21$ g/ml) were isolated from the same subject. Both the total HDL fraction and HDL_3 subfraction inhibited

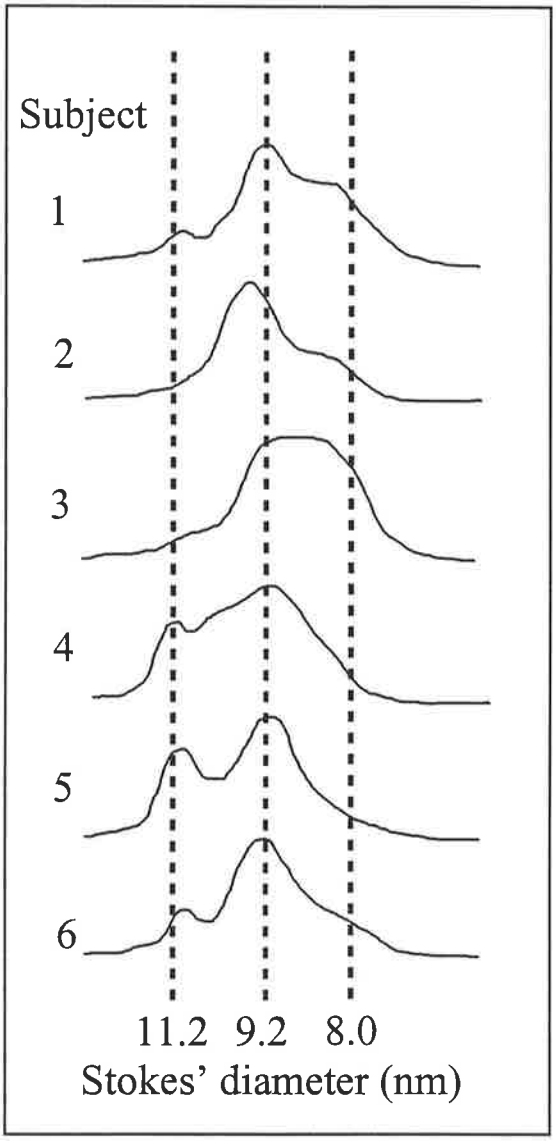

Figure 3.4. Particle size distribution of the HDL samples used in the experiment presented in figure 3.3. The HDL samples were subjected to non-denaturing polyacrylamide gradient gel electrophoresis on 3- 40 % gels. After fixing and staining, the gels were scanned with a laser densitometer. Populations of HDL2b (diameter 11.2-11.4 nm), HDL3a (9.2-9.4 nm) and HDL3b (8.0 nm) were identified.

the cytokine-induced expression of VCAM-1 in endothelial cells in a concentration dependent manner (Figure 3.5). At equivalent apoA-I concentrations, however, the HDL_3 subfraction was superior to the total HDL fraction in the inhibition of VCAM-1 expression. This result was confirmed in other similar experiments comparing total HDL and HDL_3. Studies were then undertaken to directly compare the effect on VCAM-1 expression of the two major HDL subfractions; HDL_2 and HDL_3.

3.3.5. Comparison of the Inhibition of VCAM-1 Expression by HDL_2 and HDL_3.

HDL_2 ($1.07<d<1.12$ g/ml) and HDL_3 ($1.13<d<1.21$ g/ml) were isolated from each of five subjects. Completeness of the separation of each subfraction from the other was confirmed by non denaturing polyacrylamide gradient gel electrophoresis (Figure 3.6). The composition of the HDL_2 and HDL_3 is shown in Table 3.1. As has been reported elsewhere (Patsch and Gotto, 1987), HDL_3 are enriched with apoA-II but depleted of cholesteryl esters, unesterified cholesterol and triglyceride relative to HDL_2.

The HDL_2 and HDL_3 were added to HUVECs at apoA-I concentrations of 2, 4 and 8 μmol/L. Both HDL subfractions inhibited the TNF-α-induced VCAM-1 expression in a concentration dependent fashion (Figure 3.7). However, at all concentrations of apoA-I, the inhibition mediated by HDL_3 was greater than that of HDL_2 (Figure 3.7A). The differences at 4 and 8 μmol/L apoA-I were statistically significant. At 4 μmol/L apoA-I, the inhibition of VCAM-1 expression by HDL_2 and HDL_3 was 39.8 ± 5.7% and 58.6 ± 5.4%, respectively ($p<0.05$). This difference was even greater at 8

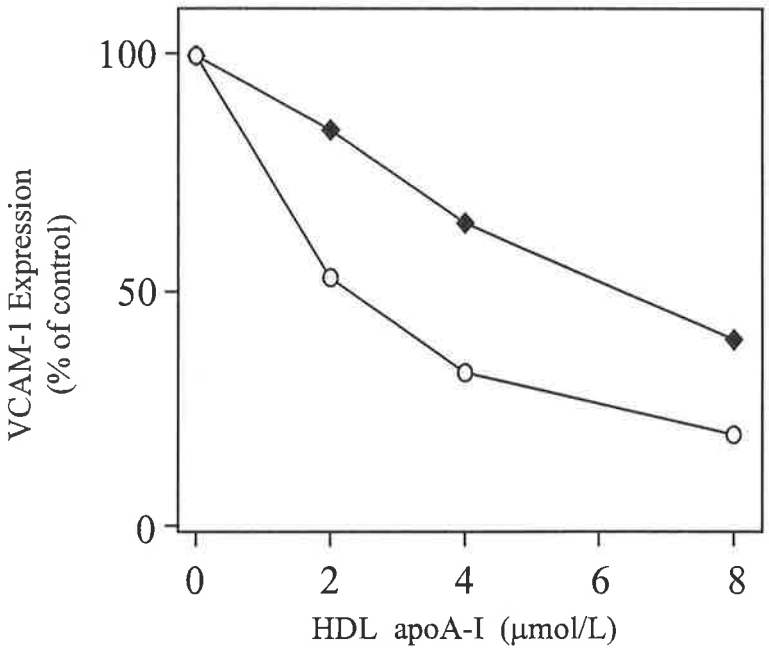

Figure 3.5 Inhibition of cytokine-induced endothelial cell VCAM-1 expression by the total HDL fraction and the HDL_3 subfraction from the same subject. Experiments were conducted as described in the legend to figure 3.1. Values are expressed as a percentage of that in a sample without HDLs. Total HDL (◆) and HDL_3 (o) were added to the HUVECs according to apo A-I concentration. Results are from a single experiment but are representative of four experiments.

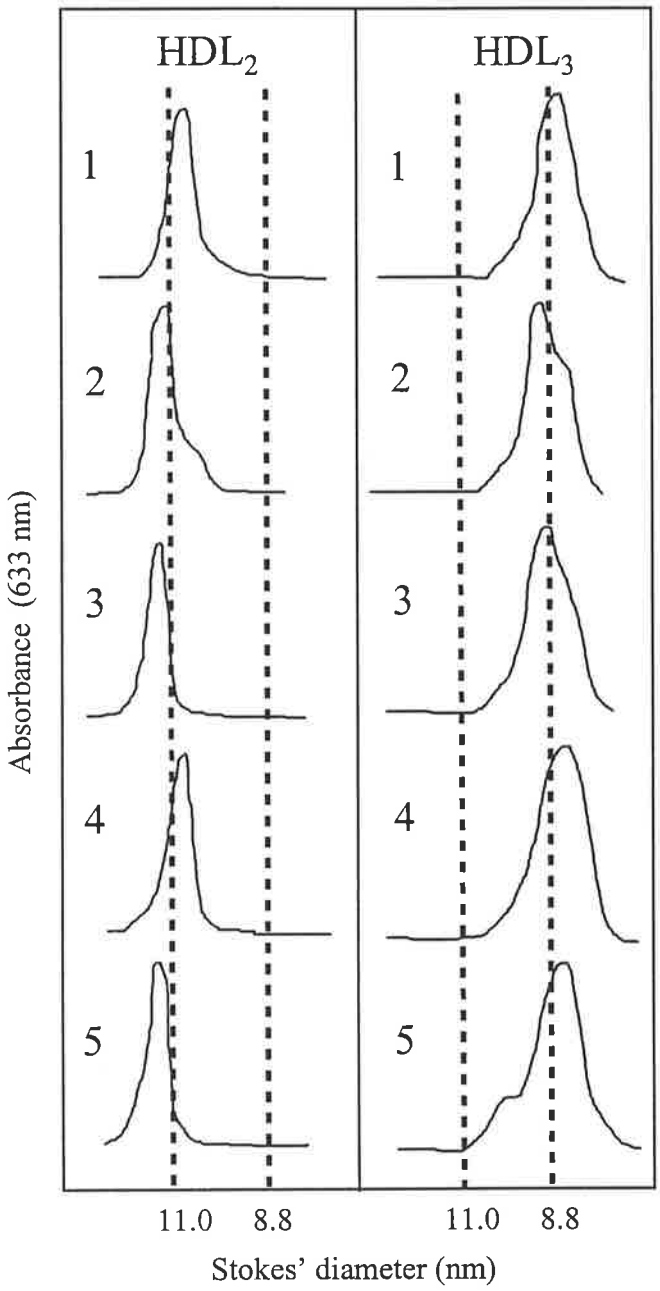

Figure 3.6 Size distributions of the HDL_2 and HDL_3. HDL_2 ($1.07<d<1.12$ g/ml) and HDL_3 ($1.13<d<1.21$ g/ml) were isolated from each of five subjects (1-5) and subjected to non-denaturing polyacrylamide gradient gel electrophoresis on 3- 40 % gels. After fixing and staining, the gels were scanned with a laser densitometer. Populations of HDL_2 (diameter 10.8-11.6 nm) and HDL_3 (diameter 8.0-9.0 nm) were identified.

Table 3.1 Composition (mass %) of HDL_2 and HDL_3.

Constituent	HDL_2	HDL_3
ApoA-1	29.4 ± 2.9	34.7 ± 0.8
ApoA-II	3.5 ± 0.4	14.6 ± 2.0
Cholesteryl Esters	29.4 ± 3.3	16.9 ± 5.0
Unesterified Cholesterol	8.6 ± 1.6	5.2 ± 2.1
Triglyceride	4.2 ± 1.5	2.4 ± 0.4
Phospholipid	24.9 ± 8.1	26.2 ± 1.9

HDL_2 ($1.07<d<1.12$ g/ml) and HDL_3 ($1.13<d<1.21$ g/ml) were isolated from each of 5 subjects by sequential ultracentrifugation. Composition was determined as described in the methods. The values are the mean ± sem, n = 5.

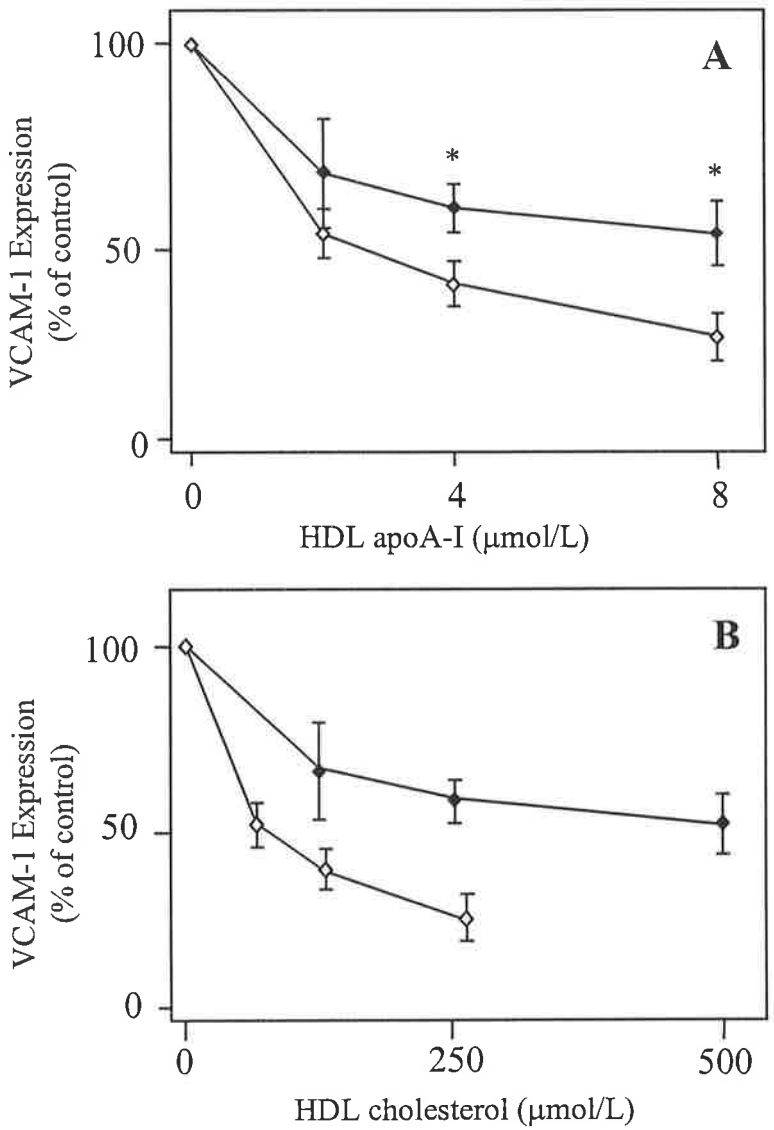

Figure 3.7 Inhibition of endothelial cell VCAM-1 expression by HDL_2 and HDL_3. HUVECs were pre-incubated for one hour with the preparations of either the HDL_2 or the HDL_3 shown in figure 3.6 before being activated by TNF-α and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The HDL_2 (♦) and HDL_3 (◇) were added to the HUVECs according to apoA-I concentration (panel A) and total cholesterol concentration (panel B). The results are the mean and standard error of the mean of the results with HDL subfractions isolated from each of 5 subjects. Differences between the means were evaluated using paired t tests. * $p < 0.05$.

μmol/L apoA-I, with HDL_2 and HDL_3 inhibiting VCAM-1 expression by 46.2 ± 8.0% and 71.4 ± 5.8% respectively ($p< 0.05$). When the inhibitory activities of HDL_2 and HDL_3 were compared in terms of their total cholesterol concentrations, the HDL_3 subfraction was still superior (Figure 3.7B).

3.3.6 Effect of removing HDL_2 and HDL_3 prior to adding TNF-α.

To determine whether the difference in the inhibition of VCAM-1 expression between HDL_2 and HDL_3 was dependent on the HDL subfractions being physically present at the time of adding the TNF-α, studies were conducted with cells that had been preincubated for two hours with each of the HDL subfractions. The HDL subfractions were then removed prior to adding TNF-α. Before adding the TNF-α, the cells were washed twice with fresh medium to ensure maximal removal of the HDLs. Using this technique more than 95% of the added HDL (expressed as apoA-I) was accounted for in the removed media.

Despite removing the HDLs prior to adding TNF-α, expression of VCAM-1 was still inhibited in a concentration dependent manner (Figure 3.8). Furthermore, the superior inhibitory activity of HDL_3 persisted. At concentrations of apoA-I in the preincubation of 2, 4 and 8 μmol/L, the subsequent inhibition of TNF-α-induced VCAM-1 expression was respectively 14%, 32% and 46% in the case of HDL_2 and 31%, 50% and 60% in the case of HDL_3 ($p < 0.05$).

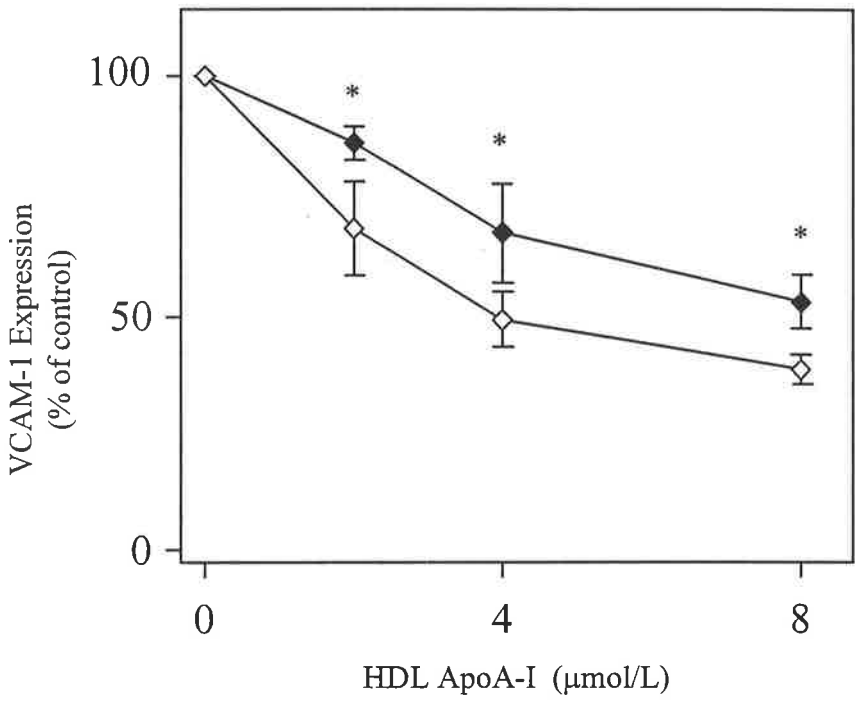

Figure 3.8 Effect of HDL_2 and HDL_3 when removed from HUVECs prior to addition of cytokine. HUVECs were preincubated for two hours with either HDL_2 or HDL_3. The media containing the HDLs was then removed and the cells were washed twice with fresh media. The cells were then stimulated with the cytokine TNF-α and the incubation continued for an additional 5 hours. Cells were then assayed for cell-surface VCAM-1 expression by flow cytometry. The HDL_2 (◆) and HDL_3 (◇) were added to the cells according to apo A-I concentration. The results are the mean and standard error of the mean from 3 experiments, where each was performed in duplicate. Differences between the means were evaluated using paired t tests. * $p < 0.05$.

3.4 DISCUSSION

These studies show that HDLs isolated from different subjects vary markedly in their ability to inhibit the cytokine-induced expression of VCAM-1 in endothelial cells. They also show that the inhibitory activity of HDL_3 is substantially greater than that of HDL_2.

In order to determine whether the apparent differences in HDLs isolated from different subjects could have been a reflection of a poorly reproducible technique it was shown that HDLs obtained from the same subject at different times produced equivalent degrees of inhibition of VCAM-1 expression. This indicated that it was differences between the HDL preparations that was causing the variation in inhibition amongst the subjects and not just assay variation. We also showed that freezing and then thawing the HDLs made no difference to their inhibitory powers. This may prove useful in the future such as in studies to compare the inhibition of adhesion molecule expression of HDLs isolated from the same subject before and after HDL modifying therapy.

The apparent superiority of HDL_3 over HDL_2 in terms of inhibiting VCAM-1 expression may be a consequence of differences in either the particle size or the composition of the subfractions. It is well known that other functions of HDLs, such as their interaction with LCAT (Barter et al, 1985) and hepatic lipase (Shirai et al, 1981), are influenced by variations in both of these parameters. Other more subtle differences between the HDL subfractions, such as variations in the conformation of

their apolipoproteins, may also impact on function by altering the surface exposure of specific apolipoprotein epitopes. It is possible, however, that the superiority of HDL_3 as an inhibitor of VCAM-1 is unrelated to any of these differences in size, composition or structure. Rather, it may be a simple reflection of the fact that when the two subfractions are equated for concentrations of either apoA-I or cholesterol there are more HDL_3 particles than HDL_2 particles.

The mechanism by which HDLs inhibit endothelial cell adhesion molecule expression is uncertain. One possibility relates to the ability of HDLs to function as antioxidants. Endothelial cell VCAM-1 expression may be induced by redox-sensitive transcriptional events (Marui et al, 1993). This raises the possibility that the ability of HDLs to inhibit endothelial cell adhesion molecule expression may be secondary to their capacity to remove lipid oxidation products that are generated by TNF-α (Matsubara and Ziff, 1986). If this were so, it could be argued that the difference between the inhibitory activities of HDL_2 and HDL_3 may reflect their relative abilities to remove such lipid oxidation products. However, this mechanism is not consistent with the observation that the inhibitory activities of the HDL subfractions remain different even when the HDLs were present only during preincubations and were removed from the endothelial cells prior to the addition of TNF-α (Figure 3.8). These preincubation studies also excluded the possibility that the HDLs may have acted by interfering with the binding of TNF-α to its cell receptors. Rather, it appears that HDLs make endothelial cells more resistant to the cytokine-induced expression of VCAM-1.

The relationship between the inhibition of endothelial cell adhesion molecule expression and the anti-atherogenic properties of HDL subpopulations is not known. This is not a trivial issue, since human and animal studies have suggested that HDL_2 may differ from HDL_3 and A-I HDLs may differ from A-I/A-II HDLs in terms of their cardioprotective properties. As discussed in the introduction, several reports have concluded that HDL_2 are superior to HDL_3 (Lamarche et al, 1997; Robinson et al, 1987; and Salonen et al, 1991), although there have also been conclusions that HDL_3 may be the superior anti-atherogenic subfraction (Kempen et al, 1987; Sweetnam et al, 1994). The observation that high levels of HDL cholesterol are generally indicative of high levels of HDL_2, highlights the importance of HDL_2 as a cardioprotective fraction. However, the possibility that HDL_3 is also cardioprotective is suggested by the fact that an inverse relationship between the concentration of HDL cholesterol and the risk of developing CHD persists even when the total HDL cholesterol concentration is below 1.0 mmol/L and there is no measurable HDL_2.

In summary, the results in this chapter confirm the ability of HDLs to inhibit the cytokine-induced expression of endothelial cell VCAM-1 and demonstrate marked differences in the inhibitory activities of the two major HDL subfractions and in HDL preparations isolated from different human subjects.

CHAPTER 4

EFFECTS OF HDL APOLIPOPROTEINS AND LIPIDS ON THE ABILITY OF HDLS TO INHIBIT THE CYTOKINE -INDUCED EXPRESSION OF VCAM-1 IN HUVECS

4.1 INTRODUCTION

In the previous chapter, it was shown that HDL_3 were superior to HDL_2 in the ability to inhibit cytokine-induced VCAM-1 expression in HUVECs, when added to the cells at equivalent concentrations of apoA-I or total cholesterol. The apparent superiority of HDL_3 over HDL_2 may be a consequence of differences in either the particle size, particle numbers or the composition of the subfractions. Other more subtle differences between the HDL subfractions, such as variations in the conformation of their apolipoproteins, may also impact on function by altering the surface exposure of specific apolipoprotein epitopes.

The experiments in this chapter investigate this issue by i) assessing whether altering the apolipoprotein composition on HDL_3 by replacing the apoA-I with apoA-II affects their ability to inhibit VCAM-1 expression, ii) determining whether the differences in the inhibition of VCAM-1 expression by HDL_2 and HDL_3 may be due to differences in their protein or lipid components and iii) investigating the effects on VCAM-1 expression of lipid-free apoA-I, di-myristoyl-phosphatidyl choline (DMPC) and discoidal rHDLs containing apoA-I and DMPC.

4.2 METHODS

4.2.1 ISOLATION AND PREPARATION OF HDLS

HDLs were isolated and prepared as described in section 3.2.1.

4.2.2 Preparation of HDL_3 Containing ApoA-II Only (A-II HDL_3)

The apoA-I in HDL_3 was displaced with lipid free apoA-II as described previously (Rye and Barter, 1994). Briefly, HDL_3 were isolated by ultracentrifugation as described above. Lipid-free apoA-II was added to the HDL_3 at an apoA-II : apoA-I molar ratio of 2:1. As a control, another aliquot of HDL_3 was supplemented with PBS in place of apoA-II. Both samples were maintained at room temperature for 30 minutes and then reisolated as the supernatant following ultracentrifugation at d=1.21 g/ml. The samples were dialysed against 3 x 1 litre of endotoxin-free PBS before use.

4.2.3. Isolation of HDL_2 and HDL_3 Proteins and Lipids

HDL_2 and HDL_3 were isolated as above and delipidated as described (Osborne, 1986). The protein constituents were resuspended in PBS and stored at 4°C. The lipids were extracted from the HDL_2 and HDL_3 by the method of Folch et al (1956) and resuspended in PBS before being sonicated under N_2 at 51°C by the method of Sparks et al (1995). The resuspended apolipoproteins and the sonicated lipid preparations were stored at 4°C and were used within one week.

4.2.4. Preparation of Discoidal rHDLs

Discoidal rHDLs containing apoA-I and DMPC were prepared by the cholate dialysis method of Matz and Jonas (1982).

4.2.5. Incubation Conditions

Incubation conditions were identical to those described in section 3.2.2.

4.3 Results

4.3.1 Effect of apolipoprotein composition on the inhibition of VCAM-1 expression by HDL_3

To determine whether changing the apolipoprotein composition of HDL_3 impacts on the ability of these lipoproteins to inhibit VCAM-1 expression, preparations of HDL_3 were modified by replacing all of their apoA-I with apoA-II to form (A-II) HDL_3. This replacement of apoA-I with apoA-II increased the size of the HDL_3 particles slightly (from 8.9 to 9.2 nm, Figure 4.1) but did not change their lipid composition (Table 4.1). The A-II HDL_3 were then compared for their ability to inhibit VCAM-1 expression with the unmodified particles which comprised a mixture of (A-I) and (A-I/A-II) HDL_3 (Figure 4.2). The two groups of HDLs were added to HUVECs according to total cholesterol concentration and after stimulation with cytokine, the VCAM-1 expression was determined as previously described.

Both the unmodified HDL_3 and the (A-II) HDL_3 inhibited the cytokine-induced expression of VCAM-1 in endothelial cells in a concentration-dependent fashion (Figure 4.2). When equated for cholesterol concentration (and thus for HDL particle concentration) there was no difference in the magnitude of the inhibition achieved by the two preparations.

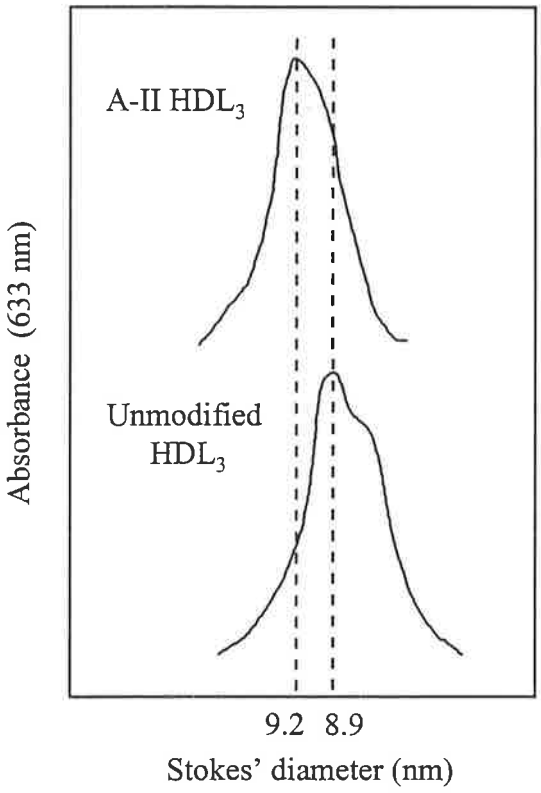

Figure 4.1 Effect of replacement of apoA-I with apoA-II on the size of human HDL_3. Unmodified HDL_3 and A-II HDL_3 (prepared as described in methods) were subjected to non-denaturing polyacrylamide gradient gel electrophoresis as outlined in the legend to figure 3.4. The A-II HDL_3 and unmodified HDL_3 had Stokes' diameters of 9.2 and 8.9 nm respectively. The profiles in figure 4.1 are from a single experiment but are representative of 3 experiments.

Constituent	Unmodified HDL_3	(A-II) HDL_3
ApoA-I	33.7	0.4
ApoA-II	13.7	50.8
Cholesteryl Esters	21.6	19.6
Unesterified Cholesterol	2.8	2.8
Triglyceride	2.0	1.9
Phospholipid	26.2	24.5

Table 4.1 Composition (mass %) of unmodified HDL_3 and AII HDL_3. Unmodified HDL_3 were isolated as the ultracentrifugal fraction of $1.13 < d < 1.21$ g/ml. A-II HDL_3 were prepared by displacing the apo A-I from HDL_3 by the addition of lipid free apo A-II as described in the methods. The values represent the means of two separate experiments.

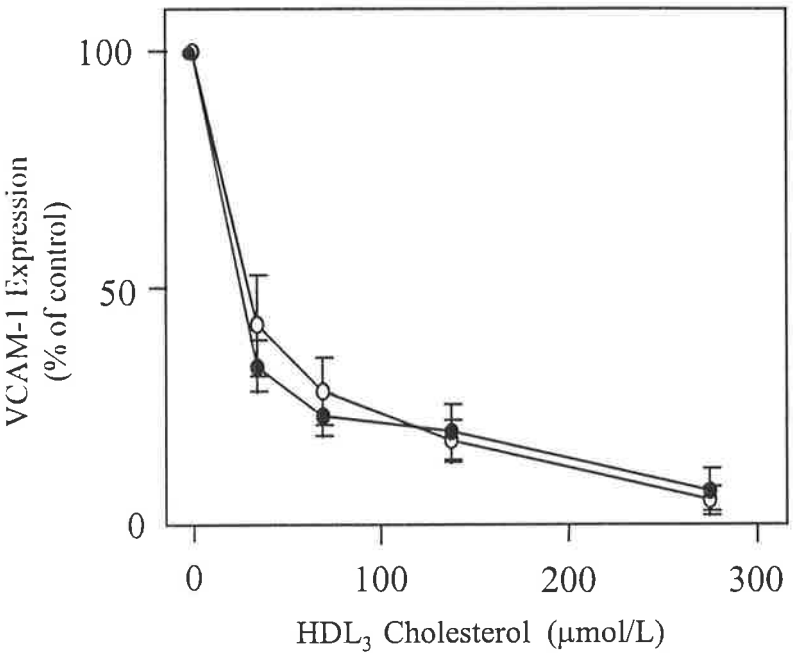

Figure 4.2. Comparison of the inhibitory activities of unmodified HDL_3 and A-II HDL_3. HUVECs were pre-incubated with unmodified HDL_3 or A-II HDL_3 (prepared as described in the Methods) for 1 h and then stimulated with TNF-α (100 U/ml). The incubation was then continued for an additional 5 hours before the cells were assayed for cell-surface VCAM-1 expression by flow cytometry. Values are expressed as a percentage of that in a sample without HDLs. Unmodified HDL_3 (●) and A-II HDL_3 (○) were added to the HUVECs according to total cholesterol concentration. The results are the mean and standard error of the mean from 3 experiments, each of which was performed in duplicate.

4.3.2. Effects of HDL_2 and HDL_3 Apolipoproteins and Lipids on VCAM-1 Expression

To investigate the possibility that the differences between HDL_2 and HDL_3 may have reflected differences in their protein or lipid constituents, their apolipoproteins and lipids were isolated and their effects on TNF-α-induced VCAM-1 expression determined. In marked contrast to the intact HDL subfractions, neither the apolipoproteins (Figure 4.3 A) nor sonicated preparations of the lipids (Figure 4.3 B) from either subfraction had any significant inhibitory activity.

Similarly, when HUVECs were incubated with purified apoA-I (up to 24 μmol/L) in the absence of lipid or with sonicated, small unilamellar vesicles prepared with DMPC (up to 2.4 μmol/L) in the absence of apolipoproteins, there was no inhibition of the TNF-α-induced expression of VCAM-1 (Figure 4.4) (Baker et al, In Press). When, however, apoA-I and DMPC were reconstituted into discoidal rHDLs, the resulting complexes promoted a concentration dependent inhibition of VCAM-1 expression comparable to that seen with native HDLs (Figure 4.4) (This final study with discoidal rHDL was conducted in collaboration with Dr. M. Clay in our laboratory).

4.4 Discussion

These studies show that major changes to the apolipoprotein composition of HDL_3 had no effect on its ability to inhibit VCAM-1 expression in endothelial cells. Unmodified HDL_3 contain both A-I HDLs and A-I/A-II HDLs. Studies of transgenic animals suggest that A-I HDLs may be more atherogenic than both A-I/A-II HDLs

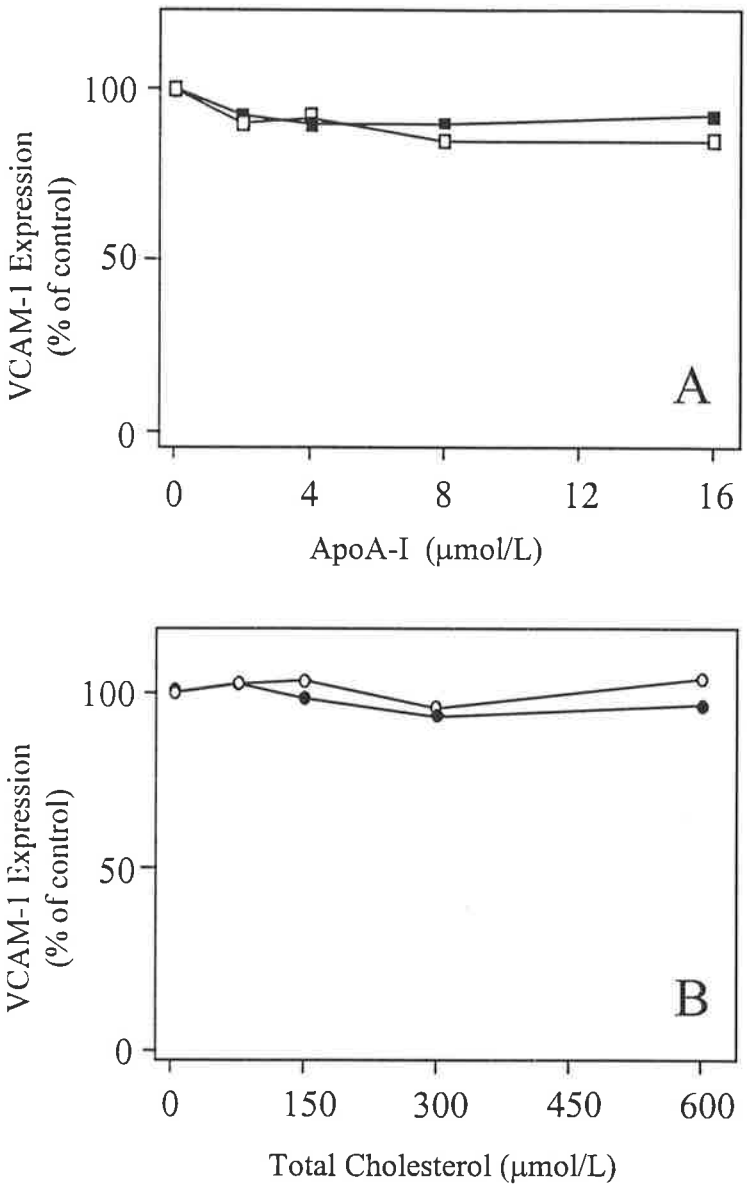

Figure 4.3. Inhibitory activities of lipid-free apolipoproteins and extracted lipids isolated from HDL_2 and HDL_3. HUVECs were pre-incubated for one hour with either the isolated apolipoproteins of HDL_2 or HDL_3 (panel A) or the extracted lipids of HDL_2 or HDL_3 (panel B) before being activated with TNF-α and assayed for cell-surface VCAM-1 expression. Values are expressed as a percentage of that in a sample without HDLs. The proteins from the HDL_2 (■) and the HDL_3 (□) were added to the HUVECs according to apoA-I concentration. The lipid components of the HDL_2 (●) and the HDL_3 (○) were added to the HUVECs according to total cholesterol concentration. The results are the means of two separate experiments each of which was performed in duplicate.

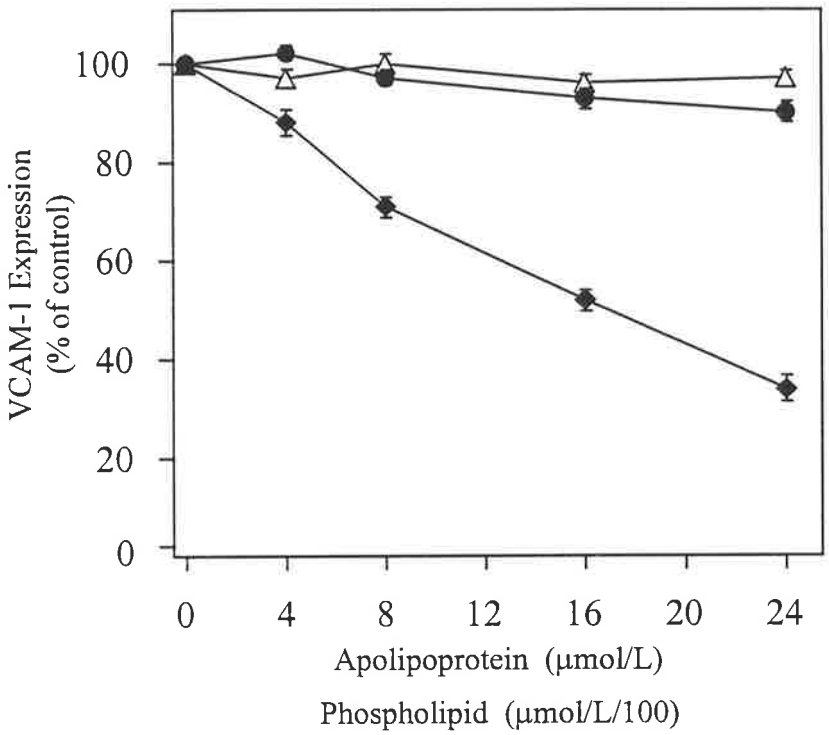

Figure 4.4. Effect of lipid-free apoA-I, DMPC vesicles and discoidal (A-I) rHDL on the TNF-α induced expression of VCAM-1 in HUVECs. Small unilamellar vesicles (SUVs) of DMPC and discoidal (A-I)rHDL containing DMPC and apo A-I in a molar ratio of 100:1 were prepared as described in the methods section. HUVECs were pre-incubated with lipid-free apoA-I, DMPC SUVs or discoidal (A-I)rHDL for 16 hours before stimulation of the cells with TNF-α. VCAM-1 expression was determined 5 hours after stimulation with TNF-α. The apolipoprotein molarity shown on the horizontal axis refers to both the lipid-free apoA-I and the rHDL, while the phospholipid molarity refers to the SUVs. Results are expressed as means +/- S.E.M. (n=6); lipid-free apoA-I (●), DMPC SUVs (Δ) and discoidal (A-I)rHDL (◆).

and A-II HDLs in their ability to protect against atherosclerosis (Hedrick et al, 1993; Schultz et al, 1993; Schultz and Rubin, 1994). However, as with HDL_2 and HDL_3, the situation in humans is uncertain, with circumstantial evidence both in favour (Amouyel et al, 1993) and against (O'Brien et al, 1996) the proposition that A-I HDLs account for most of the ability of HDLs to protect against CHD. It remains to be determined whether A-I HDLs are also more atherogenic than A-I/A-II HDLs in their ability to inhibit endothelial cell adhesion molecule expression and, if so, whether this translates into an enhanced ability to inhibit the development of atherosclerosis.

In these experiments, the A-II only HDLs produced in vitro, by the displacement of apoA-I with apoA-II, were as effective as the unmodified HDL_3 (containing A-I HDLs and A-I/A-II HDLs) at inhibiting VCAM-1 expression by endothelial cells. This raises the possibility that the protective powers of HDLs against atherosclerosis, at least in terms of adhesion molecule expression, may be independent of the apolipoprotein composition of the HDL particles. This observation is consistent with a recent report that reconstituted HDLs containing apoA-I are comparable to those containing apoA-II as inhibitors of endothelial cell VCAM-1 expression, although it does not address the possibility of differences between A-I HDLs and A-I/A-II HDLs, the two main apolipoprotein-specific HDL subpopulations in human plasma (Calabresi et al, 1997).

Insights into what components of HDLs are responsible for the inhibition of VCAM-1 expression were obtained from the studies with discoidal rHDL (Figure 4.4).

Discoidal complexes containing only apoA-I and DMPC were able to mimic native HDLs in their ability to inhibit endothelial cell VCAM-1 expression. The fact that neither lipid-free apoA-I nor DMPC vesicles had inhibitory activity indicated that complexes of both apolipoproteins and lipids are required for the effect. In retrospect, it was therefore not surprising that there was no inhibitory activity detected in incubations containing either the delipidated proteins or the extracted lipids isolated from HDL_2 and HDL_3 (Figure 4.3). This negative result frustrated attempts to determine whether the differing inhibitory activities of the two HDL subfractions resided in their lipid or apolipoprotein moieties.

The fact that lipid-free apolipoprotein A-I and DMPC vesicles do not have the ability to inhibit the cytokine-induce expression of VCAM-1, while discoidal complexes containing apoA-I and DMPC do, raises the possibility that inhibition of adhesion molecule expression in endothelial cells must require a particle that contains at a minimum apolipoproteins and phospholipids. HDLs may cause inhibition of cellular adhesion molecule expression by the apolipoprotein binding the HDL particle to a cell receptor which then allows the phospholipid contained in the HDL to come in contact with the cell. This may then activate intracellular messengers which would cause a reduction in the expression of VCAM-1, ICAM-1 and E-selectin. Discoidal complexes with either apoA-I or apoA-II have the ability to reduce adhesion molecule expression on endothelial cells (Calabresi et al, 1997; Baker et al, In Press). Hence the ability of the HDL particle to inhibit adhesion molecule expression may be independent of the type of apolipoprotein present on the particle.

In summary, the ability of HDL_3 to inhibit VCAM-1 expression in endothelial cells is unaffected by replacing all the apoA-I by apoA-II. It has also been shown that significant inhibition of VCAM-1 expression requires at least a combination of apolipoproteins and phospholipids. The apolipoproteins and lipid components of HDLs alone have no inhibitory action on VCAM-1 expression in endothelial cells.

CHAPTER 5

THE EFFECT OF SERUM AMYLOID-A ON THE ABILITY OF HDLS TO INHIBIT THE CYTOKINE -INDUCED EXPRESSION OF VCAM-1 IN HUVECS

5.1 INTRODUCTION

SAA is synthesized by the liver in substantial quantities during times of inflammation and over 90% of the SAA in plasma is associated with HDL (Brissette et al, 1989; Benditt et al, 1979; Skogen et al, 1979). SAA mainly associates with the HDL_3 subfraction (Benditt and Eriksen, 1977) and it alters both the physical and chemical properties of the HDLs as discussed in chapter 1.

The process of inflammation has an important role in the pathogenesis of atherosclerosis. The fact that plasma SAA levels are elevated in inflammatory states (Berliner et al, 1995), has lead to several theories as to how SAA could predispose to atherosclerosis. As discussed in the chapter 1, SAA may predispose to atherosclerosis by i) enhancing the binding of HDL particles to macrophages, ii) causing HDLs carrying SAA to bind preferentially to human monocytic THP-1 cells, iii) by adversely affecting reverse cholesterol transport by decreasing LCAT activity, iv) by decreasing the ability of HDLs to protect LDL against oxidation, v) by the chemoattractant properties of SAA and vi) by its ability to induce the migration, adhesion and tissue infiltration of monocytes, polymorphonuclear leukocytes and T lymphocytes and to increase the expression of cellular adhesion molecules. (Kisilevsky and Subrahmanyan, 1992; Banka et al, 1995; Steinmetz et al, 1989; Van Lenten et al, 1995; Xu et al, 1995; Badolato et al, 1994).

Human HDL, both its major subfractions, HDL_2 and HDL_3, and reconstituted HDLs have all been shown to inhibit the cytokine induced expression of cellular adhesion molecules in vitro (Cockerill et al, 1994; Ashby et al, 1998) . Given that SAA has

potential proatherogenic actions, the question arises: do HDLs that contain SAA (SAA-HDL) retain their ability to inhibit adhesion molecule expression in endothelial cells?

The experiments in this chapter investigate the ability of SAA-HDL_3 to inhibit the expression of VCAM-1 in HUVECs. The first set of experiments compare the effect on endothelial VCAM-1 expression by HDL_3 isolated from subjects before and after coronary artery bypass graft surgery. HDL_3 obtained from the subjects before surgery (pre-op HDL_3) is compared with HDL_3 obtained from the same subjects on the third day after surgery. This day 3 post-op HDL_3 contains SAA as one of its apolipoproteins (SAA-HDL_3). It is shown that the SAA-HDL_3 inhibits VCAM-1 expression to the same degree as the unmodified, pre-op HDL_3. In the next group of experiments lipid-free SAA is isolated and SAA-HDL_3 is produced in vitro by replacing the apo A-I on unmodified HDL_3 with SAA. This SAA-HDL_3 which has virtually no apo A-I also retains its ability to inhibit VCAM-1 expression in HUVECs when it is compared with unmodified HDL_3. Finally it is demonstrated that lipid-free SAA has no effect on VCAM-1 expression in HUVECs.

5.2 Methods

5.2.1. Isolation and Characterization of HDLs

Blood was collected from subjects after a 14 hour fast on the day prior to coronary artery bypass graft (CABG) surgery and again from the same subjects on the third post-operative day. Pre-op HDL_3 and post op SAA-HDL_3 were isolated as described

in chapter 2. HDL particle size distribution and concentrations of apolipoproteins and lipid constituents in the HDL were determined as described in chapter 2.

SAA was estimated by two methods: i) by SDS polyacrylamide gel electrophoresis and ii) by the measurement of total protein, apo A-I and apo A-II by Lowry and immunoturbidometric assays. Assuming dye uptake on SDS polyacrylamide gels to be proportional to apolipoprotein mass, the percentages of SAA, apo A-I and apo A-II were calculated from the integrated curves of the stained gels. In the second method of estimating the percentage of SAA that made up the HDLs apolipoproteins, the total protein, apo A-I and apo A-II levels of the HDLs were determined by Lowry and immunoturbidometric assays. By subtracting the apo A-I and A-II levels from the total protein, an estimate of the SAA concentration and hence mass ratio of SAA to the apo A-I and A-II could be made. Both methods were used on all the occasions that the percentage of total protein made up by SAA were estimated and there was never greater than 8 % difference in the calculations.

5.2.2. Isolation of Lipid-Free SAA

Plasma with high levels of SAA was obtained from CABG patients on the third post-operative day and was pooled. SAA-HDL_3 was isolated from the plasma by sequential ultracentrifugation as described previously. The SAA-HDL_3 was delipidated as described in section 2.2 and the SAA was separated from the other apolipoproteins by FPLC on a Q-sepharose column.

5.2.3. Preparation of SAA-HDL_3 by the Displacement of Apo A-I with Lipid-Free SAA

HDL_3 from healthy subjects was isolated by ultracentrifugation. Lipid-free SAA was added to the HDL_3 at a mass ratio of apo SAA:HDL_3 protein of 2:1. As a control another aliquot of HDL_3 was supplemented with PBS in place of SAA. Both samples were maintained at room temperature for 30 minutes and then reisolated as the supernatant following ultracentrifugation at 1.25 g/ml. The samples were dialysed against 3 x 1 litre of endotoxin- free PBS before use.

5.2.4. Incubation Conditions

Confluent preparations of passage 3, 4 or 5 HUVECs were incubated for 1 or 17 hours in the presence of the various preparations of HDL_3 or lipid-free SAA. TNF-α (100 U/ml) was then added to the culture medium and 5 hours later the cell surface expression of VCAM-1 was measured by FACS analysis as described in chapter 2.

5.2.5. Statistical Analysis

The data obtained in this chapter are expressed as mean ± sem. Students t test for paired samples was used to determine whether differences between values were significant. Statistical significance was set at $p < 0.05$.

5.2.6 Ethical Approval

The study protocol was approved by the Royal Adelaide Hospital human ethics committee.

5.3 Results

5.3.1 Comparison of the Inhibition of VCAM-1 Expression by Pre-Operative HDL_3 and Post-Operative HDL_3 containing SAA.

To determine whether acute phase HDL_3 that contains SAA is as effective as unmodified HDL_3 in the inhibition of cytokine induced VCAM-1 expression in HUVECs, pre-op HDL_3 containing no SAA and post-op, acute phase HDL_3 containing SAA were isolated from the same subjects before and after CABG surgery. Nineteen subjects were studied. As a control, HDL_3 from 6 healthy volunteers who did not undergo surgery were isolated. From each volunteer, two samples of plasma were taken 4 days apart and the HDL_3 were isolated as described above.

The composition of the pre-op HDL_3 and the post-op SAA-HDL_3 is shown in Table 5.1. The post-op SAA-HDL_3 had a similar percentage by mass of total protein as the pre-op HDL_3 but had significantly higher percentages by mass of unesterified cholesterol, triglycerides and phospholipids and a lower percentage by mass of cholesteryl esters. As shown in Table 5.2, there was no difference in the composition of the HDL_3 obtained from the controls 4 days apart.

The protein composition of HDLs was estimated by assessing the percentages of SAA, apo A-I and apo A-II from the integrated curves obtained after staining and scanning SDS polyacrylamide gels (Table 5.3). The means for the apo A-I, apo A-II

Table 5.1. Composition (mass %) of pre-op HDL_3 and post-op SAA HDL_3.

Constituent	Pre-Op HDL_3	Post-Op SAA HDL_3
Total Protein	52.9 ± 0.5	52.7 ± 0.34
Cholesterol Esters	16.6 ± 0.64	11.9* ± 0.95
Unesterified Cholesterol	2.6 ± 0.21	3.1* ± 0.15
Triglyceride	3.9 ± 0.31	6.1* ± 0.62
Phospholipid	24 ± 0.52	26.3* ± 0.36

The values represent the means ± SEM. n = 19. Students two-tailed t-test *signifies $p < 0.05$.

Table 5.2. Composition (mass %) of initial-HDL_3 and final-HDL_3 from normal controls.

Constituent	Initial HDL_3	Final HDL_3
Total Protein	52.2 ± 0.38	56.4 ± 2.74
Cholesterol Esters	18.6 ± 0.76	17.3 ± 1.23
Unesterified Cholesterol	2.4 ± 0.47	2.2 ± 0.49
Triglyceride	3.4 ± 0.63	2.9 ± 0.47
Phospholipid	23.5 ±0.31	21.3 ± 1.25

The values represent the means ± SEM . n = 6. Students two-tailed t-test *signifies $p < 0.05$.

Table 5.3 Percent composition of major proteins

Subject		% ApoA-I	% ApoA-II	% SAA
CABG Subjects (n=19)	Pre-Op HDL_3	74.8 ± 1.3	25.2 ± 1.3	0
	Post-Op SAA-HDL_3	*44.9 ± 1.7	*12.3 ±1.0	*42.2 ± 1.4
Normal Controls (n=6)	Initial HDL_3	71.5 ± 3.3	28.5 ± 3.3	0
	Final HDL_3	74.5 ± 2.9	25.5 ± 2.8	0

Derived from the peaks of the integrated curve obtained by subjecting the apolipoproteins to SDS polyacrylamide gel electrophoresis as described in section 5.2.1. The values represent the means ± SEM. n=19 for subjects and n=6 for controls. Students two-tailed t-test *signifies $p<0.05$.

Table 5.4 Diameter of HDL_3

Constituent	Diameter Pre HDL_3 (nm)	Diameter Post HDL_3 (nm)
CABG Subjects (n=19)	8.5 ± 0.06	*9.7 ± 0.13
Controls (n=6)	8.7 ± 0.07	8.6 ± 0.05

Diameters determined by non denaturing polyacrylamide gradient gel electrophoresis as discussed in section 5.2.1. The values represent the means ± SEM. n=19 for subjects and n=6 for controls. Students two-tailed t-test *signifies $p<0.05$.

and apo-SAA for the pre-op HDL_3 were 74.8, 25.2 and 0.0 % respectively and for the post-op SAA-HDL_3 were 44.9, 12.3, and 42.2 % respectively. In the controls, there were no significant changes in the ratios of apo A-I and A-II in the initial and final batches of HDL. There was no apo SAA detected in any of the batches of HDLs from the controls.

The pre-op HDL_3 and the post-op SAA-HDL_3 and the two samples of HDL_3 from the controls were subjected to non denaturing polyacrylamide gradient gel electrophoresis (Table 5.4). In the surgical subjects there was a significant increase in the diameter of the HDL_3 from a mean of 8.5 nm for the pre-op HDL_3 to 9.7 nm for the post-op SAA-HDL_3. There was no significant change in the diameter of the HDL_3 from the controls.

Pre-op HDL_3 and the post-op SAA-HDL_3 were added to HUVECs at total cholesterol concentrations of 75, 150, and 300 μM (Figure 5.1). Both the pre-op HDL_3 and the post-op SAA-HDL_3 inhibited the cytokine-induced expression of VCAM-1 in the HUVECs in a concentration dependent manner. The post-op SAA-HDL_3 was equally effective as the pre-op HDL_3 in the inhibition of VCAM-1 expression in the endothelial cells. As shown in Figure 5.2, the HDL_3 obtained from the controls also inhibited VCAM-1 expression in a concentration dependent manner and there was no significant difference in the degree of inhibition between the first or second batches of HDL_3.

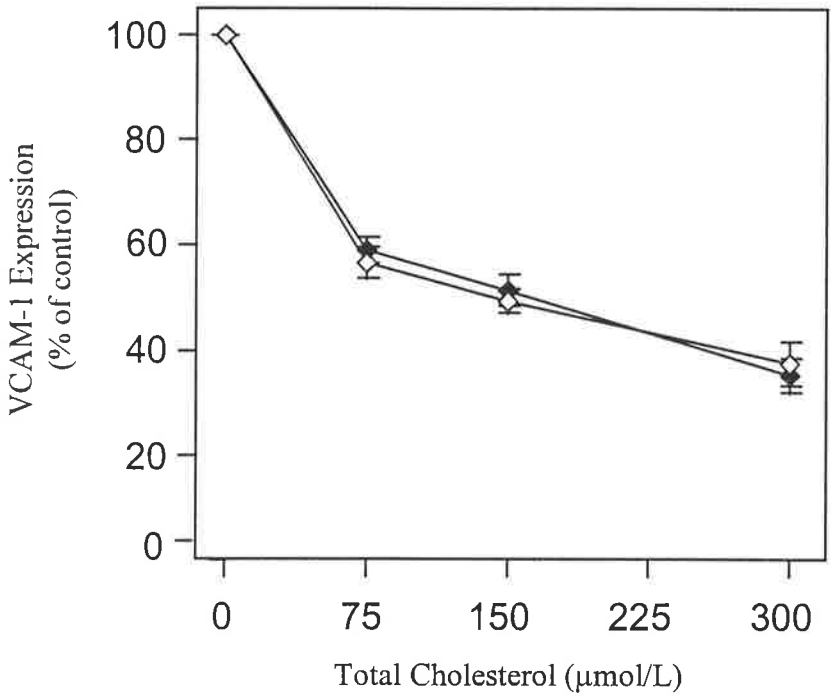

Figure 5.1 The Effect of pre-op HDL_3 and post-op SAA-HDL_3 on VCAM-1 expression in HUVECs. HUVECs were preincubated for one hour with pre-op HDL_3 and post-op SAA-HDL_3 before being activated with TNF-α (100 U/ml) and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The pre-op HDL_3 (◆) and post-op SAA-HDL_3 (◇) were added to the HUVECs according to total cholesterol concentration. The results are the mean and standard error of the mean. n = 19.

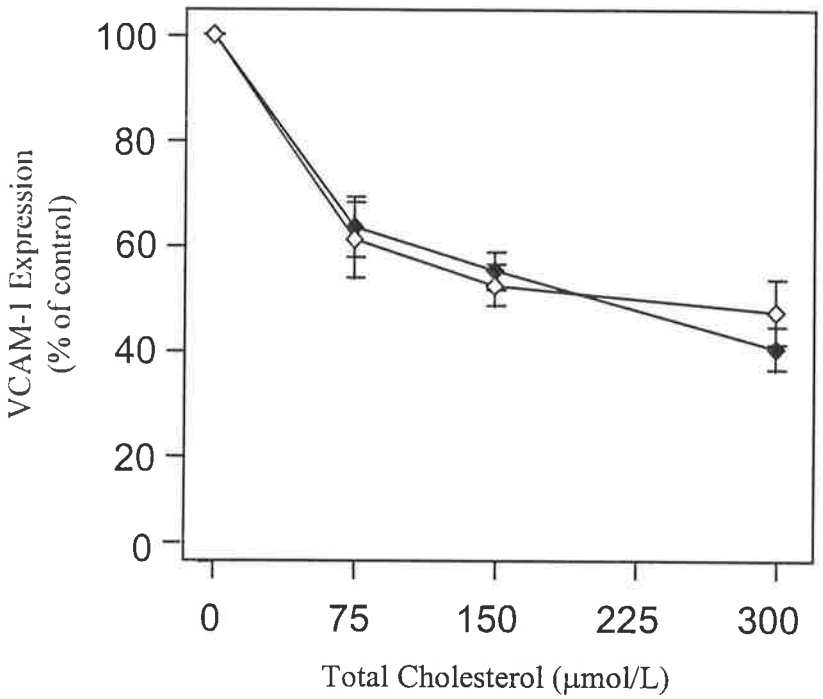

Figure 5.2. The effect of pre HDL_3 and post HDL_3 taken from normal subjects 4 days apart on VCAM-1 expression in HUVECs. HUVECs were preincubated for one hour with pre HDL_3 and post HDL_3 before being activated with TNF-α (100 U/ml) and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The pre HDL_3 (◆) and post HDL_3 (◇) were added to the HUVECs according to total cholesterol concentration. The results are the mean and standard error of the mean. n = 6.

5.3.2 Effect of SAA-HDL_3 Produced In-Vitro on the Cytokine-Induced Expression of VCAM-1 Expression in HUVECs

To assess the effect of having a greater proportion of the HDL_3 protein as SAA, SAA-HDL_3 was produced in vitro. As described in the methods, HDL_3 was isolated from healthy subjects and lipid-free SAA was added to the HDL_3 at a mass ratio of apo SAA:HDL_3 protein of 2:1. As a control another aliquot of HDL_3 was supplemented with PBS in place of SAA. The total composition of the unmodified HDL_3 and the SAA-HDL_3 are shown in Table 5.5. As seen with the SAA-HDL_3 obtained from the CABG subjects, there was no change in the percentage of protein, but there was a significant decrease in the proportion of esterified cholesterol and increase in the triglycerides in the SAA-HDL_3. Unlike the in-vivo SAA-HDL_3 isolated from the CABG subjects, there was a significant decrease in the phospholipid content in the in-vitro produced SAA-HDL_3 compared to the unmodified HDL_3.

The apolipoprotein composition of the unmodified HDL_3 and the in-vitro produced SAA-HDL_3 (as determined by SDS polyacrylamide gel electrophoresis) is shown in Table 5.6. The unmodified HDL_3 had a mean of 72.9 % apo A-I, 27.1 % apo A-II and no detectable apo SAA. The SAA-HDL_3 produced in-vitro had a mean of 81.5 % of its protein as SAA and only 1.4 % and 17.2 % apo A-I and apo A-II respectively. The unmodifed HDL_3 and in-vitro produced SAA-HDL_3 were subjected to non denaturing polyacrylamide gradient gel electrophoresis (Table 5.7). There was a significant increase in the diameter of the HDL_3 from a mean of 8.8 nm for the unmodified HDL_3 to 10.2 nm for the SAA-HDL_3.

Table 5.5. Total composition (mass %) of unmodified HDL_3 and SAA HDL_3.

Constituent	Unmodified HDL_3	SAA-HDL_3
Total Protein	51.3 ± 0.4	55.1 ± 1.9
Cholesteryl Esters	16.0 ± 0.1	*11.5 ± 1.0
Unesterified Cholesterol	2.9 ± 0.1	2.8 ± 0.3
Triglyceride	3.8 ± 1.0	*10.1 ± 1.8
Phospholipid	26.0 ± 1.3	*20.5 ± 2.0

The values represent the means ± SEM. n = 3. Students two-tailed t-test *signifies $p < 0.05$.

Table 5.6. Percent Composition of Major Proteins

Constituent	Unmodified HDL_3	SAA-HDL_3
ApoA-I	72.9 ± 1.6	*1.4 ± 0.4
ApoA-II	27.1 ± 1.6	*17.2 ± 1.3
SAA	0 ± 0	*81.5 ± 1.7

Derived from the peaks of the integrated curve obtained by subjecting the apolipoproteins to SDS polyacrylamide gel electrophoresis as described in section 5.2.1. The values represent the means ± SEM. n=3. Students two-tailed t-test *signifies $p<0.05$.

Table 5.7 Diameter of the HDL_3

Constituent	Diameter (nm)
Unmodified HDL_3	8.8 ± 0.3
SAA-HDL_3	10.2 ± 0.3

Diameters of HDL_3 were determined by non-denaturing polyacrylamide gradient gel electrophoresis. The values represent the means ± SEM. n=3. Students two-tailed t-test *signifies $p<0.05$.

Unmodified HDL_3 and in-vitro SAA-HDL_3 were added to HUVECs at total cholesterol concentrations of 75, 150, and 300 μM for one hour before the addition of the TNF-α (Figure 5.3). After 5 hours the VCAM-1 expression was determined by FACS analysis. Both the unmodified HDL_3 and the SAA-HDL_3 inhibited the cytokine-induced expression of VCAM-1 in the HUVECs in a concentration dependent manner. As with the SAA containing HDL_3 obtained from the CABG subjects, the SAA-HDL_3 that had been produced in-vitro was just as effective as unmodified HDL_3 in the inhibition of VCAM-1 expression in the endothelial cells.

To assess if a longer period of preincubation of the SAA-HDL_3 with the HUVECs would affect the subsequent inhibition of VCAM-1 expression, the unmodified HDL_3 and the SAA-HDL_3 prepared in vitro were incubated with the cells for 17 hours prior to the addition of the cytokine TNF-α. As in the earlier experiments, 5 hours after the addition of cytokine the VCAM-1 expression was determined by FACS analysis. Both the unmodified HDL_3 and the SAA-HDL_3 inhibited the cytokine-induced expression of VCAM-1 in the HUVECs in a concentration dependent manner and again there was no difference between the two preparations (Figure 5.4). The longer preincubation time of the HDLs with the endothelial cells resulted in a greater degree of VCAM-1 inhibition for a given total cholesterol concentration. For both the unmodified HDL_3 and the SAA-HDL_3 there was over 85 % inhibition of VCAM-1 expression at 75 μmol/L total cholesterol.

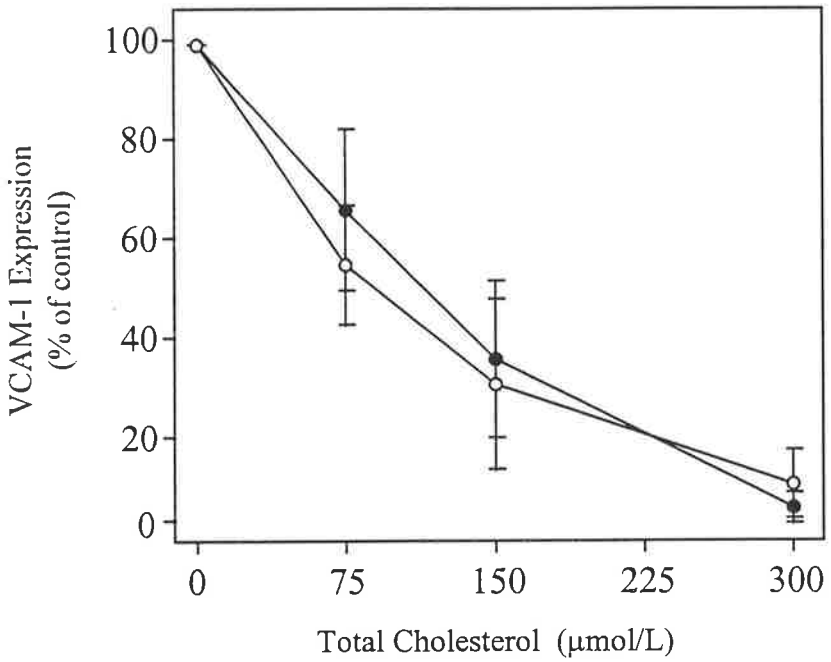

Figure 5.3. The effect of unmodified HDL_3 and in vitro SAA-HDL_3 on cytokine-induced VCAM-1 expression in HUVECs. SAA-HDL_3 was produced in vitro by displacing apo A-I with lipid-free SAA as descibed in methods. HUVECs were preincubated for one hour with unmodified HDL_3 and SAA-HDL_3 before being activated with TNF-α (100 U/ml) and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The unmodified HDL_3 (●) and in vitro SAA-HDL_3 (o) were added to the HUVECs according to total cholesterol concentration. The results are the mean and standard error of the mean from three experiments where each was performed in duplicate.

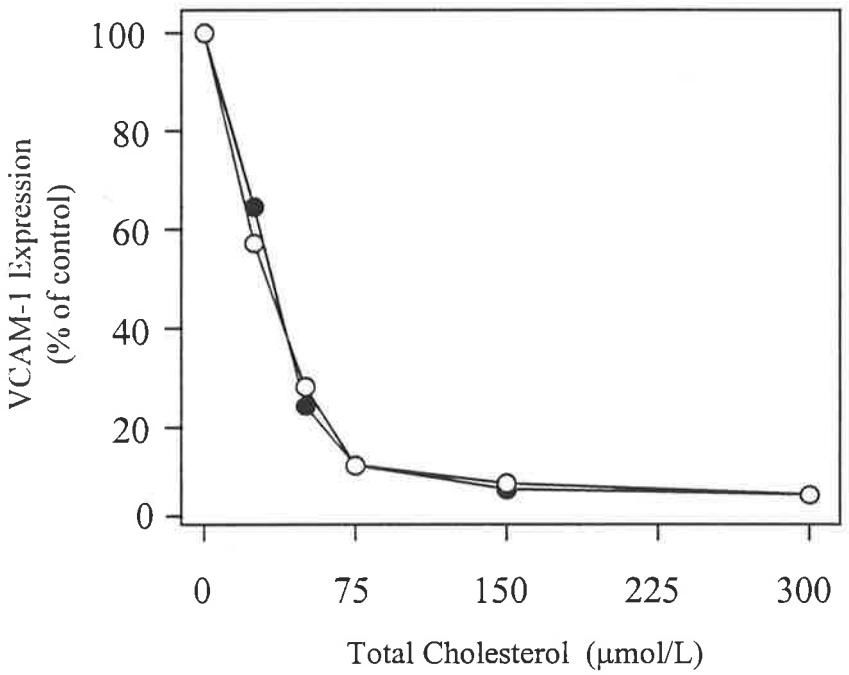

Figure 5.4. The effect of unmodified HDL_3 and in vitro SAA-HDL_3 on cytokine-induced VCAM-1 expression in HUVECs. SAA-HDL_3 was produced in vitro by displacing apo A-I with lipid-free SAA as descibed in methods. HUVECs were preincubated for 17 hours with unmodified HDL_3 and in vitro SAA-HDL_3 before being activated with TNF-α (100 U/ml) and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The unmodified HDL_3 (●) and in vitro SAA-HDL_3 (○) were added to the HUVECs according to total cholesterol concentration. The results are the means from one experiment performed in duplicate.

5.3.3. The Effect of Lipid-Free SAA on the Cytokine-Induced Expression of VCAM-1 in HUVECs

To assess the effect of lipid-free SAA on the cytokine-induced expression of VCAM-1 in endothelial cells, lipid-free SAA was isolated from SAA-HDL_3 and added to HUVECs at total protein concentrations of 2, 4, 8 and 16 μM. The cells were incubated for one hour before the addition of TNF-α. After an additional 5 hours the VCAM-1 expression was determined by FACS analysis. As shown in Figure 5.5, the lipid-free SAA had no effect on VCAM-1 expression at the concentrations tested.

5.4 Discussion

The experiments in this chapter show that altering the protein composition of HDL_3 by introducing SAA at the expense of apo A-I and to a lesser extent apo A-II, has no effect on the ability of HDLs to inhibit VCAM-1 expression in the endothelial cells. It was also shown that, as is the case with lipid-free apo A-I (Baker et al, In Press), lipid-free SAA has no effect on the cytokine-induced expression of VCAM-1 in HUVECs.

As discussed in chapter 1, SAA is potentially pro-atherogenic in both its lipid-free form and when associated with HDLs. In its lipid-free form, recombinant human SAA has been shown to be a chemoattractant and to induce the migration, adhesion and tissue infiltration of monocytes, polymorphonuclear leukocytes and T lymphocytes (Badolato et al, 1994; Xu et al, 1995). Recombinant human SAA has also been shown to increase the expression of the cellular adhesion molecules Mac-1 and leukocyte adhesion molecule-1 (Badolato et al, 1994). When associated with

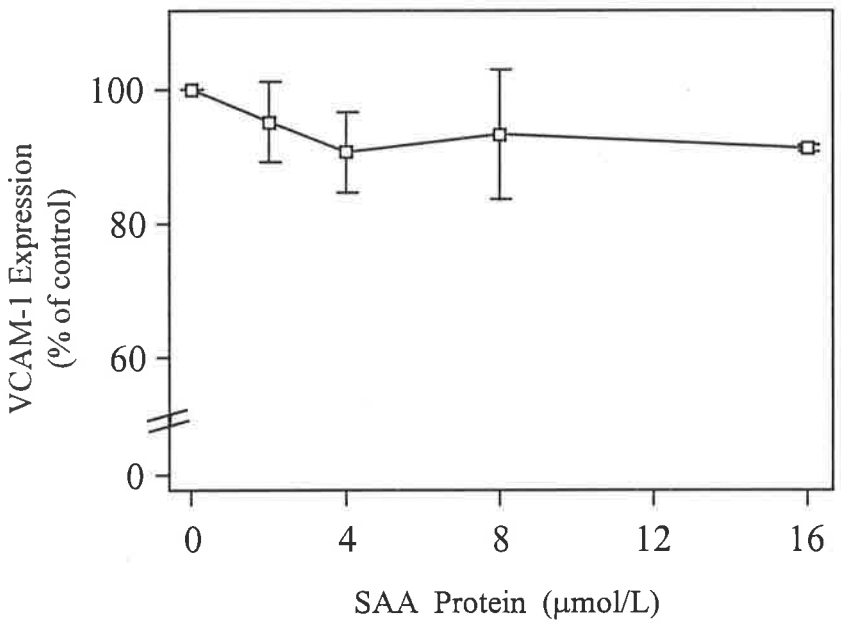

Figure 5.5. The effect of lipid-free SAA on VCAM-1 expression in HUVECs. HUVECs were preincubated for one hour with lipid free SAA before being activated with TNF-α (100 U/ml) and incubated for a further 5 hours. The expression of VCAM-1 was quantitated by flow cytometry. Values are presented as a percentage of that in a sample without HDLs. The lipid free SAA (□) was added according to total protein concentration. The results are the means and standard error of the means from three experiments, each performed in duplicate.

HDLs, SAA enhances the binding of HDL particles to macrophages (Kisilevsky and Subrahmanyan, 1992). SAA-HDLs also bind to human monocytic THP-1 cells more avidly than normal HDLs (Banka et al, 1995). The presence of SAA in HDLs adversely effects reverse cholesterol transport by decreasing LCAT activity (Steinmetz et al, 1989) and decreasing the ability of HDLs to protect LDL against oxidation (Van Lenten et al,1995).

Given these potentially pro-atherogenic effects of SAA it was important to determine whether SAA would also impair the ability of HDLs to inhibit the cytokine-induced adhesion molecule expression. To assess this we compared the inhibitory activities of unmodified HDL_3 and acute-phase SAA-HDL_3 collected from the same subjects. The SAA-HDL_3 was just as effective as the unmodified HDL_3 at inhibiting cytokine-induced VCAM-1 expression. The proportion of protein as SAA in the acute-phase HDL_3 was a median of 43.0 % with a range of range 29 - 49 %. This was not as high as that seen in some other studies. Clifton et al studied the acute-phase SAA-HDL_3 in human subjects after myocardial infarction and found that SAA made up 8-87 % (median 52 %) of the total protein in the HDL_3 (Clifton et al, 1985). The acute-phase HDL_3 in rabbits had on average 60 % of the total protein as SAA (Cabana et al, 1989). It was possible that the lower percentage of SAA in the post CABG HDL_3 in the present study, could have masked an effect of SAA on the ability of HDLs to inhibit VCAM-1 expression. However, this was excluded by finding that the greater amounts of SAA in the in vitro SAA-HDL_3 were still without effect on VCAM-1 expression in the endothelial cells.

The absence of an effect of SAA on HDL-mediated inhibition of VCAM-1 adds further support to the findings in chapter 4 that changing the apolipoprotein composition of HDLs has no effect on the ability of HDL_3 to inhibit VCAM-1 expression in HUVECs. The result is also consistent with studies showing that rHDLs containing only apo A-I are comparable to those containing only apo A-II as inhibitors of endothelial VCAM-1 expression (Calabresi et al, 1997; Baker et al, In Press).

The changes in composition of the HDL_3 after the addition of SAA in the in-vitro studies were consistent to those described elsewhere (Clifton et al, 1985; Hoffman and Benditt, 1982). There was an increase in the percentage of triglycerides and decreases in the percentages of phospholipid and esterified cholesterol. This differs from the changes seen between the pre-op and post-op HDL_3 isolated from the surgical subjects in that there was a small, but significant increase in the phospholipids in the HDL_3 that contained SAA. The explanation for this increase in the percentage composition of the phospholipids is unclear, but may relate to the smaller amounts of SAA in the post-op SAA-HDL_3 compared with the in-vitro produced SAA-HDL_3. Other studies in which human and rabbit acute-phase HDL_3 (SAA-HDL_3) were used also showed no significant variation in the phospholipid content despite increases in the percentage of triglycerides (Coetzee et al, 1986; Cabana et al, 1989).

In the final series of experiments in this chapter it was shown that lipid-free SAA had no significant effect on the cytokine-induced expression of VCAM-1 in HUVECs.

intriguing observation is clearly worth further investigation, as is the intracellular signaling processes that lead to inhibition of adhesion molecule expression.

This finding was surprising given that Badolato et al showed that reconstituted SAA caused an increase in the expression of the adhesion molecules Mac-1 and leukocyte adhesion molecule-1 in HUVECs (Badolato et al, 1994). The differing results may be due to the fact that Badolato did not stimulate the HUVECs with cytokine and Mac-1 and leukocyte adhesion molecule-1 are from the selectin family of adhesion molecules and not the immunoglobulin superfamily like VCAM-1. Our finding that lipid-free SAA had no effect on VCAM-1 expression was similar to the lack of effect on VCAM-1 expression seen with lipid-free apo A-I and A-II (Baker et al, In Press).

In chapter 4 it was demonstrated that the ability of HDLs to inhibit the cytokine-induced expression of VCAM-1 in endothelial cells was independent of the apo A-I and A-II ratio of the particle. In this chapter it is shown that when HDL_3 particles carry SAA, an apolipoprotein that possesses a similar amphipathic α-helical structure to apo A-I and A-II, there is no affect on the ability of the HDL_3 to inhibit the cytokine-induced expression of VCAM-1. This supports the proposition that the ability of HDLs to inhibit adhesion molecule expression may depend on the binding of apolipoproteins to a cell surface receptor. It may be argued that such binding enables the HDL phospholipids to interact with the cell membrane in an as yet undetermined mechanism which results ultimately in the inhibition of the expression of cellular adhesion molecules. While these studies indicate a lack of specificity for the protein moiety of the HDL, preliminary work in our laboratory (Dr. P. Baker, unpublished) has shown that different phospholipid species differ markedly in their abilities to inhibit the cytokine-induced expression of VCAM-1 in HUVECs. This

CHAPTER 6

EFFECT OF FENOFIBRATE ON PLASMA LEVELS OF LIPIDS AND SOLUBLE ADHESION MOLECULES IN DIABETIC SUBJECTS

6.1 INTRODUCTION

Soluble forms of cellular adhesion molecules can be found in the circulation. These soluble adhesion molecules are thought to arise from membrane bound versions of the adhesion molecule that have been shed into the circulation. These shedded soluble adhesion molecules are probably generated by cleavage at a site close to the membrane insertion and they lack the membrane-spanning and cytoplasmic domains that are present in the membrane-bound forms (Pigott et al, 1992). The metabolism and functional significance of the soluble adhesion molecules are not yet known. It is known, however, that the levels are altered in a range of diseases and that the treatment of these underlying diseases may modify the levels. Disease states in which soluble adhesion molecules are elevated include sepsis, autoimmune diseases, malignancies, organ failure and chronic inflammatory diseases such as atherosclerosis (Gearing and Newman, 1993) (Table 1.3).

Soluble adhesion molecules are markers for atherosclerosis. As was discussed in chapter 1, sVCAM-1, sICAM-1, sE-selectin and sP-selectin all correlate with the degree of atherosclerosis in humans (McCollum, 1994; Nakai et al, 1995; Blann and Blann et al, 1996; Peter et al, 1997; Ridker et al, 1998). There are also elevations in circulating adhesion molecules in conditions that are known to predispose to atherosclerosis such as dyslipidemia (Hackman et al, 1996), diabetes mellitus (Lampeter et al, 1992) and hypertension (Lip et al, 1995). As discussed previously, studies have shown that improved diabetic control and treatment of dyslipidemias can move soluble adhesion molecule levels back towards the normal range (Ceriello et al, 1996; Sampietro et al, 1997; Hackman et al, 1996; Abe et al, 1998).

The elevated levels of soluble adhesion molecules in type II diabetes mellitus may reflect the dyslipidemia in this condition. Subjects with type II diabetes typically have raised triglycerides, low HDL-C and relatively normal levels of LDL cholesterol (Wilson et al, 1985; Howard, 1987). Given that treatment of hypertriglyceridemia reduces sE-selectin and sICAM-1 (Abe et al, 1998), it may be hypothesised that improving the plasma lipid profile in diabetic subjects by reducing triglycerides and raising the HDL-C will result in a reduction of the soluble adhesion molecules important in atherosclerosis.

As a preliminary test of this hypothesis, 20 of the subjects enrolled in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) Study were recruited. The FIELD Study is a randomised, multicentre, double-blind, placebo-controlled trial of the effects on coronary mortality and morbidity of comicronised fenofibrate in patients with type II diabetes. The run-in phase of the FIELD Study is shown in table 6.1. The initial visit (visit 1) is to screen for eligibility and to give dietary advice. If suitable, the subjects return 4 weeks later for a second visit (visit 2) where they commence a 6 week run-in phase on placebo medication. They return 6 weeks later for their third visit (visit 3) after which they commence a 6 week active run-in phase on comicronised fenofibrate therapy at a dose of 200 mg per day. After 6 weeks on fenofibrate they return for visit 4 at which time they are randomised in a double-blinded manner to fenofibrate 200 mg per day or placebo which they will continue for the duration of the study (estimated to be 5-6 years).

Table 6.1 Schedule of visits for the FIELD study.

Visit Number	Conditions
Visit 1 (-16 weeks)	Screening for eligibility and safety. Given dietary advice.
Visit 2 (-12 weeks)	Run-in placebo medication for 6 weeks. Single blinded.
Visit 3 (- 6 weeks)	Run-in fenofibrate 200 mg/day for 6 weeks. Single blinded.
Visit 4 (0 weeks)	Randomisation to fenofibrate or placebo.

The experiments described in this chapter are the results of a preliminary study to determine if the 6 week run-in phase on 200 mg daily comicronised fenofibrate therapy reduces the serum levels of sE-selectin, sICAM-1 and sVCAM-1. For this preliminary study, 20 subjects were recruited and plasma and serum were collected after visit 3 (after 6 weeks of placebo) and visit 4 (after 6 weeks of 200 mg daily comicronised fenofibrate). Levels of sE-selectin, sICAM-1 and sVCAM-1 were determined from each of the serum samples and the lipid profile, apoA-I and A-II levels and HDL size distributions determined from the plasma samples.

6.2. METHODS

6.2.1 SUBJECT RECRUITMENT AND ISOLATION OF SERUM AND PLASMA

Twenty subjects were recruited from the FIELD Study and informed consent obtained. Blood was collected by venesection at Visits 3 and 4 from subjects after a 14 hour fast and placed into tubes containing disodium EDTA (final concentration 1 mg/ml) for plasma and into tubes with no additions for serum. After the serum samples had clotted, the blood was placed on ice. The plasma samples were placed immediately on ice. The plasma and serum were isolated by centrifugation at 4°C and then stored frozen at –70°C until needed.

6.2.2 MEASUREMENT OF PLASMA LIPIDS, APOA-I AND A-II AND DETERMINATION OF HDL SIZE DISTRIBUTION.

Analysis of plasma lipids were performed at the Institute of Medical and Veterinary Science (Adelaide, South Australia) on a Hitachi 917 automatic analyser.

Concentrations of apoA-I and A-II were determined as described in chapter 2. To determine the HDL particle size and distribution, plasma was subjected to ultracentrifugation at a density of 1.21 g/ml in a Beckman TL-100 Ultracentrifuge at 100,000 r.p.m. at 4°C for 17 hours. The supernatant was collected and the HDL particle size distribution was assessed by electrophoresis on 3-40 % nondenaturing polyacrylamide gradient gels as described in chapter 2. The proportions of HDL_2 and HDL_3 were estimated by running the total HDL fraction on a 3-40% gradient gel, scanning the HDL profile from the gel and then estimating the areas under the curve.

6.2.3. Determination of sE-Selectin, sICAM-1 and sVCAM-1 in the Serum.

Levels of sE-selectin, sICAM-1 and sVCAM-1 were determined by using monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (R & D Systems, U.S.A.) on frozen serum that had been collected at visit 3 (after 6 weeks placebo) and visit 4 (after 6 weeks fenofibrate) from all subjects. Assays of all samples, controls and standards were performed in duplicate. Concentrations of soluble adhesion molecules were determined by analysing standards with known concentrations of recombinant adhesion molecules coincident with samples and plotting of signal versus concentration.

6.2.4. Statistical Methods

The data obtained in this chapter are expressed as mean ± sem. Students' t test for paired samples was used to determine whether differences between values were significant. Statistical significance was set at $p < 0.05$.

6.3 RESULTS

6.3.1. SUBJECT DEMOGRAPHICS

The baseline characteristics for the 20 subjects are shown in Table 6.2. The average age was 63 years and 14 of the 20 subjects were male. All subjects had type II diabetes mellitus (this was an entry criteria for the FIELD Study), 14 of the 20 had a history of hypertension and 3 of the 20 were smokers. There was objective evidence of CHD in only one of the subjects but there were clinical manifestations of atherosclerosis in 4 of the 20 subjects.

6.3.2. EFFECTS OF FENOFIBRATE THERAPY ON PLASMA LIPIDS, PLASMA APOLIPOPROTEINS AND HDL PARTICLE SIZE DISTRIBUTION.

Plasma lipid, apoA-I and apoA-II levels were determined after visit 3 (after 6 weeks of placebo therapy) and after visit 4 (after 6 weeks of fenofibrate therapy) in the 20 subjects (table 6.3). There was a 40% reduction in triglycerides ($p < 0.05$), an 11.4% reduction in total cholesterol ($p < 0.05$), a nonsignificant fall of 10.6% in the LDL-C and a significant elevation of 13.5% in the HDL-C level ($p < 0.05$). There were rises in both the plasma apoA-I and apoA-II levels of 6.9% and 9.6% respectively, but this was only statistically significant for the apoA-II ($p<0.05$).

Table 6.4 shows the effects on HDL particle size and distribution of the six weeks of fenofibrate therapy. After 6 weeks of placebo therapy, the mean proportion of the total HDL that was in the HDL_2 size range was 18.8% with the remaining 81.2%

Table 6.2. Baseline characteristics of the 20 subjects recruited from the F.I.E.L.D. Study.

Pararmeter	Subjects (n = 20)
Age	63 ± 1.5
Sex, M / F	14 / 6
Diabetes	20
Hypertension	14
Smoking	3
Objective C.H.D.	1
Clinical Atherosclerosis	4

Values for age are expressed as mean ± SEM.

Table 6.3 Effects of fenofibrate 200 mg daily on plasma lipids and apolipoproteins after 6 weeks of treatment.

	Visit 3 (After 6 weeks placebo)	Visit 4 (After 6 weeks fenofibrate)	Percent Change
Triglyceride (mmol/L)	1.85 ± 0.18	*1.11 ± 0.08	– 40 %
Total Cholesterol (mmol/L)	5.18 ± 0.16	*4.59 ± 0.14	– 11.4 %
LDL-C (mmol/L)	3.31 ± 0.15	2.96 ± 0.14	– 10.6 %
HDL-C (mmol/L)	1.04 ± 0.04	*1.18 ± 0.05	+ 13.5 %
ApoA-I (mg/ml)	1.31 ± 0.03	1.40 ± 0.05	+ 6.9 %
ApoA-II (mg/ml)	0.73 ± 0.03	*0.80 ± 0.02	+ 9.6 %

Values represent the mean ± SEM for the 20 subjects. Students two-tailed t-test. *signifies $p \leq 0.05$.

Table 6.4. Effects of fenofibrate 200 mg daily on HDL particle size distribution.

	Visit 3 (After 6 weeks placebo)	Visit 4 (After 6 weeks fenofibrate)	Percent Change
% of Total HDL as HDL_2	18.8 ± 1.4 %	20.4 ± 1.3 %	+ 1.6 %
% of Total HDL as HDL_3	81.2 ± 1.4 %	79.6 ± 1.3 %	− 1.6 %

The proportions of HDL_2 and HDL_3 were estimated from the areas under the curve obtained after the electrophoresis of the HDL on 3-40 % nondenaturing polyacrylamide gradient gels and scanning the gels to determine the HDL subpopulations. Values represent the means ± S.E.M. for 20 subjects. Students two-tailed t-test.

existing as HDL_3. After 6 weeks of fenofibrate therapy there was a small, nonsignificant 1.6% increase in the proportion of the HDL that was in the HDL_2 size range.

6.3.3. Effects of Fenofibrate Therapy on the Levels of Serum sE-Selectin, sICAM-1 and sVCAM-1.

The mean baseline (visit 3) levels of the soluble adhesion molecules were elevated compared with levels seen in normal, nondiabetic subjects (Gearing et al, 1992)(Table 6.5). The baseline sE-selectin was 54.6 ± 3.8 ng/ml, sICAM-1 was 239.6 ± 7.2 ng/ml and sVCAM-1 was 714.4 ± 29.2 ng/ml. After 6 weeks of fenofibrate therapy there was a significant 19.9% ($p \leq 0.05$) reduction of sE-selectin to a mean of 43.8 ± 1.9 ng/ml. There was a non-significant 3.8% ($p = 0.19$) reduction in sICAM-1 to a mean of 230.9 ± 7.2 ng/ml and there was no significant effect on sVCAM-1 with a 1.7% reduction ($p = 0.39$) to 702.3 ± 29.6 ng/ml.

Figure 6.1 shows the levels of sE-selectin, sICAM-1 and sVCAM-1 for each of the twenty subjects at baseline and after 6 weeks of fenofibrate therapy. Those subjects with the highest baseline levels of serum soluble adhesion molecules tended to have a greater percentage fall in their levels after the six weeks of fenofibrate therapy.

Table 6.5 Effects of micronised fenofibrate 200 mg daily on the serum soluble adhesion molecules sE-selectin, sVCAM-1 and sICAM-1 after 6 weeks of treatment.

	Normal Values[a]	Visit 3 (After 6 weeks placebo)	Visit 4 (After 6 weeks fenofibrate)	Percent Change
sE-selectin (ng/ml)	46.3 ± 17	54.6 ± 3.8	43.8 ± 1.9	–*19.9 %
sICAM-1 (ng/ml)	211 ± 96	239.6 ± 7.2	230.9 ± 6.6	– 3.8 %
sVCAM-1 (ng/ml)	553 ± 158	714.4 ± 29.2	702.3 ± 29.6	– 1.7 %

[a]Normal values are from R and D systems data and represents the mean ± 1 S.D. for 131 healthy subjects. Values for visits 3 and 4 represent the mean ± SEM for the 20 subjects. The percent change is the percentage difference between the values from visits 3 and 4. Students two-tailed t-test. *signifies $p \leq 0.05$.

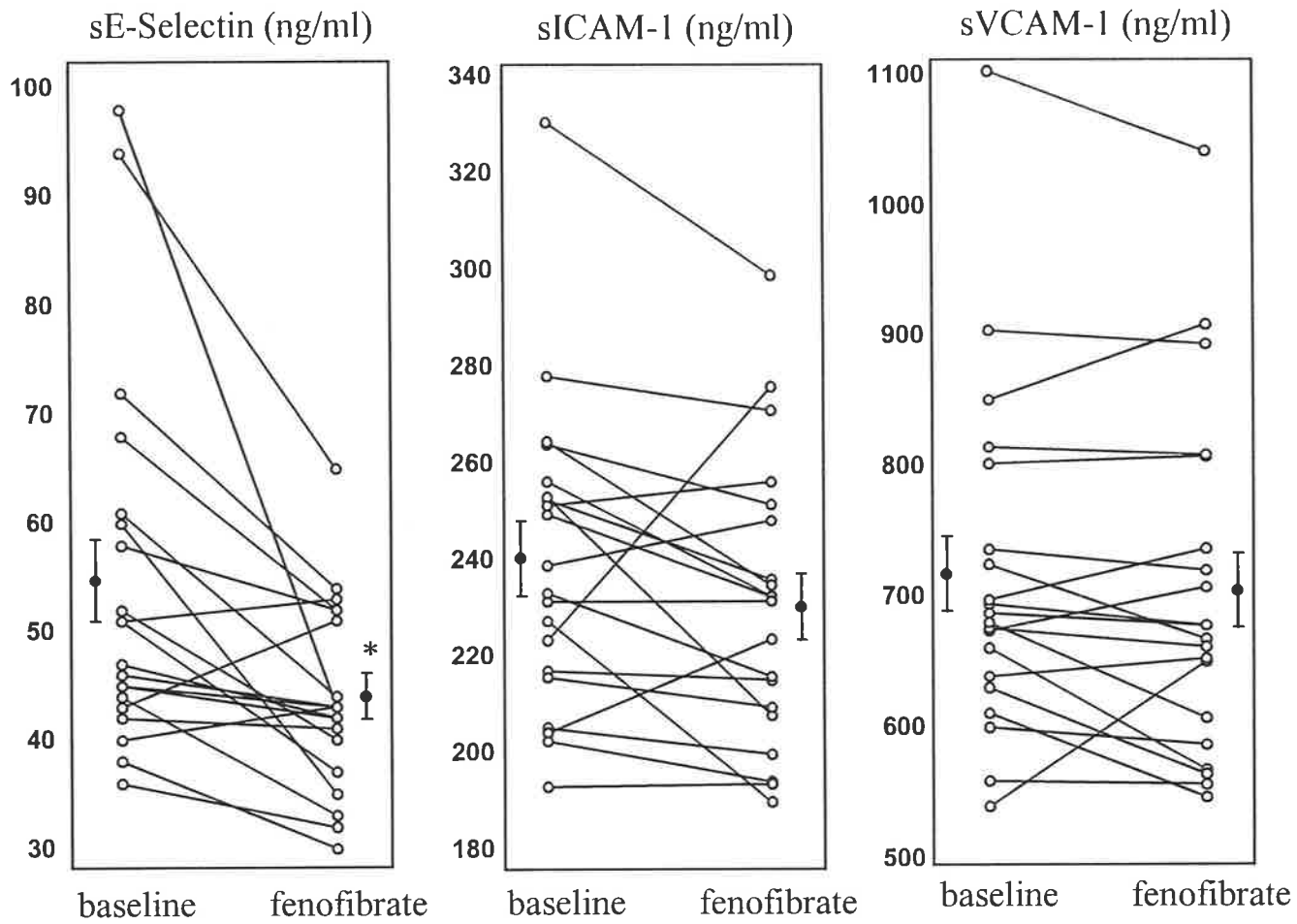

Figure 6.1. Levels of sE-selectin, sICAM-1 and sVCAM-1 in the serum of 20 subjects after 6 weeks of placebo and then 6 weeks of treatment with fenofibrate. Serum levels of soluble adhesion molecules were determined by monoclonal antibody-based ELISA kits on frozen serum collected from all subjects after 6 weeks of placebo and then 6 weeks of fenofibrate as described in methods. (∘) indicates the individual values and (•) the arithmetic mean and the error bars the SEM. * signifies $p < 0.05$

6.3.4 Relationship Between the Percent Change in Plasma HDL-C and the Percent Change in the Serum Levels of Soluble Adhesion Molecules after 6 Weeks of Fenofibrate Therapy.

The percent change in plasma HDL-C and the percent change in serum levels of sE-selectin, sICAM-1 and sVCAM-1 after 6 weeks of fenofibrate therapy were calculated for each of the 20 subjects. A linear regression analysis of the relationship between the percent changes in plasma HDL-C and percent changes in each of sE-selectin, sICAM-1 and sVCAM-1 was performed. There was no correlation observed between the percent change in HDL-C and the percent change in the soluble adhesion molecules. The correlation coefficients (R) for sE-selectin, sICAM-1 and sVCAM-1 were 0.03, 0.07 and 0.02 respectively.

6.4. Discussion

This preliminary study of twenty diabetic subjects shows that 200 mg micronised fenofibrate taken for a period of 6 weeks has a favourable effect on the plasma lipid profile and results in a significant reduction in serum sE-selectin levels. The changes in plasma lipids and lipoproteins seen in this study are consistent with other reported data on the effects of micronised fenofibrate. (Brown et al, 1986).

Previous reports have shown that subjects with type II diabetes mellitus have elevated levels of circulating sE-selectin, sICAM-1 and sVCAM-1 (Cominacini et al, 1995; Ceriello et al, 1996; Gearing et al, 1992). This was confirmed in this study

with the baseline levels of sE-selectin, sICAM-1 and sVCAM-1 being higher than those reported in non-diabetic subjects. Improved glycaemic control in subjects with type II diabetes mellitus leads to significant reductions in serum sE-selectin (Cominacini et al, 1997). This preliminary study of fenofibrate in type II diabetics showed a significant reduction in sE-selectin, but little effect on sICAM-1 and sVCAM-1 after 6 weeks of therapy. The differences between the adhesion molecule responses may reflect the differing distribution of the membrane bound adhesion molecules and the short duration of fenofibrate therapy. E-selectin is only found on activated endothelial cells whereas ICAM-1 and VCAM-1 are expressed not only on endothelial cells but on a wide variety of other cell types such as leukocytes and smooth muscle cells (Faull, 1995). Thus any change in the activation state of the endothelial cells would best be reflected in the levels of sE-selectin. Hence while improving the lipoprotein profile by a six week course of fenofibrate may inhibit the endothelial expression of E-selectin, ICAM-1 and VCAM-1, this short course of therapy may not have affected the level of expression of adhesion molecules within the intima of the atherosclerotic plaque where much of the ICAM-1 and VCAM-1 are expressed. This may explain why there was a significant reduction in sE-selectin in our study with less effect on sICAM-1 and sVCAM-1.

To actually reduce the burden of atherosclerosis there may need to be a prolonged period of lipid lowering. The Scandinavian Simvastatin Survival Study did not show a reduction in the number of CHD events in the simvastatin group until after approximately 1 year of treatment and in angiographic coronary regression studies it usually takes at least 2 years to show even modest decreases in the level of

atherosclerosis (Scandinavian Simvastatin Survival Study Group, 1994; Gotto, 1995). Thus to observe a significant fall in serum level of sICAM-1 and sVCAM-1 there may need to be not only a reduction in the endothelial expression of VCAM-1 and ICAM-1, but also a reduction in the size of the plaques as most of the VCAM-1 and ICAM-1 is found within the plaque itself (Hackman et al, 1996).

The theory that a prolonged period of therapy to improve plasma lipids is necessary to reduce the overall atherosclerotic burden is consistent with results seen in a study by Abe et al which measured serum soluble adhesion molecule levels in subjects with hypertriglyceridemia after therapy with n-3 fatty acids (Abe et al, 1998). After 6 weeks of therapy there was a significant fall in sE-selectin but not sICAM-1 or sVCAM-1. After 6 months of n-3 fatty acids in the hypertriglyceridemic subjects there was still a reduction in sE-selectin, but sICAM-1 levels had also had a significant reduction. Thus 6 weeks of therapy may have been long enough to reduce the endothelial expression of adhesion molecules, but 6 months was needed to start to reduce the level of adhesion molecule expression from within the plaque itself.

Linear regression analysis was used assess the relationship between the percent changes in plasma HDL-C and the percent changes in serum soluble adhesion molecule levels, with the hypothesis that the larger the increase in plasma HDLs in any given subject, the larger the reduction in serum soluble adhesion molecules. In this small study there was no correlation seen to support this hypothesis. Further larger studies with a longer duration of fenofibrate therapy are needed to test this hypothesis.

The mechanism by which the changes in the plasma lipid profile may reduce the serum levels of the soluble adhesion molecules is not known. On the basis of my studies, one possibility is that the effects maybe secondary to an elevation in HDL. As shown in chapters 3 and 4, HDLs inhibit the cytokine-induced expression of cellular adhesion molecules on cultured endothelial cells. If this occurs in vivo, then raising the plasma HDL level may reduce the endothelial expression of adhesion molecules and thus may cause a reduction in their soluble counterparts in the serum.

This preliminary study has obvious shortcomings. It is an open-labeled study of a small number of subjects with each subject acting as his or her own control. Despite this, the results are intriguing and clearly support the need for a larger, randomised, double blind, placebo controlled study.

CHAPTER 7

GENERAL DISCUSSION

The inhibition of cytokine-induced adhesion molecule expression in cultured endothelial cells is one of the potential mechanisms to explain HDLs antiatherogenic properties. As discussed in chapter one, there are other possible ways that HDLs may protect against atherosclerosis, including HDLs role in reverse cholesterol transport and the antioxidant functions of HDLs. It is probably the combination of all these mechanisms in vivo that give HDLs their protective properties.

Adhesion molecules are important in the initial stages of the formation of the atherosclerotic plaque and expression of adhesion molecules are essential in order for the monocytes to make their way from the blood vessel lumen into the intima where they can take up lipid to become foam cells and start the fatty streak. By inhibiting the expression of adhesion molecules, HDLs are acting at one of the earliest stages of atherosclerosis and thus this mechanism maybe one of the major methods by which HDL protects.

Although the mechanism by which HDLs inhibit cellular adhesion molecule expression has not yet been determined, insights have been obtained by the studies in this thesis. The insights of interest and potential importance to emerge include: i) HDLs isolated from different subjects may vary markedly in their ability to inhibit the cytokine-induced expression of VCAM-1 in endothelial cells, ii) the inhibitory activity of HDL_3 on VCAM-1 expression in HUVECs is substantially greater than that of HDL_2, iii) the apolipoprotein composition of HDLs do not affect their ability to inhibit VCAM-1 expression in HUVECs, iv) when isolated from HDLs, the

apolipoproteins or lipids alone have no effect on VCAM-1 expression and HDLs require at a minimum apolipoproteins and phospholipids to inhibit VCAM-1 expression, v) in a preliminary study it was shown that 6 weeks of fenofibrate therapy in diabetic subjects resulted in a significant reduction in the serum levels of sE-selectin.

One potential mechanism that may have explained HDLs' inhibitory effect on adhesion molecule expression, that of the ability of HDLs to remove lipid oxidation products, was not supported by experiments discussed in chapter 3. These studies showed that HDLs were still able to cause the inhibition of VCAM-1 expression in HUVECs after they had been removed from the media prior to cytokine stimulation of the HUVECs. These studies in which the HDLs were removed prior to stimulation of the cells with TNF-α also excluded the possibility that the HDLs may cause their inhibition of adhesion molecule expression by interfering with the binding of TNF-α to its cell receptors.

Studies in which the apolipoprotein composition of HDLs were modified provided evidence that the ability of HDLs to inhibit VCAM-1 expression in HUVECs appears to be independent of the apolipoprotein composition of the HDLs. When HDL_3 was modified by replacing the apoA-I with apoA-II or with SAA, the modified HDL_3 inhibited VCAM-1 expression in HUVECs to the same extent as unmodified HDL_3. This similarity in the inhibition of VCAM-1 expression in HDL_3 particles with differing apolipoprotein composition is consistent with the findings by Calabresi et al (1997) in which there was no difference in endothelial VCAM-1 inhibition using

rHDLs containing either apoA-I or apoA-II. This raises the possibility that the protective powers of HDLs against atherosclerosis, at least in terms of adhesion molecule expression, may be independent of the apolipoprotein composition of the HDL particles.

Experiments with native HDLs showed that the individual lipid and apolipoprotein components of HDL_2 and HDL_3, when added to the cells in isolation, had no inhibitory effect on the expression of VCAM-1. This was consistent with studies from our laboratory using lipid-free apoA-I and DMPC vesicles to inhibit VCAM-1 expression in HUVECs. Neither the lipid-free apoA-I or DMPC vesicles alone had any effect on cytokine-induced VCAM-1 expression, but when combined as simple discoidal rHDLs they mimicked native HDLs in their ability to inhibit VCAM-1 expression.

These findings allowed us to propose a theoretical model on the potential mechanism by which HDLs inhibit adhesion molecule expression in endothelial cells. It appears that an HDL particle must contain at least apolipoproteins and phospholipids for the inhibition of adhesion molecule expression to take occur. Our proposed model involves apolipoproteins binding the HDL particle to an HDL receptor on the cell which then allows the phospholipid contained in the HDLs' outer polar membrane to get in close enough proximity to act on the cell.

HDLs exist in the intravascular space whereas the cells of the atherosclerotic plaque exist in the intima, in the extravascular space. HDLs have been shown in vitro to

inhibit the expression of adhesion molecules on endothelial cells which are the natural barrier between the intra- and extra-vascular space. Two of the cellular adhesion molecules important in atherosclerosis, VCAM-1 and ICAM-1, are not only expressed on the endothelium, but are also expressed by leukocytes and smooth muscle cells in the intima. It is not known whether the HDLs from the intravascular space have any inhibitory effects on these extravascular intimal cells that express VCAM-1 and ICAM-1. Further studies are needed to assess the effects of HDLs on the adhesion molecule expression of these intimal leukocytes and smooth muscle cells.

Another interesting observation to come from these studies is the finding that HDL_3 are superior to HDL_2 in the inhibition of VCAM-1 expression in cultured HUVECs. As previously discussed in chapters 1 and 3, it has been suggested that HDL_2 may be superior to HDL_3 and A-I HDLs may be superior to A-I/A-II HDLs in terms of their cardioprotective properties. Studies of transgenic mice suggest that A-I HDLs may be superior to both A-I/A-II HDLs and A-II HDLs (Hedrick et al, 1993; Schultz et al, 1993; Schultz and Rubin, 1994). The results of human studies are less clear with respect to the superiority of the HDL subpopulations, with conflicting evidence both in favor and against the superiority of HDL_2 over HDL_3 and A-I HDLs over A-I/A-II HDLs. The studies in this thesis indicate that HDL_3 are superior to HDL_2 in terms of the inhibition of cytokine-induced cellular adhesion molecule expression in an in vitro model. Whether this superiority of adhesion molecule inhibition of HDL_3 over HDL_2 exists in vivo is not known at present.

The major points of interest to come from the studies of the acute-phase protein SAA and its relationship to HDLs were the discoveries that the presence of SAA in HDL_3 ($SAA\text{-}HDL_3$) did not alter the ability of the HDLs to inhibit VCAM-1 expression in endothelial cells. The finding was unexpected given that other workers have shown SAA to have potential pro-atherogenic properties. Badolato et al showed recombinant SAA caused an increase in the expression of the selectins Mac-1 and leukocyte adhesion molecule-1 in HUVECs (Badolato et al, 1994). In future studies it will be fascinating to repeat Badolatos' work in our model assessing Mac-1 and leukocyte adhesion molecule-1 expression in HUVECs after exposure to both lipid-free SAA and SAA-HDLs. It will be interesting to hypothesize that, as with Badolatos' studies, lipid-free SAA will increase the expression of Mac-1 and leukocyte adhesion molecule-1 but when it is associated with HDLs, the SAA will lose its stimulatory effect and perhaps inhibit the adhesion molecule expression.

The preliminary study on the effect of 200 mg fenofibrate on 20 subjects with type II diabetes gave some intriguing results which will be followed up in a larger, randomised, double-blind, placebo controlled trial. After only 6 weeks of therapy with fenofibrate there was a significant reduction in sE-selectin. The fact that there was no reduction in levels of sVCAM-1 and sICAM-1 was not surprising, as while 6 weeks of fenofibrate therapy may have been long enough to downregulate the expression of the adhesion molecules on the endothelial cells, it may not have had the same impact on the cells within the intima of the atherosclerotic plaques, which is where the majority of VCAM-1 and ICAM-1 is expressed. It appears that while the current state of activation of adhesion molecules on the endothelial cells is best

reflected in the levels of sE-selectin, the overall atherosclerotic burden may be best assessed by measuring the serum levels of sICAM-1 and sVCAM-1. This correlates with clinical studies comparing the serum soluble adhesion molecule levels with the degree of atherosclerosis in an individual. In these studies it is the serum levels of sVCAM-1 and sICAM-1 that are correlated with the degree of atherosclerosis (Peter et al, 1997; Morisaki et al, 1997).

The studies in this thesis have extended the initial work by Cockerill et al on the inhibition of cellular adhesion molecule expression by HDLs (Cockerill et al, 1995). The present studies have shown that all types of native and reconstituted HDLs tested are able to inhibit the cytokine-induced expression of adhesion molecules in HUVECs. The major limitation of the studies described in this thesis is that, while we have shown the inhibition of adhesion molecules by HDLs in vitro, there is no firm proof that HDLs actually inhibit endothelial adhesion molecule expression in vivo. Our strongest indirect evidence that they do inhibit comes from studies with soluble adhesion molecules. These studies, including the preliminary study in this thesis, show that improving the lipoprotein profile by increasing plasma HDLs results in a fall in the soluble adhesion molecules important in the process of atherosclerosis. The level of these soluble adhesion molecules in the serum are thought to approximate the level of expression of membrane bound adhesion molecules. Hence if a more favourable plasma lipid profile in subjects results in lower soluble adhesion molecule levels, then it would follow that membrane bound adhesion molecule levels would also be decreased.

While in vivo studies to assess the direct effect of altering plasma HDL levels on the level of expression of membrane bound adhesion molecule are needed, they would be difficult in human studies, as short of infusing HDLs over a prolonged period of time, it is not possible to raise the plasma HDL in isolation without causing significant changes to the levels of the other lipoproteins. The usual methods to increase HDL-C in humans such as drug therapy and life-style factors (diet, alcohol and exercise) will also cause a reduction in plasma triglycerides and often a reduction in plasma LDL-C. Thus the reduction in the serum level of soluble adhesion molecules seen in studies after lipid lowering therapy is probably multifactorial and is related to a shift in the balance of the regulators of cellular adhesion molecule expression. Lipid lowering therapies give rise to an increase in the plasma HDLs and a reduction in the plasma LDLs. This may cause a shift in the balance of stimulatory and inhibitory influences of adhesion molecule expression. The net result maybe inhibition of cellular adhesion molecule expression with a subsequent fall in levels of the serum soluble adhesion molecules.

One method of assessing the effect of an isolated increase in the plasma HDLs on serum soluble adhesion molecules in an in vivo model would be to compare the serum soluble adhesion molecule levels in transgenic mice that overexpress apoA-I (and hence have high plasma HDL-C) with control mice that have normal levels of HDL-C. An early reduction in serum soluble adhesion molecules in the over-expressing apoA-I group could be attributable to the high plasma HDLs inhibiting the expression of adhesion molecules on the endothelium thus resulting in lower serum soluble adhesion molecule levels (this would be in vivo evidence for our in

vitro observations that HDLs inhibit adhesion molecule expression in HUVECs). On the other hand, if the high plasma HDLs reduced the overall level of atherosclerosis through other mechanisms not related to adhesion molecules, it would take a longer period for the reduction in the serum soluble adhesion molecule levels to occur as their reduction would occur only after reduction in size of the atherosclerotic plaques.

In conclusion, this thesis has investigated the effects of various types of HDL particles and differing components of the HDL particle on the inhibition of the expression of cellular adhesion molecules in endothelial cells. In doing so a possible model to explain how HDLs inhibit cellular adhesion molecule expression has been developed which involves apolipoproteins binding to a cellular receptor which allows phospholipids from the HDLs outer polar membrane to activate a signaling pathway within the endothelial cell that results in the decreased expression of cytokine-induced cellular adhesion molecules. The investigation of this signaling mechanism could be the subject of future studies.

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