INSIGHTS INTO REGULATION OF LIPOPROTEIN RECEPTORS IN HUMAN MESANGIAL CELLS: THE EFFECTS OF INFLAMMATORY CYTOKINES AND CALCIUM CHANNEL BLOCKERS

Thesis submitted for the degree of Doctor of Philosophy

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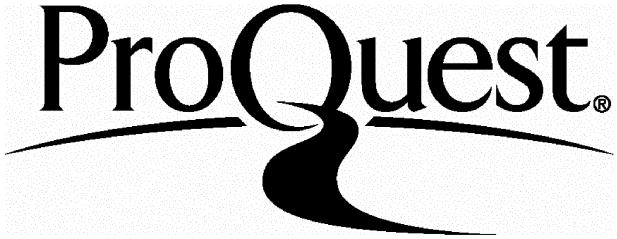
August, 2000



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ABSTRACT

The involvement of abnormal lipid metabolism in the progression of renal disease and the pathogenesis of chronic graft dysfunction is generally accepted. The dysregulation of lipoprotein homeostasis is involved in the incidence of accelerated cardiovascular disease in this population. Chronic renal dysfunction is also an inflammatory condition. Therefore, the present study was undertaken to investigate various mechanisms involved in the receptor-mediated regulation of intracellular lipoprotein transport and its interactions with inflammatory cytokines.

Using human mesangial cell line (HMCL) culture, we demonstrated that HMCL express native LDL receptors . Phorbol 12-myristate 13-acetate (PMA), Angiotensin II (Ang II), TNF-α, and IL-1β induced acetylated-LDL internalisation, scavenger receptor mRNA expression and promoter activity. Both AP-1 and ets motifs were specific response elements to PMA-induced scavenger receptor expression.

Conventionally, LDL receptor pathway is not thought to be involved in foam cells formation due to the tight metabolic control through a feedback regulation. However, our studies demonstrated that TNF-α, TGF-β, PDGF, or IL-1β increased LDL binding, LDL receptor mRNA expression and LDL receptor promoter activity. Both TNF-α, and IL-1β overrode the suppression of LDL receptor activity caused by a high concentration of native LDL and caused foam cell formation in HMCL. TNF-α, and IL-1β also increased the expression of a cleavage activating protein (SCAP) for sterol regulatory element binding proteins (SREBP). Diltiazem and verapamil, not nifedipine increased LDL binding, LDL receptor mRNA expression and LDL receptor promoter activity, but they were not able to override sterol-induced inhibition.

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Our results suggest that inflammatory cytokines may contribute to lipid deposition and foam cell formation in HMC through following pathways: 1) inducing scavenger receptor expression; 2) disregulating LDL receptor gene expression by increasing sterol-independent and mitogenesis-independent gene transcription. The implications of these findings are that inflammatory cytokines are important risk factors for glomerular atherosclerosis. Therefore, future strategies for controlling progression of renal and cardiovascular diseases should include anti-oxidants, lipid-lowering, and anti-inflammatory drugs.

Acknowledgements

The experimental studies described in this thesis were carried out at the Renal Research Laboratory, Royal Free and University College Medical School. The work was conducted by the author and was supervised by Dr. Zac Varghese and Professor John F. Moorhead. I am also grateful to Professor Stephen H. Powis for his kind help and support during the execution of this work.

I thank Dr. Christopher K. Glass (University of California, San Diego) for his kind gift of the human scavenger receptor promoter, Professor David W. Russell (Department of Molecular Genetics, University of Texas) for his kind gift of the human LDLr promoter, and Professor J. D. Sraer (Hopital Tenon, Paris) for providing the immortalised human mesangial cell line.

Finally I am indebted to Professor Moorhead and Dr. Varghese for the financial support, advice and encouragement provided while I was conducting my experimental work and preparing this thesis.

PUBLICATIONS

1. **Ruan XZ**, Varghese Z, Powis SH, Moorhead JF Human mesangial cell express inducible macrophage scavenger receptor. *Kidney Int 56:440-451, 1999*

2. **Ruan XZ**, Varghese Z, Powis SH, Moorhead JF Human mesangial cell express inducible macrophage scavenger receptor: an Ap-1 and ets mediated response. *Kidney Int 56. Suppl. S163-S166, 1999.*

3. **Ruan XZ**, Varghese Z, Fernando R, Moorhead JF. Cytokines regulate LDL receptor gene transcription in human mesangial cells. *Nephrol Dial Transplant* 13:1391-1397,1998

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6. **Ruan XZ**, Kang ZQ, Li XW, Zheng FL. Effects of LDL on mesangial cells proliferation and mesangial extracellular matrix expansion. *Chin J Nephrol 12(2):89-92,1996.*

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8. Varghese Z, **Ruan XZ**, Fernando R, Moorhead JF. The effects of 1,25-dihydroxyvitamin D_3 on inhibition of growth of human mesangial cells (abstract). *(Presented in International Congress of Nephrology, Sydney 1997).*

9.**Ruan XZ**, Kang ZQ, Li XW, Zheng FL. Minimally oxidized low density lipoprotein stimulates the secretion of tumor necrosis factor of mesangial cells (abstract). *Kidney Int 48. Suppl.595 1995.*

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LIST OF ABBREVIATIONS

ACAT	**Acyl Co A:Cholesterol acyl transferase**
Ac-LDL	**Acetylated LDL**
Ang II	**Angiotensin II**
Apo	**Apolipoprotein**
AP-1	**Activator protein-1**
A-SAA	**Acute-phase serum amyloid A proteins**
bFGF	**Basic fibroblast growth factor**
BHT	**Butylated hydroxytoluene**
CBP	**CREBP binding protein**
CE	**Cholesterol ester;**
CCBs	**Calcium channel blocks**
CETP	**Cholesterol ester transfer protein**
CHO	**Chinese hamster ovary**
CIAP	**Calf intestinal phosphatase**
CM	**Chylomicrons**
CREBP	**cAMP response element-binding protein**
CRF	**Chronic renal failure**
CyA	**Cyclosporin A**
DiI-Ac-LDL	**Ac-LDL labelled with 1,1'-dioctadecy-3,3,3',3',-tetramethylindocarbocyanine**
EDTA	**Ethylene diamine tetra acetic acid**
EGF	**Epidermal growth factor**
ESRD	**End-stage renal disease**

ET-1	**Endothelin-1**
FACS	**Fluorescence-activated cell sorter analysis**
FSGS	**Focal and segmental glomerulosclerosis**
FSH	**Follicle-Stimulating hormone**
GAPDH	**Glyceraldehyde phosphate dehydrogenase**
GM-CSF	**Granulocyte monocyte colony stimulating factor**
HDL	**High density lipoprotein**
Hep	**Heparin**
HMCL	**Human mesangial cell line cells**
HMCL-Scr	**Human mesangial cell line transfected by human scavenger receptor full cDNA**
HMG CoA	**3-Hydroxy-3-methyl glutaryl-Coenzyme A**
hsDNA	**Herring sperm DNA**
IL-1β	**Interleukin-1β**
IGF	**Insulin-like growth factor**
LDL	**Low density lipoprotein**
LCAT	**Lecithin:cholesterol acyl transferase**
Lp(a),	**Lipoprotein (a)**
LPL	**Lipoprotein lipase**
LRP	**LDL receptor-related protein**
LOX-1	**Lectin like Ox-LDL receptor**
MFI	**Mean fluorescence intensity**
M-CSF	**Monocyte colony stimulating factor**
MCP-1	**Monocyte chemotactic protein**

mOx-LDL	**Minimally oxidised LDL**
m-CSF	**Monocyte colony stimulating factor**
Ox-LDL	**Oxidised low-density lipoprotein**
P300:	**adenovirus E1A- associated protein**
PBS	**Phosphate buffered saline**
PDGF	**Platelet derived growth factor**
PGL3SCR	**Scavenger receptor promoter reporter gene**
PGE	**Prostaglandin E**
pmp	**Per million population**
PMA	**Phorbol 12-myristate 13-acetate**
Poly I	**Polyinosinic acid**
PRD	**Progression of renal disease**
PPAR	**Peroxisome proliferator activated receptor**
ROS	**Reactive oxygen species**
RRT	**Renal replacement therapy**
SCAP	**SREBP cleavage-activating protein**
Scr	**Scavenger receptors class A**
SDS-PAGE	**Sodium dodecyl sulphate-polyacrylamide Gel electrophoresis**
ssDNA	**Single strand DNA**
STAT	**Signal transducer and activator of transcription**
SRE-1	**Sterol regulatory element-1**
SMC	**Smooth muscle cells**
SRB-1	**Scavenger receptor class B-1**

TG	**Triglyceride**
TPA:	**12-O-Tetradecanoyl-phorbol 13- acetate**
TNF-α	**Tumour necrosis factor-α**
TGF-β	**Transforming growth factor-β**
TBARS	**Thiobarbituric acid reactive substances**
VLDL	**Very low density lipoprotein**
WHHL	**Watanabe heritable hyperlipidaemic rabbit**

CHAPTER 1- GENERAL INTRODUCTION

The incidence and prevalence of end-stage renal disease (ESRD) are increasing world-wide. Information available for the European Union from ERA-EDTA Registry for 1995 indicated that the stock of patients receiving renal replacement therapy (RRT) was 644 per million population (pmp), with nearly 250,000 patients receiving treatment in the European Union population of 373 million, with a mean expansion rate of about 8.2 % per year (Berthoux et al. 1999). The RRT population of the United States is approximately 300,000 against a background of 2 million patients with chronic renal disease. The mean incidence of new patients starting RRT in Europe is 120 pmp and the mean incidence of death among patients receiving RRT is 67 pmp (Berthoux et al. 1999). Considerable public health concern arises from the high mortality rate and impaired quality of life of RRT patients. In the United Kingdom RRT consumes nearly 2% of the NHS budget at a projected cost of £25,000 per patient per annum. Therefore, one of the major strategies of nephrology today is to prevent the progression of renal disease (PRD) and extend the time to reach ESRD and place patients on RRT.

In many patients decline in renal function occurs at a constant rate and may continue despite the remission of the pathological processes which initiated kidney damage. Studies over the last two decades have identified a large number of risk factors, thought to be important in contributing to the rate of progression of chronic renal disease and this has led to the formulation of several ideas which emphasise one contributory factor or another. However, a unifying hypothesis is emerging which indicates that ESRD progression occurs through a primary renal insult that irreversibly damages a significant number of nephrons. In response to this decline in function, surviving nephrons undergo a variety of compensatory adaptations such as

glomerular hyperfiltration and hypertrophy (Hostetter, 1995). In the short term, compensatory adaptations tend to normalise biochemical parameters of renal function. However, other changes such as endothelial injury, tubulo-interstitial damage, hypertension, diabetes, hyperlipidaemia, platelet activation, oxidative stress and proteinuria may cause long-term damage. In the long-term, factors contributing to compensatory changes in the glomerulus and tubulo-interstitial damage may feed on each other to create further glomerular and tubulo-interstitial damage. El Nahas has postulated the involvement of two pathways in the pathogenesis of glomerulosclerosis: an intrinsic pathway involving infiltrating and resident glomerular cells and a second extrinsic pathway involving the trans-differentiation of tubular epithelial cells to myofibroblasts causing tubulo-interstitial damage (El Nahas, 1996). From all these different ideas a consensus has emerged which suggests that over a period of adaptive changes, glomerulosclerosis and tubular atrophy reduce nephron number, fuelling a self-perpetuating cycle of nephron destruction culminating in fibrosis, scarring and uraemia.

Abnormalities of lipid metabolism are seen in a variety of renal diseases, particularly in those associated with nephrotic range of proteinuria. These lipid abnormalities persist during all stages of renal disease and during RRT, including renal transplantation, although the pattern of dyslipidaemia changes when patients move from one modality of RRT to another (Chan et al. 1982). Cardiovascular disease is usually well established by the time RRT starts, but is the most important cause of death at all stages of PRD, accounting for approximately 50% of the mortality among patients on both dialysis and allograft recipients (Raine et al. 1992). The prevalence of left ventricular hypertrophy, coronary artery disease, and congestive heart failure in

patients with ESRD is in far excess of that in control population (Spencer, 1980; Foley and Parfrey, 1998; Kasiske, 1998; Harris and Brown, 1998). As a result of the magnitude of these cardiovascular complications, there is much interest in risk factors associated with the development of atherosclerosis in progressive renal disease. Hyperlipidaemia is now well established as an important risk factor for coronary artery disease and the therapeutic value of normalising plasma lipids by clinical intervention has been demonstrated (Tyroler, 1987; Anonymous, 1985).

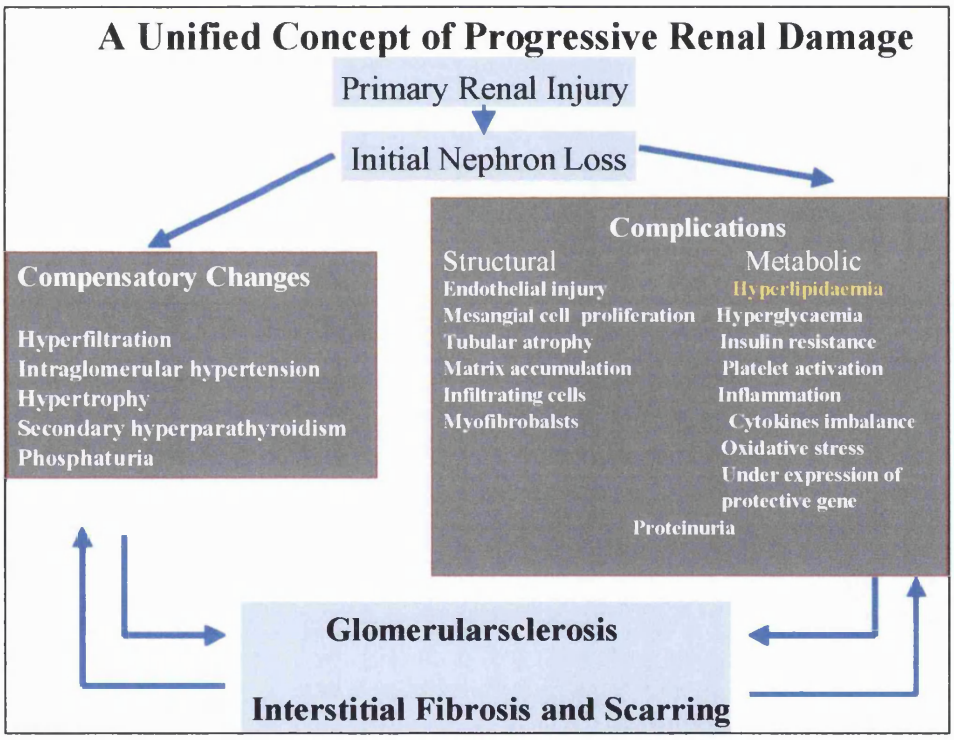


Fig 1.1. A unified concept of progressive renal damage

Atherosclerotic renal artery stenosis is a contributory factor for ESRD and is particularly significant in the elderly group of patients starting RRT (Scoble et al. 1989). However, only limited studies directed at the treatment of the lipid abnormalities of chronic renal failure patients are available, and major efforts are needed in controlling hyperlipidaemia in this vulnerable group of patients. It is logical to pose the question: is hyperlipidaemia the cause or the consequence of renal diseases? Before the publication of the lipid nephrotoxicity hypothesis of Moorhead and colleagues in 1982 (Moorhead et al. 1982), it was generally considered that abnormal lipid metabolism was a consequence of renal disease. They pointed out that in some circumstances, circulating lipoproteins may directly damage glomerular structures and suggested that hyperlipidaemia is an aggravating factor in the progression of initial glomerular injury to glomerulosclerosis. A large number of animal experiments, cell culture and clinical studies have suggested an association between abnormal lipid metabolism and PRD. In both the remnant kidney model and in obese Zucker rats, lowering lipids with drugs lessens injury.

In the rest of this introductory chapter 1 will briefly discuss normal lipoprotein metabolism, lipoprotein abnormalities in renal diseases, involvement of lipids in PRD, and the pathogenesis of lipoprotein-induced glomerulosclerosis. Finally the specific purpose of the project will be discussed.

1.1. NORMAL LIPOPROTEIN METABOLISM

Cholesterol, cholesterol esters and triglyceride are virtually insoluble in aqueous environments and are transported in plasma in association with proteins in

macromolecular complexes called lipoproteins. Five major classes of lipoproteins are recognised; chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL), Lipoprotein (a) (Lp(a)), and high density lipoprotein (HDL). These five classes of lipoproteins are heterogeneous in terms of their size, lipid and apoprotein content and can be fractionated by using techniques such as electrophoresis, gradient ultracentrifugation and affinity chromatography (Table 1.1). Typical values for the major composition of the five classes of lipoproteins are shown in Table 1.2 and the apo content is described in Table 1.3. (Illingworth, 1993). The main triglyceride-carrying lipoproteins are chylomicrons and VLDL. Chylomicrons are not normally present in blood after a 12 h fasting. In the fasting state VLDL account for approximately 60% of the total plasma triglyceride (Powell et al. 1987; Young, 1990; Illingworth, 1993). Lp(a) is an LDL-like particle in which apo-B100 is linked to another apoprotein-apo(a) (Kronenberg et al. 1996).

1.1.1. Lipoproteins, lipolytic enzymes and transfer proteins.

Lipids, being insoluble in aqueous solution, are transported in plasma in association with specialised proteins. Lipoproteins are composed of an inner core of non-polar neutral lipids (esterified cholesterol and triglyceride) surrounded by an outer coating of polar molecules (phospholipids, free cholesterol and apoproteins) (Fig.1.2). They are synthesised in the liver and intestine and transport dietary and endogenously synthesised lipids in the circulation.

Lipid synthesis and assembly into lipoproteins, and the transport, storage and metabolism of lipoproteins are regulated by apoproteins, lipid modifying enzymes,

cholesterol ester transfer protein and membrane receptors. All regulatory mechanisms are potential sites for generating imbalances between synthesis and catabolism, thereby giving rise to a variety of dyslipidaemia. The genes for most apoprotein and membrane receptors have been isolated, sequenced and mapped in the human genome.

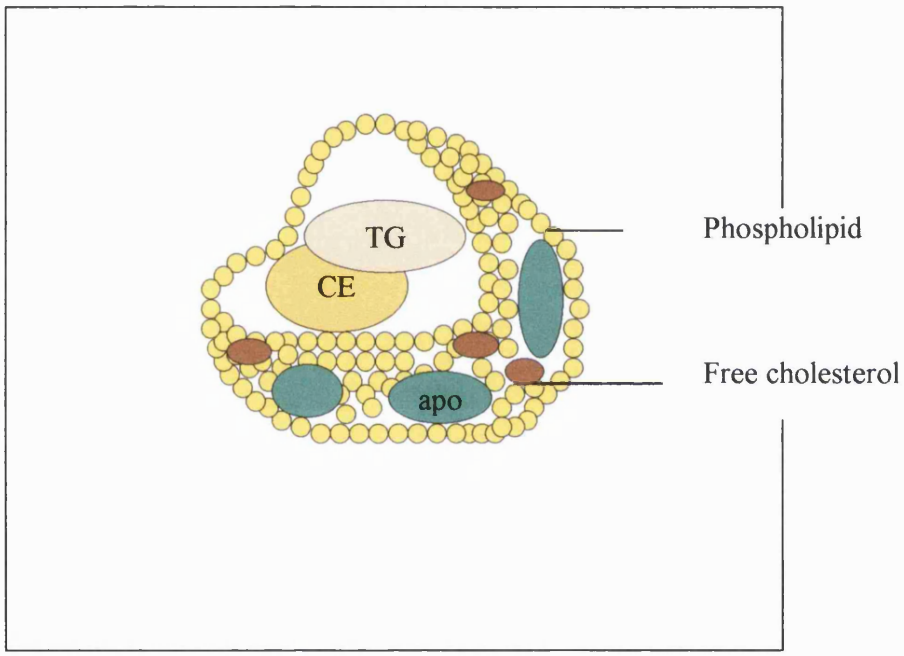


Fig.1.2. The structure of lipoprotein. TG=triglyceride; CE=cholesterol ester; apo=apolipoprotein

Chylomicrons (CM) are rich in triglycerides derived from food. They are synthesised in small intestine, then secreted into the lymphatics and subsequently enter the circulation. They become emulsified through the addition of apo-B48, apo-AI, apo-AII and apo-AIV, then acquired apo-CI, -CII, -CIII and apo-E from HDL. They are available for early metabolism at the endothelial surface where 80% of triglycerides in

chylomicrons are removed by the action of lipoprotein lipase (LPL). Apo-CII activates LPL and apo-CIII inhibits this enzyme. Some apoproteins namely apo-AI and apo-AII are transferred to HDLs during this delipidation process and the partially delipidated chylomicrons are known as chylomicron remnants. The chylomicron-mediated pathway represents exogenous lipoprotein metabolism (Windler and Havel, 1985).

VLDL also is triglyceride-rich lipoprotein which synthesised in the liver. The delipidation pathway of VLDL is similar to that of chylomicrons in that LPL on the endothelial surface concerts VLDL to IDL. About 50% of IDL are removed directly by the liver and remaining fraction is further delipidated by hepatic lipase and converted to LDL (Young, 1990). During the delipidation process of VLDL, surface phospholipids and apoproteins are transferred to HDL as they are from chylomicrons. Cholesteryl ester, which is formed in the plasma as a result of the action of lecithin:cholesterol acyl transferase (LCAT) on the HDL particle, is transferred from HDL to VLDL through the mediation of cholesterol ester transfer protein (CETP).

LDL is the major lipoprotein fraction of fasting plasma and it carries approximately 75% of the total cholesterol in plasma. A single molecule of apo-B100 is the only apoprotein present in LDL. In normal situation, there is a substrate product relationship between VLDL and LDL and almost all LDL apo-B can be accounted for as a product of VLDL metabolism.

HDL particles are synthesised and secreted by the liver and the small intestine. Newly secreted particles appear as lamellar discs made up of phospholipid, free cholesterol,

apo-A1 and apo-E. This is an ideal substrate for LCAT which converts free cholesterol, acquired from extrahepatic tissue and other lipoproteins, into cholesteryl esters. During this process HDL becomes a spherical particle having cholesteryl ester as its core lipid. HDL particles undergo a series of remodelling processes in the plasma. In normal human plasma, the major species are the smaller HDL3 and the larger HDL2. The continued LCAT activity in HDL3 converts these particles to cholesteryl ester rich HDL2. This mature HDL2 is necessary for the transport of the lipoprotein lipase activator apo-CII to nascent chylomicrons and VLDL. CETP can exchange cholesteryl ester from HDL for triglycerides in VLDL and IDL. Hepatic lipase can act on HDL triglycerides and convert HDL2 to HDL3. Thus during the hydrolysis of chylomicrons and VLDL, excess surface components are transferred to HDL2 and this increases the size of these particles. In addition, VLDL triglycerides can exchange with cholesteryl esters in the presence of CETP and the hydrolysis of resulting HDL2 triglycerides by hepatic lipase can cause a decrease in HDL2 size and convert HDL2 to HDL3. Therefore, HDL is a key player in modulating the delipidation cascade of chylomicrons, VLDL and IDL, and also provides a medium for the reverse transport of cholesterol from extrahepatic tissue to the liver.

1.1.2. Lipoprotein receptors

The removal of lipoprotein from the circulation occurs largely by a receptor-mediated process in the liver and extrahepatic tissues. Evidence for the existence of cell surface receptors for apo-B was first provided by Goldstein and Brown in studies of human skin fibroblasts (Brown and Goldstein, 1975). Over last two decades, a number of lipoprotein receptors have been identified. These receptors include LDL receptor, scavenger receptor(s), HDL receptor, VLDL receptor, and LDL receptor-related

protein/alpha2-macroglobulin receptor (LRP). As the present study is designed to investigate involvement of lipoprotein receptors in lipid accumulation in the progression of renal dysfunction using HMC in culture, a brief description of current knowledge of lipoprotein receptors is therefore apposite.

Table 1.1. The five major density classes of lipoproteins

	Density	Sources	electrophoretic	Mean diameter (nm)
Chylomicrons	<0.95	Intestine	origin	500
VLDL	<1.006	liver	Pre-β	43
IDL	1.006-1.019	Catabolism of VLDL and chylomicrons	Broad-β	27
LDL	1.019-1.063	Catabolism of VLDL	β	22
Lp(a)	1.063-1.107			
HDL	1.107-1.21	Catabolism of chylomicrons& VLDL; liver & intestine	α	8

Table 1.2. Composition of human plasma lipoproteins.

(% of total dry weight of the lipoprotein)

	Chylomicrons	VLDL	LDL	Lp(a)	HDL2	HDL3
Protein	2-4	8-12	20-25	26-36	42-45	50-55
Phospholipid	8-10	16-20	20-24	22-24	25-30	22-25
Free cholesterol	1-3	5-10	7-10	6-10	5	3-4
Cholesterol ester	2-4	10-15	35-40	26-38	12-18	10-12
Triglyceride	85-90	50-65	6-10	4-9	4-6	3-5
MW	$0.4x10^9$	$5\text{-}10x10^6$	$2.8x10^6$	$3\text{-}8x10^6$	$3.6x10^5$	$1.8x10^5$

Table 1.3. Apoprotein content of human plasma lipoproteins

Chylomicrons	VLDL	LDL	Lp (a)	HDL
Major apoproteins				
Apo B-48	Apo B-100	Apo B-100	Apo B-100	
Apo C-I	Apo C-I		Apo (a)	
Apo C-II	Apo C-II			
Apo C-III	Apo C-III			
Apo E	Apo E			
Minor apoproteins				
Apo A-I	Apo D			Apo C-I
Apo A-II				Apo C-II
Apo A-IV				Apo C-III
Apo D				Apo E

Table 1.4. Function of Apoproteins

Apoprotein	lipoprotein	Function
A-1	CM, VLDL, HDL	Lecithin cholesterol acyltransferase (LCAT) activator
AII	CM, VLDL, HDL	?
A-IV	CM, VLDL, HDL	LCAT activator
B48	CM	Structural component of CM
B100	VLDL, IDL, LDL	Structural component of LDL Ligand for LDL receptor
C-I	VLDL,CM, IDL, HDL	LCAT activator
C-II	CM, VLDL	Lipoprotein lipase (LPL) activator
C-III	CM, VLDL, IDL, HDL	Inhibitor of LPL
E	CM, VLDL, IDL, HDL	Ligand for LDL receptor

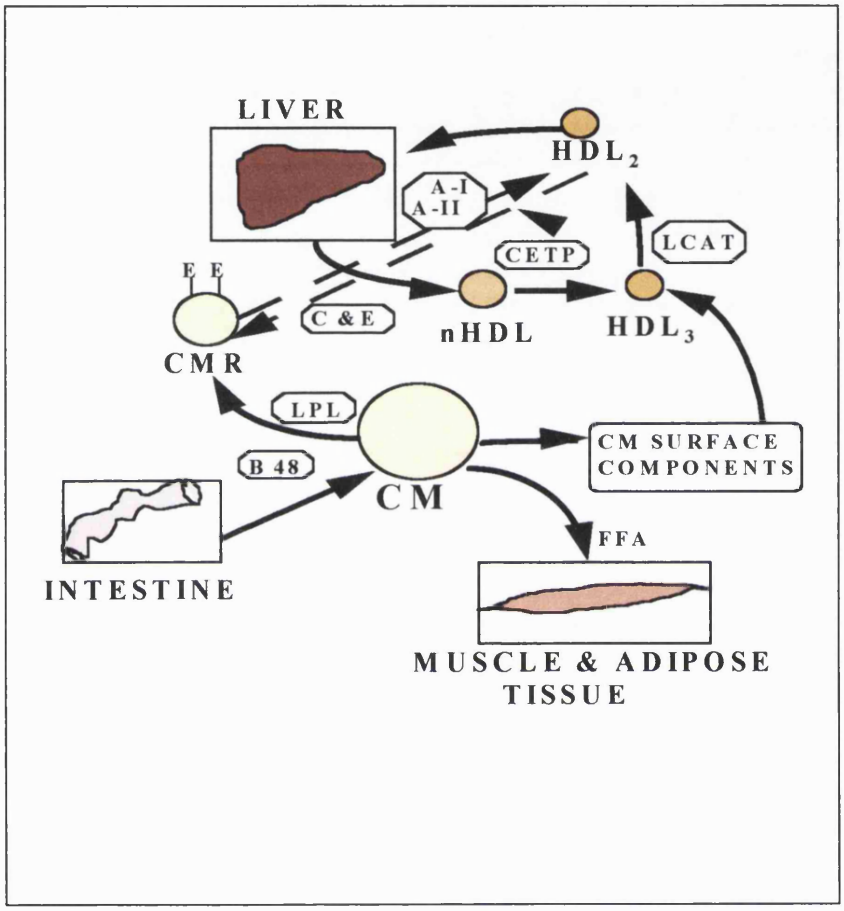


Figure 1.3. Chylomicron metabolism and HDL chylomicron interactions

HDL2 = HDL with a density between 1.063 g/ml and 1.125 g/ml; HDL3 = HDL with a density between 1.125 and 1.21 g/ml; FFA = free fatty acids, LPL= lipoprotein lipase; CETP = cholesterol ester transfer protein; LCAT= Lecithin cholesterol-acyltransferase

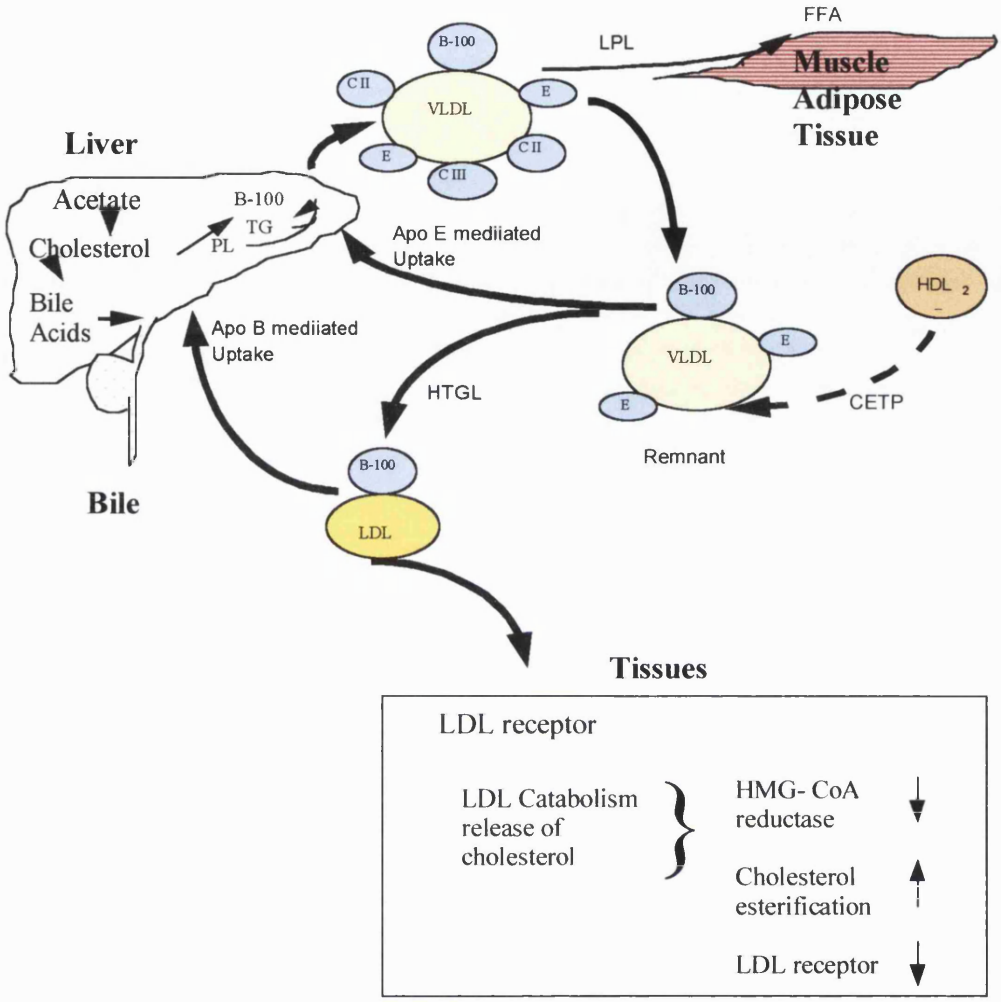


Figure 1.4. VLDL and LDL metabolic pathways and the consequences of receptor mediated LDL uptake

FFA=free fatty acids; LPL=lipoprotein lipase; HDL2=HDL with a density between 1.063 and 1.125 g/ml; HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A; PL=phospholipid; TG=triglyceride; HTGL=Hepatic triglyceride lipase

1.1.2.1 The LDL receptor

The LDL receptor is the primary receptor for binding and internalising plasma-derived LDL-cholesterol and regulates plasma LDL (Goldstein and Brown, 1985; Brown and Goldstein, 1986a). In normal lipidaemic subjects 60-80% of LDL is removed through the LDL receptor pathway. Defect of LDL receptor in familial hypercholesterolaemia (FH) patients leads to raise level of plasma cholesterol and atherosclerosis. The transport of macromolecules into cells by receptor-mediated endocytosis first emerged as a distinct mechanism following studies carried out on human fibroblasts by Brown and Goldstein (Brown and Goldstein, 1975). On exposure of human fibroblasts to high concentrations of LDL, cellular endogenous cholesterol synthesis was decreased while the intracellular content of cholesterol remained largely unchanged. Following further biochemical studies it emerged that in mammals, the delivery of LDL-derived cholesterol into hepatic and extra-hepatic cells was mediated by a specific cell surface receptor known as the apo B/E receptor. The sequential process of receptor-mediated endocytosis of LDL and the subsequent regulation of cellular synthesis of cholesterol has been termed the LDL receptor pathway. This important receptor pathway is represented schematically in fig.1.5. It depicts the events that are involved in the receptor-mediated endocytosis of LDL, which allows cells to control their intracellular cholesterol content and establish intracellular cholesterol homeostasis.

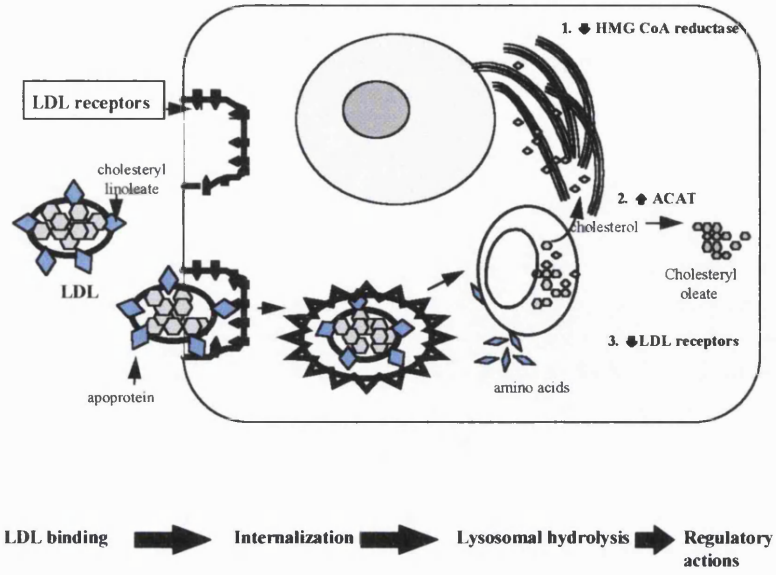


Fig.1.5. The LDL receptor pathway. See text for details of the pathway.

The LDL receptor gene locus is located on the distal short arm of chromosome 19 and spans about 45 kb of DNA. The gene consists of 18 exons which are separated by 17 introns (Sudhof et al. 1985). In LDL receptor 5'-flanking region contains three imperfect 16-base pair repeats and a TATA box reside. The first and third of these repeats bind the positive transcription factor SP-1. The second repeat is crucial in maintaining sterol-mediated repression of LDL receptor transcription and contains the octameric sequence, designated the sterol regulatory element-1 (SRE-1) (Sudhof et al. 1987; Chang et al. 1996; Kawabe et al. 1994). These three repeats are located in close proximity in a region approximately 150 base pairs upstream from the major transcription initiation sites between positions -93 and -79. There is a strong correlation between the proposed structural arrangement of the receptor protein and the sequence of exons on its gene. The first exon encodes the non-translated signal sequence and exons 2-6 code the 7 cysteine-rich repeats of the LDL binding domain (Sudhof et al. 1985; Schneider, 1989). The next 8 exons code for the EGF precursor homology domain and the third receptor domain (O-linked sugar cluster) is translated from a single exon found

between introns 14 and 15. The membrane spanning and cytoplasmic domains are encoded by 2 exons, and the 18th exon is translated into the carboxyl end amino acids of the receptor protein and also contains a 2.5 kb non-translated stretch of mRNA (Yamamoto et al. 1984; Schmid and Jelinek, 1982).

The structure of the LDL receptor from four species (human, rabbit, bovine and hamster) has been well characterised (Schneider et al. 1982) (Fig.1.6.). It is a highly conserved integral membrane glycoprotein with five main domains. These domains listed in order of the appearance from the amino terminus of the protein are: 1) The LDL binding domain, 2) a domain which has a strong homology to the epidermal growth factor (EGF) precursor, 3) a domain in which there is a cluster of o-linked carbohydrate chains, 4) a transmembrane domain and 5) a short region that extends into the cytoplasm (Schneider, 1989).

A detailed description of LDL receptor regulation is given in Chapter 4.

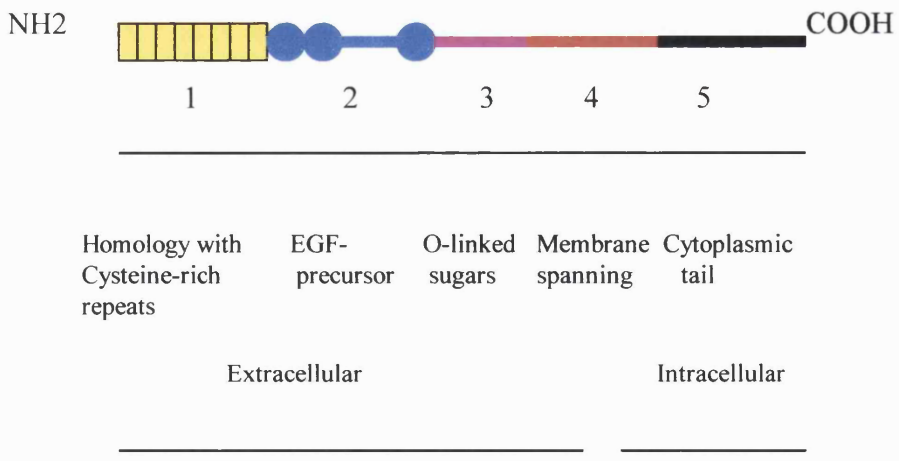


Fig.1.6. The structure of the LDL receptor protein

1.1.2.2 Scavenger receptors class A

Brown and Goldstein found that cholesterol uptake by the LDL receptor pathway did not lead to massive accumulation of cholesterol in cells because the uptake was tightly coupled to the concentration of intracellular cholesterol. They noticed however, when LDL was chemically modified, macrophages in culture were able to accumulate large amounts of lipid and convert into cholesterol ester droplet-filled cells. These cells show a striking morphological similarity to foam cells found in atherosclerotic plaques (Goldstein et al. 1983; Brown and Goldstein, 1983; Brown and Goldstein, 1986a; Goldstein and Brown, 1977). These receptors were first termed acetyl LDL receptors but are now known as macrophage scavenger receptors because of their multi-ligand binding capacity.

Scavenger receptor class A cDNA has been cloned in bovine, mouse, rabbit and human. Human scavenger receptor genomic DNA is located on chromosome 8. Two mRNA, 4.0 and 3.2 kb, have been detected in human liver, placenta, and brain (Matsumoto et al. 1990). The two isoforms of human scavenger receptor class A, SR-AI and SR-AII are produced by alternative splicing of a message encoded by a single gene located on chromosome 8 in humans (Kodama et al. 1990; Naito et al. 1992; Matsumoto et al. 1990).

Analysis of the ligand-binding properties of scavenger receptor activities on macrophages and endothelial cells lead to the suggestion that there are multiple classes of scavenger receptors. Scavenger receptor class A was identified as the first family in 1990 (Kodama et al. 1990; Rohrer et al. 1990). Two types of scavenger receptors class A (SR-AI and SR-AII) have been characterised (Fig.1.7) (Naito et al.

1991; Matsumoto et al. 1990). Although their normal physiological role remains uncertain, biochemical studies have demonstrated that both isoforms of scavenger receptor class A are capable of binding and internalising acetylated LDL (Ac-LDL) and oxidised LDL (Ox-LDL) (Kodama et al. 1990; Goldstein et al. 1979; Freeman et al. 1991; Steinbrecher et al. 1989; Parthasarathy et al. 1986). Unlike LDL receptor, the activity of scavenger receptor is not suppressed by rising intracellular cholesterol concentrations, thus providing a mechanism for unregulated cholesterol uptake. The massive accumulation of cholesterol in foam cells present in atherosclerotic plaques is thought to involve scavenger receptors class A. Several lines of evidence support this view: firstly, macrophage-like cells, and CHO cells transfected with scavenger class A receptor accumulate modified LDL and become lipid-laden foam cells in vitro (Freeman et al. 1991); secondly, scavenger receptor class A mRNA is expressed and the ligand for scavenger receptor class A (Ox-LDL) is present in atherosclerotic plaques (Hiltunen et al. 1998; Hiltunen and Yla-Herttuala, 1998); finally, the antioxidant drug probucol inhibits formation of atherosclerotic plaques in animal models of atherosclerosis (Donetti et al. 1998; Braesen et al. 1995).

Type I receptors made up of 451-454 amino-acids with an elongated homotrimeric integral membrane protein structure. This protein is organised as 6 distinct domains (Fig 1.7.).

I: The N-terminal cytoplasmic domain [amino acid (aa) residues 1-50].

II: A single transmembrane domain per chain (aa 51-76).

III: A spacer region (aa 77-150).

IV: An a-helical coiled coil domain composed of three helices, each consisting of a series of up to 16, seven amino acid repeats known as heptads (aa 151-271).

V: A second coiled-coil domain composed of a right handed, collagenous triple helix containing 23 or 24 uninterrupted Gly-X-Y triplet repeats (aa 272-343).

VI: A C-terminal cysteine-rich domain (SRCR) which is thought to fold into a globular structure (aa 344-453).

Type II receptors have domains I -V found in type I receptors but lack the cysteine-rich domain VI which is replaced by a truncated C-terminus consisting of 6-17 aa. Although type II receptors lack SRCR they still have a broad ligand-specificity suggesting that this region is not essential to ensure binding of multiple ligands.

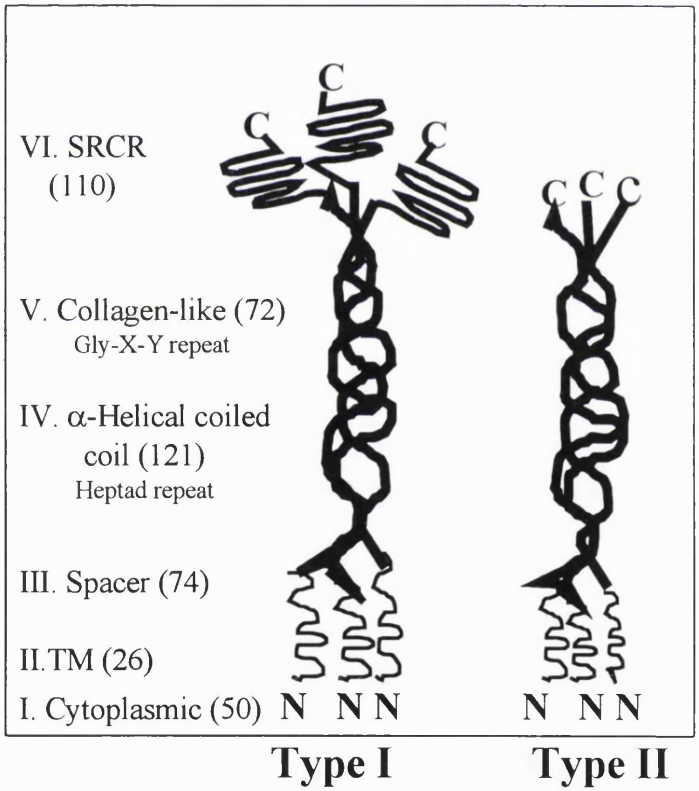


Fig.1.7 Models of the predicted quaternary structure of macrophage scavenger receptors.

Type I and type II receptors consist of 6 domains (see text). Type II receptors share domains I-V but the C-terminal domain VI (the SRCR region) is replaced by a short oligopeptide (6-17 aa residues depending on the species). The aa residues of the bovine type I receptor are indicated in parenthesis.

Initial studies indicated that scavenger receptors class A found on mouse peritoneal macrophages were capable of high affinity binding, internalisation and degradation of ^{125}I-labelled Ac-LDL. A wide variety of compounds were found to be able to

competitively inhibit this binding. Using direct binding assay and competitive inhibition studies a variety of compounds that can bind scavenger receptor class A with a high affinity have been identified (Table.1.5.). Ligands that bind macrophage scavenger receptor class A identified so far are either polyanionic molecules or macromolecular complexes. The regulation of scavenger receptor class A is discussed in chapter 3.

Table 1.5. Macrophage scavenger receptor ligands

Effective competitors	**Ineffective competitors**
Modified proteins Ac-LDL, Ox-LDL, mLDL, M-HDL, M-albumin	**Native and modified proteins** Poly (D-glutamate), Phosvitin, thyroglobulin, orosomucoidin, fetuin, asialoorosomucoidin, lysozyme, acetylated proteins including albumin, g-globulin, a-1-antitrypsin, transferrin, ovalbumin, histones, ovomucoid, a-1-acid glycoprotein, HDL and methylated LDL
Four stranded nucleic acids Polyinosinic acid (poly I), poly G, poly G:I, polyxanthinylic acid, telomere models [$d(G_4T_4)_5$]	**Non-four stranded nucleic acids** poly A, poly C, poly U, single and double stranded DNA
Polysaccharides Dextran sulphate, Fucoidin, carragheenan	Polysaccharides Heparin, chondroitin sulphate A and C, colominic acid (polysialic acid), yeast mannan
Phospholipids Phosphatidylserine	**Phospholipids** Phosphatidylcholine
Others Bovine sulfatides, polyvinyl sulphate, endotoxin, lipoteichoic acid, crocidolite asbestos	**Others** Polyphosphates

1.1.2.3 Scavenger receptors class B

The class B scavenger receptor family is divided into two: CD36 and scavenger receptor class B-1 (SRB-1). Endemann and colleagues reported the identification of the first class B scavenger receptor, CD36 (Endemann et al. 1993). The cell surface protein of CD36 family was shown to bind modified lipoprotein proteins (acetylated LDL, oxidised LDL), but not the broad array of other polyanions which are ligands of the class A receptors (Acton et al. 1994; Endemann et al. 1993). CD36 may play a quantitatively significant role in modified LDL binding to macrophages (Endemann et al. 1993). In addition to binding modified LDL, CD36 binds thrombospondin (Asch et al. 1987), anionic phospholipids (Rigotti et al. 1995), long-chain fatty acids (Abumrad et al. 1993), collagen (Tandon et al. 1989), and plasmodium falciparum-infected erythrocytes. CD36 is expressed in a variety of tissues, including adipocytes, macrophages, epithelial cells, monocytes, endothelial cells, platelets, and a wide variety of cultured lines (Abumrad et al. 1993). CD36 has been reported to be clustered in specialised domains of the plasma membrane (Lisanti et al. 1994). Although the physiological functions of CD36 have not been fully described, it may serve as an adhesion molecule owing to its collagen-binding properties. CD36 may also serve as a receptor on macrophages for damaged or senescent neutrophils (Savill et al. 1992).

Another member of the class B scavenger receptor SR-BI was isolated by expression cloning from a Chinese hamster ovary cell variant Var-261, which expresses a scavenger receptor activity distinct from that scavenger receptor class A (Acton et al. 1994). The protein sequence of SR-BI (509 amino acids) is approximately 30% identical to CD36. SR-BI is a multiligand receptor. Like CD36, SR-BI displayed high

affinity binding for acetylated LDL, oxidised LDL, maleylated bovine serum albumin, but not the broad array of other polyanions (e.g. fucoidin, polyguanosinic acid, carragheenan) which are ligands of the class A receptors. It also binds anionic phospholipid suggesting that SR-BI might be involved in recognising senescent or apoptotic cells (Acton et al. 1994; Rigotti et al. 1997).

Recently SR-BI has been identified as HDL receptor (Acton et al. 1996; Stangl et al. 1998). Cholesteryl ester delivery from HDL to cells through SR-B1 receptor pathway is fundamentally different from that of the LDL receptor pathway, because it does not involve endocytosis and degradation of the entire lipoprotein particle (Krieger, 1999). Instead, HDL binds to the cell surface and transfer cholesteryl esters to the cell and then the lipid-depleted HDL dissociates from the cell surface and re-enters the circulation. This novel receptor exchange mechanism for HDL cholesterol uptake is called selective lipid uptake (Krieger, 1999). Human SR-BI has been mapped to human chromosome 12 (12q24.2-qter) (Acton et al. 1994). The partial and complete genomic structures for the murine and human SR-BI homologues have been reported (Cao et al. 1997). Northern blot analysis of murine tissues showed that SR-BI was most abundantly expressed in adipose tissue and was present at moderate levels in lung and liver. Furthermore, SR-BI mRNA expression was induced upon differentiation of 3T3-L1 cells into adipocytes. Thus, the tissue distribution of expression and ligand binding properties of SR-BI raise the possibility that this cell surface receptor may play an important role in lipid metabolism.

1.1.2.4 Lectin like receptor – a novel endothelial receptor for Ox-LDL

Vascular endothelial cells in culture and in vivo internalise and degrade Ox-LDL through a putative receptor-mediated pathway that does not involve macrophage scavenger receptors. Sawamura reported a molecular cloning of an Ox-LDL receptor from vascular endothelial cells. The cloned receptor is a membrane protein that belongs structurally to the C-type lectin family, and is expressed in vivo in vascular endothelium and vascular-rich organs (Sawamura et al. 1997). Lectin like Ox-LDL receptor (LOX-1) is a receptor for Ox-LDL but not for Ac-LDL. LOX-1 recognizes protein moiety of Ox-LDL, and its ligand specificity is distinct from other receptors for Ox-LDL, including class A and B scavenger receptors (Moriwaki et al. 1998).

1.1.2.5 VLDL receptor

The VLDL receptor has been described as a new member of the LDL receptor supergene family. The predicted human VLDL receptor protein shows approximately 75% sequence homology to LDL receptor proteins (Webb et al. 1994). Isolation and characterisation of cDNAs encoding human very low density lipoprotein (VLDL) receptor revealed the presence of two forms of the receptor: one consists of five domains that resemble the LDL receptor, and a variant form which lacks an O-linked sugar domain (Sakai et al. 1994). The ligands for VLDL receptor are apolipoprotein E-rich lipoproteins such as β-migrating VLDL, IDL, chylomicron remnants, LRP receptor-associated protein (RAP). In human tissues in vivo, the mRNA was expressed predominantly in heart and skeletal muscle, and also in ovary and kidney, but not in the liver (Takahashi et al. 1992). Based on the structural features, ligand specificity, and tissue expression of the mRNAs, VLDL receptor may mediate uptake

of apolipoprotein E-containing lipoproteins enriched with triglyceride in non-hepatic tissues that are active in fatty acid metabolism (Takahashi et al. 1992).

1.1.2.6 The LDL receptor-related protein.

LDL receptor-related protein (LRP) is primarily expressed in the liver, brain and placenta. The cDNA for this receptor was cloned in 1988 using a homology screening approach. The predicted protein structure of this receptor includes many structural motifs found in the LDL receptor. These include clusters of ligand binding (complement-type) domains which contain repeats of cysteine-rich regions, cysteine-rich EGF repeats, EGF-precursor homologous domains (Herz et al. 1988). The main ligands for LRP are thought to be apo E containing lipoprotein "remnants" that are formed from VLDL and chylomicrons by modifications caused by removal or addition of apoproteins by the action of LPL. There is strong evidence however that LRP is also a multiligand receptor for other important ligands such as β-VLDL, lactoferrin, a_2-macroglobulin and complexes of plasminogen activator/inhibitor (Krieger and Herz, 1994).

1.1.2.7. Lipoprotein receptor in mesangial cells

Lipoprotein receptors have variable degree of expression in different tissues and cell types. Its expression, centrally, in the hepatic tissue is important in the modulation of the concentration of plasma lipids. However, the expression of various lipoprotein receptors and their physiological functions in the peripheral tissue has not been

completely elucidated. Our group have previously showed that human and rat mesangial cells have LDL receptors (Wheeler et al. 1991a; Wheeler et al. 1990b). Furthermore we showed that rat mesangial cells have the ability to oxidise native LDL and they have receptors for modified LDL (Fernando et al. 1993). Wanner and Anami demonstrated that human mesangial cells (HMC) express VLDL receptors (Anami et al. 1997; Quaschning et al. 1997). The evaluation of the expression and regulation of these receptors on peripheral tissues may help in the understanding of the influence of lipid-mediated injury.

1.2. LIPOPROTEIN ABNORMALITIES FOUND IN RENAL DISEASE

1.2.1 Nephrotic syndrome

The nephrotic syndrome is characterised by proteinuria of greater than 3.5 g/day, hypoproteinaemia, oedema, and hyperlipidaemia and is a result of an alteration in glomerular filtration barrier selectivity and permeability (Cameron, 1987). The nephrotic plasma lipid profile is characterised by an increase in plasma cholesterol concentration and an elevated plasma triglyceride levels, particularly in patients with heavy proteinuria (>10 g/day). This characteristic profile is a result of an increase in LDL, VLDL and/or IDL particles (Joven et al. 1990) and a decreased or unchanged level of the HDL fraction (Joven et al. 1990; Kaysen, 1991). There is also evidence that the relative levels of HDL subtypes also undergo change in the nephrotic patient (Muls et al. 1985). The HDL_3:HDL_2 ratio increases due to a small elevation in HDL_3 levels and a more dramatic decrease in HDL_2 levels (Muls et al. 1985; Short et al. 1986). The HDL_2 subclass is thought to be protective against atherosclerosis and this

factor together with the increase in VLDL, IDL and LDL cholesterol puts these patients in to a group which has a high risk of developing premature cardiovascular disease (Miller et al. 1981).

Qualitative and quantitative changes in the composition of the plasma lipoprotein fractions in nephrotic syndrome increased ratio of cholesterol to TG and of free cholesterol, cholesterol esters and phospholipid to protein in these fractions (Gherardi et al. 1977). There is also an accumulation in the plasma of lipoprotein particles that are similar to VLDL, IDL and CM remnants which are rich in esterified and non-esterified cholesterol and phospholipids. ApoB and C-III levels increase in nephrotic syndrome while apo A-I, A-II and C-II remain unchanged (Joven et al. 1990). Apo C-II is an essential co-factor involved in LPL activity and apo C-III is a competitive inhibitor of the action of apo C-II. The increase in the apo CIII/apo C-II ratio may reduce LPL activity resulting in a delayed clearance of triglyceride rich lipoprotein particles (Brown and Baginsky, 1972).

There is general agreement that the hyperlipidaemia of the nephrotic syndrome is a result of both increased hepatic synthesis of lipids and apolipoproteins (Makar et al. 1998) and decreased lipoprotein catabolism (Garber et al. 1984). Low plasma oncotic pressure and viscosity are thought to be the reason for increasing the synthesis of albumin and apoprotein B (Yedgar et al. 1982; Conwill et al. 1977). Delayed clearance of lipoproteins due to defective catabolism may also cause hyperlipidaemia (Garber et al. 1984; Staprans et al. 1987; Davies et al. 1990; Mene et al. 1989)(Fig.1.8).

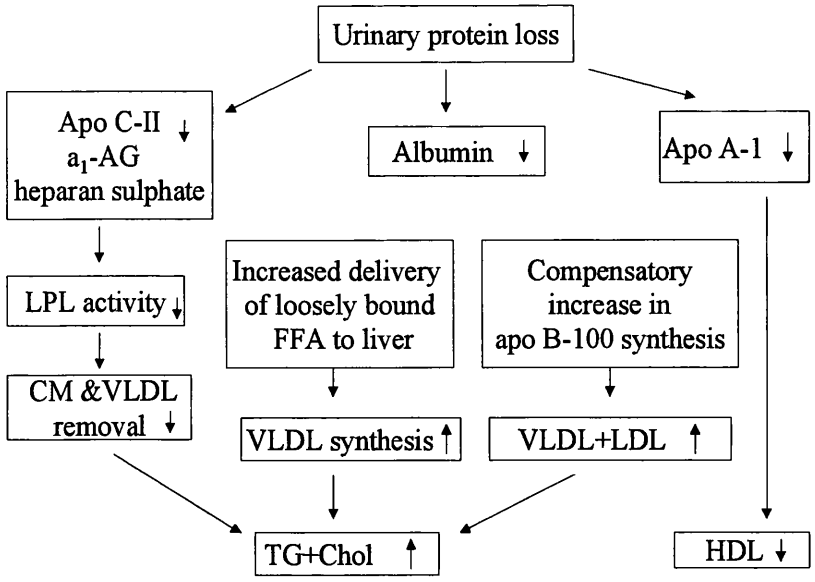


Fig 1.8 Mechanisms of hyperlipidaemia in nephrotic syndrome

1.2.2. Lipid abnormalities in chronic renal insufficiency

Chronic renal failure (CRF) is frequently associated with disturbances in lipoprotein metabolism. Although these lipoprotein metabolic disorders are more prevalent with advancing chronic renal failure, the mechanisms linking the two processes are poorly understood. The most common abnormality in plasma lipids of adult and paediatric patients with CRF is moderate hypertriglyceridaemia (Brown and Goldstein, 1986b; Grutzmacher et al. 1988; Alaupovic et al. 1988). Alterations in cholesterol metabolism in chronic renal insufficiency is thought to be largely secondary to abnormal triglyceride metabolism (Attman and Alaupovic, 1991). Triglyceride concentrations seem to be increased not only in the VLDL fraction but in other lipoprotein sub-fractions, IDL, LDL, and HDL as well (Dieplinger et al. 1986; Attman and Alaupovic, 1991). The cholesterol content of VLDL, IDL and LDL may also elevated (Norbeck and Carlson, 1981). This means that the LDL cholesterol:HDL cholesterol ratio is increased enhancing the risk of cardiovascular morbidity. The total

mass of VLDL and IDL is increased and that of HDL decreased (Attman and Alaupovic, 1991). These patients frequently have low HDL_2 levels (Rubies-Prat et al. 1987) and higher Lp (a) levels (Kronenberg et al. 1996).

Apolipoprotein	Plasma levels in CRF patients
apo A-I, apo A-II	Reduced
apo B apo, C-I	normal or slightly elevated
apo E	normal/ slightly elevated (males)
apo C-II	Decreased
apo C-III	Highly increased

Table 1.6 Plasma profile of the major apolipoproteins in patients with CRF

Lipid abnormalities (table 1.6) are also reflected in the apolipoprotein distribution in lipoprotein sub-fractions (Attman et al. 1987). These changes result in reduced apo A-I/apo C-III and apo A-I/apo B ratio and an increased ratio of apo C-III/apo-E ratio (Attman et al. 1987; Rubies-Prat et al. 1987). The apo C-III ratio (the ratio of apo C-III in heparin supernatant versus apo C-III in heparin precipitate) is also reduced in hypertriglyceridaemic CRF patients and is indicative of a triglyceride-rich lipoprotein degradation defect (Attman et al. 1987). Apolipoproteins B, C and E has been shown to increase threefold in VLDL and twofold in IDL (Attman and Alaupovic, 1991; Attman et al. 1992; Nestel et al. 1982). The apoC, apoE, apo A-I and apo A-II concentrations have been found to be reduced in HDL (Attman and Alaupovic, 1991;

Grutzmacher et al. 1988; Dieplinger et al. 1986). There is a decreased post heparin LPL activity in CRF patients the reasons for which are not fully understood (Chan et al. 1984). However insulin resistance and a non-dialyzable LPL inhibitor may play a role in this inhibition (Attman and Alaupovic, 1991).

In conclusion CRF is associated with alterations in the metabolism of lipoproteins, which results decreased removal and increased synthesis. Although these lipoprotein abnormalities are thought to be an important contributory factor in the development of cardiovascular disease in CRF patients, therapeutic intervention to correct dyslipidaemia has not been generally implemented.

1.2.3 Lipid abnormalities following renal transplantation

Hyperlipidaemia is a common problem following successful kidney transplantation (Ong et al. 1994; Moore et al. 1993). Lipid abnormalities have been reported to be present in 16%-70% of these patients, the prevalence depends on at what time point after transplantation the lipid profiles were investigated (Cattran et al. 1979; Drueke et al. 1991). The type of abnormalities seen include increases in cholesterol and triglyceride (TG) concentrations (Kasiske and Umen, 1987).

In a recent long-term longitudinal study that included a large number of renal transplant recipients followed up for up to 14 years, 70% had hypercholesterolaemia 3 years following transplantation and 59% 5 years post-transplant (Ong et al. 1994). Hypertriglyceridaemia following transplantation has been reported to be present in some studies and correlated with an excessive relative weight gain and elevated serum creatinine (Vathsala et al. 1989). It is generally thought that hypercholesterolaemia is

the more common problem in these patients. The effect of transplantation on HDL cholesterol levels remains unclear with decreased, (Bagdade and Albers, 1977; Brown et al. 1997) normal (Kasiske and Umen, 1987; Jung et al. 1982) or elevated (Gunjotikar et al. 1994).

Table 1.7. Factors that contribute to changes in triglyceride metabolism in chronic renal insufficiency.

Impaired catabolism and removal of triglycerides	**Increased synthesis of triglycerides**
Decreased activity of lipolytic enzymes	***Increased dietary carbohydrate***
Lipoprotein lipase (LPL)	
Insulin deficiency or resistance	
Increased PTH	***Uptake of glucose from dialysate in CAPD***
Inhibitors in uraemic plasma	
Reduced apo C-II/apo C-III ratio	
Hepatic lipase (HTL) and Lecithin	***Hyperinsulinaemia?***
cholesterol acyl transferase (LCAT)	
Alteration of lipoprotein substrate	
Triglyceride enriched LDL	
Altered apolipoprotein composition	
Increased apo C-III/apo E in IDL and LDL	
Modifications of lipoproteins	
Decreased removal by receptor and non-receptor mediated pathways	

Post renal transplant Apo CII, CIII and E have been found to be raised or normal in some studies and the apo CII/CIII ratio found to be decreased (Massy and Kasiske, 1996). In general Lp(a) levels in renal transplant patients have been reported to be normal although there is some evidence that patients on CyA had higher levels (Massy and Kasiske, 1996).

An initial increase in plasma cholesterol (within the first 3 months after transplantation) has been attributed to the higher doses of steroids used immediately following transplantation (Vathsala et al. 1989). The fall in serum cholesterol levels in long-term transplant patients may be due to the lower dependence on steroid immunosuppression (Vathsala et al. 1989). The immunosuppressive regimes employed may also have a bearing on the magnitude of hyperlipidaemic problems these patients may experience, for example, cholesterol levels are generally lower in tacrolimus-treated patients in comparison with cyclosporin-A (CyA)-treated patients. Some studies have reported that the use of CyA adversely affects lipid profiles and that withdrawal leads to an improvement in serum cholesterol and triglyceride (Harris et al. 1986; Hilbrands et al. 1995). LDL isolated from CyA treated patients has been shown to be more susceptible to oxidation *in vitro*, and their levels of auto-antibodies against Ox-LDL raised (Apanay et al. 1994; Ghanem et al. 1996). Our group has recently demonstrated that calcineurin-inhibiting drugs, CyA and tacrolimus, have pro-oxidant activity and they increase the susceptibility of LDL to oxidation. Neoral formulation is fortified with DL-alpha tocopherol and therefore provides protection against oxidation. The above study clearly demonstrated the benefit of giving vitamin C and E supplements to patients taking tacrolimus and this seems to be particularly important during the early period after transplantation (Varghese et al. 1999). β-

blockers and diuretics also have an adverse effect on lipid profiles (Kasiske and Umen, 1987).

1.3. INVOLVEMENT OF LIPIDS IN PROGRESSIVE RENAL DISEASES

1.3.1. Hypothesis

In 1982 Moorhead and colleagues suggested that the compensatory hepatic synthesis of lipoproteins in response to the urinary loss of albumin could cause progressive renal disease (Moorhead et al. 1982) (Fig.1.9.). This hypothesis is focused on the possibility that nephron may be damaged by lipoproteins resulting in progressive renal disease. Since smooth muscle cells (SMC) proliferated and became foam cells (Ross and Glomset, 1973; Ross, 1993) after exposure to LDL. It was thought that mesangial cells which have smooth muscle cell characteristics, would react similarly, resulting in 'glomerular atherosclerosis'. Therefore, in this 'two-hit' model, the original disease could be replaced by lipid-mediated disease. Persistent albuminuria stimulates excess lipoprotein synthesis by the liver so maintaining the lipid injury cycle. Although the lipid nephrotoxicity hypothesis is supported by numerous animal studies which implicate a role for hyperlipidaemia in the pathogenesis of progressive renal disease (Moorhead et al. 1982), it has been difficult to translate this into patients with progressive renal disease with lipid lowering drugs for the following reasons: firstly, hyperlipidaemia in nephrotic syndrome can seldom be normalised; secondly, treatment trials with lipid lowering drugs were very few and for shorter duration in comparison to trials carried out in cardiovascular diseases; finally, persistent inflammation may have a major role in aggravating lipid-mediated renal injury. It is becoming increasingly clear that inflammation is a major contributory factor in the

pathogenesis of atherosclerosis. The influence of inflammation and its interaction with lipid-mediated renal injury is discussed further in chapter 3, 4 & 6.

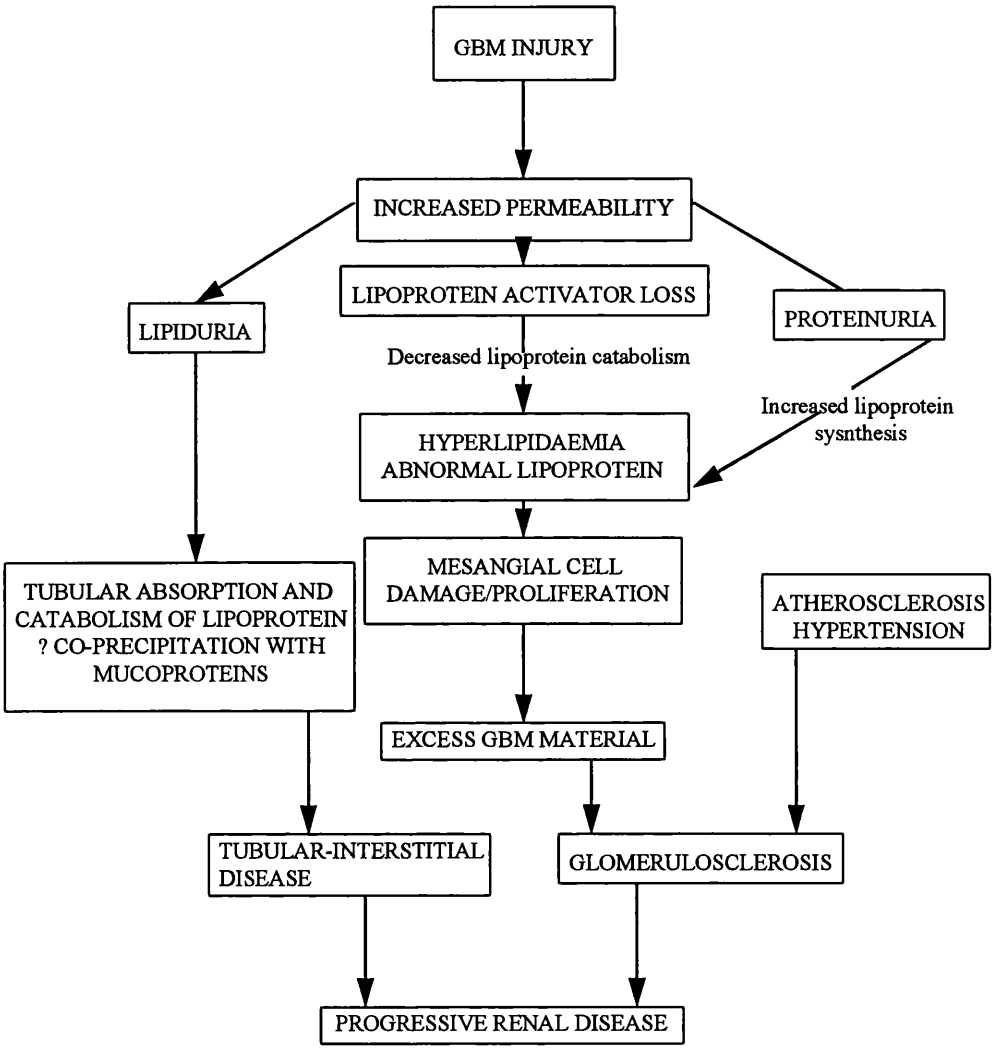


Figure. 1.9 Proposed mechanisms for the pathogenesis of lipid-induced glomerular atherosclerosis and tubulo-interstitial damage in chronic progressive renal disease (Moorhead et al. 1982).

1.3.2. Experimental evidence

The possible link between renal disease and hyperlipidaemia was made over a century ago when Virchow commenting in 1860 on the "Fattydegeneration of the renal epithelium in Brights disease", speculated whether this degeneration was primary or secondary to the disease state (Virchow, 1860). In 1916, Munk described the extensive glomerular and tubular lipid deposition in the nephrotic syndrome as "Lipid Nephrosis"(Munk, 1913). Wilens, studying hypertensive diabetic patients in 1951, noted the association between intercapillary glomerulosclerosis and "glomerular lipidosis" (Wilens et al. 1951). He concluded that the combination of hyperlipidaemia and elevated intraglomerular pressure might be responsible for lipid containing material penetrating "the intercapillary substance of the tufts".

A striking histological finding in animal studies was the dense inflammatory infiltrate. It has been established that cholesterol supplementation of the diets of several animal species leads to focal and segmental glomerulosclerosis (FSGS). French et al showed that feeding guinea pigs a diet containing 1 % cholesterol caused severe glomerular disease (French et al. 1967), with cholesterol crystals in glomerular capillaries and the proliferation of monocytes within the glomerulus. Subsequent studies using guinea pigs demonstrated that there was glomerular hypercellularity mesangial matrix expansion within a few weeks of starting high cholesterol diet (Brenner, 1985). Peric-Golia et al have demonstrated that feeding normal male Sprague-Dawley rats a 3% cholesterol or 3% cholic acid and taurine diet for 2-80 weeks resulted in hypercholesterolaemia within 4 weeks and within 1 year, histological evidence of aortic abnormalities and kidney abnormalities localised in the glomerulus including lipid droplets. They observed hyalinosis, glomerulosclerosis and interstitial fibrosis

(Peric-Golia and Peric-Golia, 1983). This study demonstrates that hypercholesterolaemia may cause renal lesions in the absence of a pre-existing glomerular injury if dietary supplementation with cholesterol is continued for a sufficiently long period. A study carried out by Kasiske et al. showed that FSGS resulted within three months of Sprague-Dawley rats being fed with a diet supplemented with 4% cholesterol (Kasiske et al. 1990). Cholesterol feeding also induced a slight increase in glomerular capillary pressure in the absence of an increase in systemic blood pressure. Isolated perfused kidney preparations from cholesterol fed animals have been shown to display higher whole kidney vascular resistance than kidneys from control animals. These hydraulic pressure changes may be modulated by vasoactive substances produced by infiltrating or intrinsic glomerular macrophages and mesangial cells whose numbers increase in cholesterol fed animals. Hypercholesterolaemia has been shown to reduce vascular production of vasodilatory substances such as prostacyclin and nitric oxide and to increase synthesis of vasoconstrictor factors such as thromboxane A_2 and endothelin (Kaplan et al. 1990; Triau et al. 1988; Verbeuren et al. 1986). The severity of glomerular injury is increased greatly if dietary induced hyperlipidaemia is combined with either a loss of functioning nephrons, nephrectomy or the presence of hypertension (Kaplan et al. 1990; Triau et al. 1988). Rats fed a diet consisting of 4% cholesterol which had a unilateral nephrectomy at 1 month developed significantly higher glomerular scarring than cholesterol fed rats with two kidneys (Liang and Vaziri, 1997). This increase in FSGS in nephrectomised animals could not be attributed to changes in glomerular haemodynamics as sham operated animals had similar increases in glomerular capillary pressures. Studies using the puromycin amino nucleoside (PAN) nephrotic rat model have also shown that cholesterol feeding increases the severity of proteinuria and

FSGS (Kasiske et al. 1990; Pavlovic et al. 1984). Glomerular injury is also higher when an increase in systemic hypertension is induced in the presence of hyperlipidaemia (Grone et al. 1989).

Animals that have endogenous hyperlipidaemia also develop progressive glomerular damage. Several models exist and these include hyperlipidaemic SD rat developed by Imai (Imai et al. 1977), hyperlipidaemic, spontaneously hypertensive rat model described by Koletsky (Koletsky, 1975), and the obese Zucker rat model (Kasiske et al. 1985).

Recently studies have been carried out to investigate the role of glomerular macrophage together with hyperlipidaemia in promoting glomerulosclerosis following an initial glomerular injury (Diamond et al. 1989). Diamond et al using the PAN rat model, demonstrated that during the acute nephrotic phase when hyperlipidaemia and proteinuria was at its peak, peritoneal macrophage phagocytosis, basal eicosanoid production and numbers in the glomerulus were significantly increased compared with control animals (Diamond et al. 1989). Cholesterol and cholic acid supplementation of the diet resulted in a much greater increase in the three parameters described above. These investigators concluded that dietary induced hypercholesterolaemia and the secondary hypercholesterolaemia of nephrosis may act in synergy to cause alterations in macrophage function and numbers in the glomerulus (Diamond and Pesek-Diamond, 1991; Pesek-Diamond et al. 1992). However, these works failed to point out the influence of inflammatory cytokines produced by infiltrating macrophages in these models.

1.3.3. Clinical evidence

An analysis of data collected in 1979 for the Diabetic Retinopathy Study emphasised that only hypertension and hypercholesterolaemia were positively linked with rapid progression of renal disease and that hypercholesterolaemia was the major predictor of death (Krolewski et al. 1994). Patients with hereditary LCAT enzyme deficiency are unable to esterify cholesterol normally and have defectively matured, abnormally large lipid laden HDL. These individuals have been shown to have lipid deposition in the glomerulus and progressive renal insufficiency (McIntyre, 1988). Some patients with hepatorenal syndrome and Fabry's disease who have lipoproteins with abnormal morphologies have been reported to have progressive damage in their glomeruli (Schatzki et al. 1979). A unique form of the nephrotic syndrome was reported in Japanese patients, where mesangial proliferation, mesangial expansion, glomerular deposition of lipoproteins, and FSGS occurred in association with high levels of circulating apolipoprotein E (Koitabashi et al. 1990; Saito et al. 1989). There have been several descriptions of an abnormal accumulation of lipids in glomeruli and tubules (Lim et al. 1974). Recently Lee et al. found that 8.4% of 631 patients had ultrastructurally detectable extracellular lipid in non-sclerotic glomeruli which suggests that there may be an early pre-sclerotic stage of lipoprotein-mediated damage (Lee et al. 1991).

Another important clinical area where lipid mediated renal injury may have an influence is chronic graft dysfunction, it is becoming apparent that both alloantigen-dependent and independent factors may contribute to tissue injury, remodelling and repair. The vascular lesions of chronic graft dysfunction are thought to progress through cyclical episodes of endothelial injury followed by inflammation and repair.

This process involves vascular smooth muscle cell and fibroblast proliferation and extracellular matrix deposition and all of which are similar to naturally occurring atherosclerosis (Varghese, 1999). A characteristic histopathological manifestation of chronic graft dysfunction is graft vascular lesion known as transplant-associated arteriosclerosis; this lesion is characterised by a diffuse, concentric intimal thickening of arteries in the graft. The high prevalence of lipoprotein abnormalities in transplant patients and the generally accepted involvement of dyslipidaemia in atherosclerosis suggest that lipid abnormalities may play a significant role in the development of chronic graft dysfunction. However, any explanation for the involvement of lipid in the development of transplant-associated arteriosclerosis must explain the selective involvement of the engrafted vessels with sparing of the recipient's native vessels. This probably suggests that immune-mediated mechanisms in the graft may potentiate the influence of lipid in accelerating the development of chronic graft dysfunction. This potentiation may be mediated through inflammatory cytokines.

1.3.4. Pathogenesis of lipid mediated renal injury

The pathological effects of lipoprotein in progressive renal disease are similar to those in atherosclerosis but occur in the microvasculature (Moorhead et al. 1982; Ross, 1993). In recent years, improved understanding of atherosclerosis has illuminated the pathology of glomerulosclerosis and supported the concept of lipoproteins as mediators of renal disease (Ross, 1993; Moorhead et al. 1982; Diamond, 1991). The possible roles of lipoproteins in progressive renal disease may be understood in the more familiar context of atherosclerosis. In major arteries atherosclerosis results from a complex sequence of events in which normal cycling of LDL through vascular endothelium is altered in favour of LDL trapping and oxidation, aided by change of

haemodynamic, increased oxidisability of LDL, recruitment of monocytes, proliferation of SMC and formation of fibrous tissue. The similarity between atherosclerosis and glomerulosclerosis is based on the assumption that the glomerulus possesses cell types which are known to respond to lipoprotein injury in large arteries, namely endothelium and monocytes, or which resemble smooth muscle, namely the mesangial cell. Moreover the glomerular basement membrane (GBM) is capable of binding human plasma lipoproteins (Iverius, 1972). The mechanisms involved in atherosclerosis also apply to glomerulosclerosis. Morphological and immunocytochemical studies of hypercholesterolaemic animal models have now clearly established the chronological patterns of cellular interactions that occur during the initial and transitional phases of the atherogenic process. These include: adherence of leukocytes to the endothelial surface, chemotactic attraction of the leukocytes into the arterial intima, conversion of monocytes to foam cells, proliferation of smooth muscle cell or mesangial cell, connective tissue synthesis, inflammatory and immune activation of macrophages and T lymphocytes, and the necrosis or apoptosis of cells within the developing lesions (Ross, 1993).

1.3.4.1 Oxidation of LDL

Though initial event involved in lipid-mediated renal damage is unclear, oxidative modification is thought to be of particular importance in atherogenesis. Another is non-enzymatic glycation, which may be a very important mechanism linking diabetes mellitus with some of the basic processes involved in the pathogenesis of atherosclerosis. Glycated LDL is also more susceptible to oxidation, a phenomenon known as glycoxidation (Lyons, 1993; Colaco and Roser, 1994). All major cell types in the artery wall, endothelial cells, SMC and monocyte-macrophages have been

shown to be able to effect oxidative modification of LDL in vitro (Parthasarathy et al. 1986; Henriksen et al. 1981; Heinecke et al. 1986). The mesangial cell may also participate directly in this process since rat mesangial cells were shown to promote lipoprotein oxidation and similar data exists for HMC (Fernando et al. 1993; Wheeler et al. 1994).

There is evidence for the importance of oxidative stress in the pathogenesis of atherosclerosis from numerous studies. Studies have suggested that oxidative stress is increased in uraemic patients. Ox-LDL has been identified in the lesions of FSGS (Lee, 1999). Oxidative stress is also implicated in the mediation of ischemia/reperfusion injury and it is possible to reduce its impact with superoxide dismutase (SOD). Our group has demonstrated that Ox-LDL induced vasoconstriction in an isolated perfused rat kidney model is mediated by decreased activity of nitric oxide (NO), probably due to the inactivation of NO by ROS (Reactive Oxygen Species). Free radical scavengers, SOD, catalase, and L-arginine provided protection against Ox-LDL-induced vasoconstriction. Hypercholesterolaemia and atherosclerosis are strongly associated with impairment of endothelium dependent relaxation and arteries from hypercholesterolaemic animals produced significantly higher rates of oxygen radical, than control arteries. Ox-LDL induces apoptotic cell death in endothelial cell and this pathway is considered to be important for the cellular turnover in atherosclerotic arteries (Rahman et al. 1999; Varghese, 1999).

Both OX-LDL and minimally oxidised LDL (mOx-LDL) have also been found to have a role in the recruitment of circulating monocytes directly (Quinn et al. 1987;

Berliner et al. 1990) and by inducing SMC, mesangial cells and/or endothelial cells to produce chemotactic and adhesive factors such as monocyte chemotactic protein (MCP-1) (Cushing et al. 1990), monocyte colony stimulating factor (m-CSF) (Rajavashisth et al. 1990; Kamanna et al. 1996), Interleukin-1β (IL-1β) (Ku et al. 1992), and other adhesion molecules (Frostegard et al. 1993; Cominacini et al. 1997). Post-secretory modification of LDL could confer chemoattractant properties, and, as in atherosclerosis, stimulate glomerular resident monocytes to produce chemoattractants leading to further influx of monocytes (Schreiner, 1991; Pesek-Diamond et al. 1992). Furthermore, Rosenfeld have also recently demonstrated that components of Ox-LDL maximally induce the production of IL-1 by macrophage-derived foam cells. These observations suggest that there may be a common intracellular signal transduction pathway that is responsive to oxidative mechanisms and which underlies some of the key cellular events in the atherogenic process (Rosenfeld, 1996). These events have the hallmarks of an on-going inflammatory process.

Ox-LDL is demonstrably more cytotoxic when compared with unmodified native LDL (Hessler et al. 1983). Ox-LDL may be extremely important in lesion progression, specifically in the development of the extracellular lipid core as the foam cell undergoes necrosis (Schwartz et al. 1991). Minimally oxidised LDL may initiate the enhanced uptake by monocytes of cholesterol-containing particles derived from atherosclerotic lesions, a possible cause of foam cell generation (Hoff et al. 1990). Ox-LDL binds preferentially to the glomerulus when injected intra-arterially in the rat and binds to mesangial cells *in vitro* (Coritsidis et al. 1991). Modified LDL may also

inhibit the motility of resident monocytes once they have differentiated into macrophages within the intima (Quinn et al. 1987).

Modified LDL is not recognised by the "native" LDL receptor, but by the scavenger receptor. The scavenger receptor therefore provides a pathway for the unregulated uptake of modified lipoproteins with eventual foam cell formation (Goldstein et al. 1979). This mechanism has been thought to play an important role in the process of lipid-mediated glomerulosclerosis. This process is described further in chapter 3.

Under physiologic conditions, there is a balance between the production of reactive oxygen species (ROS) and the activity of antioxidant enzymes; an imbalance characterises a state of oxidative stress. Inflammatory mediators, including tumour necrosis factor and interleukin-1 may induce oxygen radical production by mesangial cells (Radeke et al. 1990). Immune-mediated mesangial injury causes increased oxygen radical and eicosanoid synthesis (Oberle et al. 1992; Kelley et al. 1985). However, minimally oxidised LDL may also induce protective molecules against products of moderately or maximally Ox-LDL (e.g. Haemoxygenase, glutathione transferase, Bcl-2 and members of serum amyloid A family proteins). Some of these proteins have anti-oxidant and anti-apoptotic function and block activation of NFkB. It seems these protective genes and their proteins can be induced by TH2 cytokines. Therefore a balance between inflammatory cytokines and protective gene may by important in the regulating oxidative stress induced damage to vessel walls (Varghese, 1999).

1.3.4.2 Recruitment of monocyte-macrophages

It is a feature of many chronic progressive renal diseases and is evidenced histologically by the accumulation of macrophages, cholesterol and cholesteryl esters in sclerotic glomeruli (Ichikawa and Fogo, 1996). Recent studies have begun to provide mechanistic explanations for that the adherence of leukocytes to the endothelium appears to be dependent on the increased expression of adherence molecules by endothelial cells.

When glomeruli are damaged, evidenced clinically by albuminuria, endothelial and mesangial cells produce chemotactic and adhesive factors such as monocyte chemotactic protein (MCP-1) (Cushing et al. 1990), monocyte colony stimulating factor (m-CSF) (Rajavashisth et al. 1990), interleukin-1β (IL-1β) (Ku et al. 1992), and other adhesion molecules (Frostegard et al. 1993). There is an influx of circulating monocytes into damaged glomeruli, which then take up cholesterol and cholesteryl ester and become foam cells. Intrinsic glomerular cells of both mesangial and macrophage origin may also become foam cells (Goldszer et al. 1984a). Lipid-stimulated monocyte influx was first shown by French (French et al. 1967) in cholesterol-fed rabbits and later characterised more fully (Schreiner, 1991). Minimally oxidised LDL stimulates monocytes endothelial cell interaction, and is chemotactic for monocytes which secrete the inflammatory cytokines TGFβ (Berliner et al. 1990) and TNFα (Ruan et al. 1995).

1.3.4.3 Mesangial cell proliferation and matrix expansion

The proliferation of intrinsic glomerular cells and the accumulation of extracellular matrix proteins are principal histopathological features seen in glomerular injury.

Native LDL (20 to 200 μg/ml) caused a dose-dependent increase in (^{3}H)-thymidine incorporation and increased human mesangial cell numbers over 96 h. Higher LDL concentrations (1,000 to 2,000 μg/ml) inhibited (^{3}H)-thymidine incorporation and reduced cell numbers, possibly as a result of the oxidative modification of LDL, indicated by an increase in thiobarbituric reactive substances (Keane et al. 1993). This peroxidation of LDL involved superoxide, because superoxide dismutase and butylated hydroxytoluene prevented it, whereas hydroxyl radical scavengers were without effect. Native LDL subjected to chemical oxidation by copper sulphate also inhibited mesangial cell proliferation. These results suggest that low concentrations of LDL may stimulate human mesangial cell proliferation, which may, in turn, cause the production of reactive oxygen molecules. Moreover, the oxidative modification of LDL may mediate the toxic effects of high LDL concentrations on HMC (Keane et al. 1993). Wheeler also reported that at greater concentrations (more than 200 μg/ml), cell proliferation was inhibited and above 500 μg/ml cells sustained visible morphological injury when assessed under phase contrast microscopy. Estimation of ^{51}Cr release from pre-labelled cells confirmed that LDL was cytotoxic in these greater concentrations (Wheeler et al. 1990). Mesangial cells proliferate at a similar rate to that of vascular SMC (Hyman and Burkholder, 1973). A similar pattern of proliferation and toxicity has been observed in vascular smooth muscle cell cultures over a corresponding range of LDL concentrations. These results strengthen the analogy between glomerulosclerosis and atherosclerosis and provide further evidence that lipoproteins may contribute directly to glomerular scarring (Wheeler et al. 1990).

Not only LDL but also triglyceride (TG)-rich lipoproteins VLDL, IDL promoted the proliferation of mesangial cells up to certain concentrations, but cell growth was

actually decreased at higher concentrations. Ox-LDL caused a concentration-dependent decrease of ^{3}H-thymidine incorporation. HDL had no proliferative effect at any concentration (Nishida et al. 1999).

The mechanism by which lipoproteins stimulate mesangial cell proliferation is unclear. Exposure of HMC to LDL resulted in a transient elevation of mRNA that encodes c-fos and c-jun, with a maximal effect seen after 30-60 min. In addition, PDGF A- and B-chain mRNAs were transiently elevated, peaking at 3 h in response to LDL and continued to increase in a concentration-dependent manner (Grone et al. 1992). Exposure to VLDL, IDL, LDL, or a high concentration of HDL enhanced the secretion of IL-6, PDGF-AB, and TGFβ by mesangial cells, whereas TNFα secretion was stimulated by Ox-LDL (Nishida et al. 1999). This data suggested that some cell cycle protein and inflammatory cytokines were involved.

One prominent feature of lipid-induced glomerular injury in animal models is the accumulation of mesangial matrix. Proliferative mesangial cell produced excess deposition of collagenous and non-collagenous extracellular matrix material in both the inflamed glomerular mesangium (Hyman and Burkholder, 1973) and atheromatous artery (Ross et al. 1984). Lee has showed that LDL stimulates collagen I and collagen IV mRNA synthesis in HMC through induction of PKC and TGFβ expression, suggesting TGFβ is a important mediator in matrix generation (Lee et al. 1999). LDL during its incubation with HMC was peroxidatively modified and stimulated collagen I, collagen III and collagen IV mRNA expression. Vitamin E, an antioxidant, and antibody against Ox-LDL caused a marked reduction in collagen mRNA stimulated by LDL. These findings suggest that LDL deposited and oxidised in the glomeruli may

be implicated in the development of glomerulosclerosis by facilitating excessive mesangial matrix generation (Lee, 1999; Lee et al. 1996). Chana recently reported that LDL selectively enhances the synthesis of specific proteoglycans and hyaluronan by HMC (Chana et al. 2000). The incubation of murine mesangial cells with LDL (25-100 μg/ml) increased the synthesis and secretion of both fibronectin and laminin in a dose-dependent manner. Similarly, oxidised forms of LDL (25-100 μg/ml) increased fibronectin and laminin synthesis and secretion dose dependently. However, oxidatively modified forms of LDL had a greater effect on increasing ECM protein synthesis than their native counterpart (Roh et al. 1998). Ox-LDL also stimulates matrix protein fibronectin in human glomerular epithelial cells (Ding et al. 1997), mesangial cell (Studer et al. 1995) by a mechanism involving expression of TGFβ which in turn stimulates fibronectin production by glomerular cells, and collagen and proteoglycan synthesis. Therefore, lipoproteins and modified lipoproteins get trapped intracellularly in foam cell and extracellularly in the matrix, this lipoprotein trapping is an essential step in the pathogenesis of lipid-mediated injury.

1.3.4.4 Tubulo-interstitial lesions

It was first observed by Risdon *et al.* (Risdon et al. 1968), and subsequently confirmed by others, that tubulo-interstitial disease was a better predictor of progression than glomerular disease (Wehrmann et al. 1990; Bohle et al. 1990). The concept of lipoprotein-related tubulo-interstitial disease derived from histopathological findings of tubular lipidosis and the clinical examination of nephrotic urine for oval fat bodies which are presumably formed in the renal tubules. The original observations of Munk were in fact on fat bodies in the urine and fatty changes in the tubules (Munk, 1913). In nephrotics, fatty acids are presented in substantial

amounts via albumin, their major carrier, which are then probably metabolised into neutral lipids in the tubules. The accumulation of lipids in renal tubular cells was suggested as being potentially toxic, perhaps contributing to tubulo-interstitial disease (Warwick et al. 1991).

In a study of the hyperphagic fatty rat, Zucker observed proteinuria and progressive 'glomerulonephrosis' for the first time and described severe interstitial renal disease especially in males (Zucker, 1965). These early descriptions provide clinical and experimental support for a causative link between hyperlipidaemia and progressive tubulo-interstitial disease. Despite the fact that the mass of LDL is probably too large to enable significant filtration of intact particles in measurable quantities in nephrotic urine, both apo-A and apo-B, major apoproteins of LDL have been found in the tubules and interstitium of nephrotic patients (Streather et al. 1993). HDL is excreted in measurable amounts in normal subject and in larger amounts in nephrotics (Moorhead et al. 1983; Short et al. 1986). Studies in rabbit proximal straight tubule segments microperfused *in vitro* have shown progressive uptake and cellular accumulation of radiolabeled HDL (Peterson et al. 1984).

In order to clarify tubule/lipoprotein interactions, our group examined human proximal tubular cells in culture (Ong and Moorhead, 1994) and found that native LDL was not toxic to human proximal tubular cells, but both Ox-LDL and minimally oxidised LDL caused cell detachment and death, possibly because of the presence of cytotoxic oxidation products in LDL particles. This study also suggested that LDL may be oxidised by proximal tubular cells, suggesting that any filtered lipid may become toxic in its passage through the tubule. Since mesangial cells which are upstream of the

tubules also have this capacity (Fernando et al. 1993), the possibility exists that some oxidatively modified LDL will pass into the tubular lumen. This stimulated us to examine renal tubular cell cytokine response, in the form of endothelin-1 (ET-1) synthesis, to *in vitro* challenge to HDL. Ong et al found that HDL was mitogenic for these cells and caused a four-fold increase in ET-I secretion. Since ET-I is secreted basolaterally, the high quantities of HDL filtered in nephrotic syndrome could have adverse effects on the renal microcirculation, interstitial fibroblasts and interstitial macrophages (Ong et al. 1994).

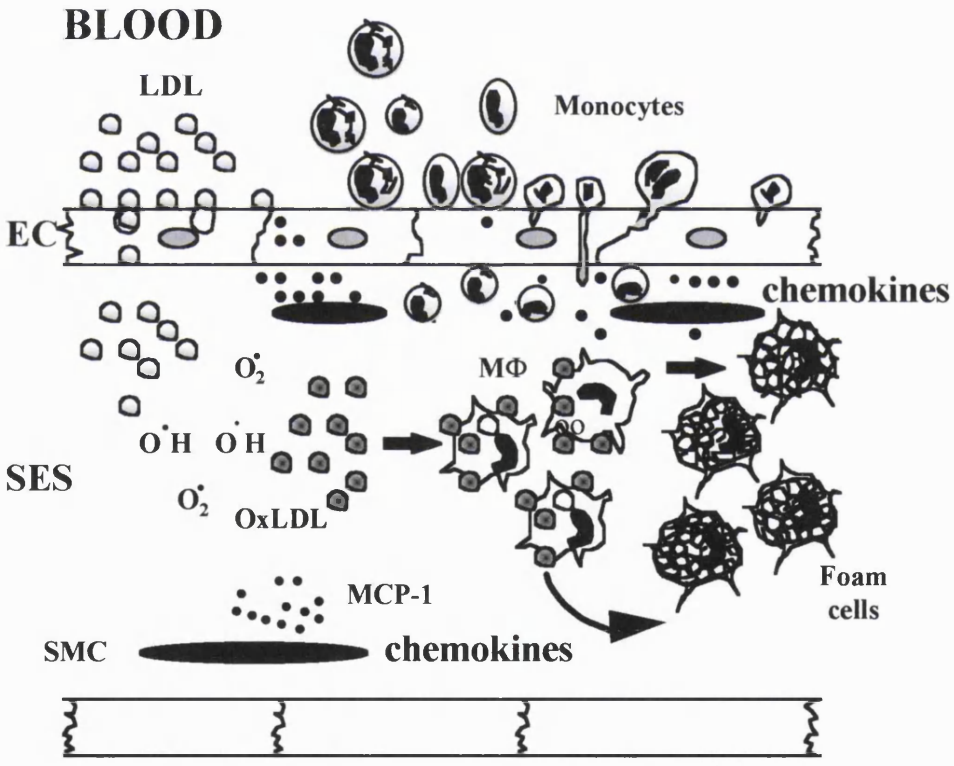


Figure 1.10. A schematic of the basic mechanisms involved in the pathogenesis of atherosclerosis. See text for details. EC= Endothelial cells, SES= sub endothelial space, SMC= Smooth muscle cells

In summary, when glomeruli are damaged, evidenced clinically by albuminuria, there is an influx and accumulation of plasma lipoproteins and /or circulating monocytes into damaged glomeruli under action of inflammatory mediators. Loss of endogenous lipoprotein antioxidants and generation within the intima and mesangium of reactive oxygen species or free radicals by macrophages, endothelial cells and mesangial cells result in an oxidative modification of lipoprotein. Intrinsic glomerular cells of both mesangial and macrophage origin take up cholesterol and cholesteryl ester and become foam cells (Goldszer et al. 1984). Autoimmune inflammation, a likely result of the antigenic epitopes of Ox-LDL produce inflammatory cytokines. Glomerular mesangial cells proliferate at a similar rate to that of vascular SMC and there is an excess deposition of collagenous and non-collagenous extracellular matrix material in both the inflamed glomerular mesangium (Hyman and Burkholder, 1973; Ross et al. 1984). Finally, hypercholesterolaemia accelerates both glomerulosclerosis and atherosclerosis (Steinberg, 1983; Moorhead et al. 1982; Keane et al. 1991). Inflammatory cytokines play an important role during the progression of glomerulosclerosis (Litalien et al. 1999) (Fig.1.10.)

1.3.5 Treatment of hyperlipidaemia in progressive renal diseases

Lipid lowering drugs have also been used to assess the contribution of hyperlipidaemia to the progression of renal damage. A range of drugs including clofibric acid, cholestyramine, HMG-CoA reductase inhibitors and fish oils have been used in several animal models such as the obese Zucker rat model, the PAN nephrosis model and the renal ablation model (Wheeler et al. 1991b; Harris et al. 1990; Diamond et al. 1990). Lipids and immune complexes acted synergistically in lupus nephritis in mice, and fish oil rich in eicosapentaenoic acid prolongs the life of the

animals (Kelley et al. 1985). Total deprivation of lipid mediators through essential-fatty-acid-deficient diets has also shown to prevent progression in glomerulonephritis in rats. Most of these studies demonstrated that reducing serum lipids ameliorated the degree of glomerular injury and had a positive effect on renal function without significantly altering glomerular haemodynamics.

In spite of strong clinical and experimental links between hyperlipidaemia and progressive renal disease, it has been tantalisingly difficult to ameliorate human renal disease with lipid-lowering drugs. Lipid-lowering drugs are effective in reducing plasma cholesterol in nephrotic patients. Our group conducted a double-bind, placebo controlled trial of the HMG-CoA reductase inhibitor simvastatin in patients with the nephrotic syndrome or significant proteinuria and hypercholesterolaemia. The results showed that total and LDL cholesterol levels fell by a mean of 33 and 31% respectively, in simvastatin group. Apolipoprotein B100 levels fell by a mean of 31% in the simvastatin group but rose 0.3% in the placebo group. There were no significant changes in HDL levels. There were no significant differences between the groups in their urine protein levels, their rise in plasma creatinine, or decline in plasma insulin clearance (Thomas et al. 1993). In an uncontrolled clinical trial where simvastatin was used in a group of nephrotic syndrome for 48 weeks LDL cholesterol was reduced by over 50 % (Rabelink et al. 1990). This was accompanied by a partial remission of the nephrotic syndrome with a decrease in albuminuria from 5.8 to 2.3 g/24h, with a corresponding increase in plasma protein concentrations while plasma creatinine levels remained unchanged. A retrospective study carried out patients with type 1 diabetic nephropathy, showed that plasma cholesterol was partly correlated with the decline in renal function (Krolewski et al. 1994). However, prospective,

controlled trials using lipid-lowering drugs, with clinically appropriate functional and morphological end points will be needed to properly evaluate the clinical implications of these experimental studies.

Another approach to reduce cholesterol is LDL aphaeresis in nephrotic patients. This rapidly reduces the LDL concentration. Selective elimination of LDL, VLDL and Lp(a), achieved by passing hyperlipidaemia plasma over negatively charged dextran sulphate bound to porous cellulose beads, is valuable in the management of familial hypercholesterolaemia; weekly treatments effectively maintain LDL-cholesterol within the normal rang (Olbricht and Schulzeck, 1991). Recently, Sakai reported a multicentre trial of LDL aphaeresis in 16 drug-resistant nephrotics (Sakai et al. 1992). Treatment was given twice weekly for 3 weeks, 2.5-4 litres of plasma being treated per session. Total cholesterol and triglyceride were reduced by 60-70% after treatment. HDL-cholesterol remaining unchanged. One month later, mean total cholesterol was 30-40 % of the initial level, albumin increased by 10-30%, and in 8 patients, urine protein fell to less that 1 g/day. No improvement was shown in membranous nephropathy, but 9 patients with focal segmental glomerulosclerosis had improvement ranging from 'very useful' to 'slight'. In a 15-year-old child with drug-resistant FSGS, a combination of LDL aphaeresis and pravastatin resulted in a fall in plasma total cholesterol and urine protein. Urinary excretion of thromboxaneB2 fell strikingly after the initiation of aphaeresis, suggesting a decrease in the supply of substrate for inflammatory prostaglandins (Hattori et al. 1993). While this technique offers a new approach, judgement as to its efficacy must await a controlled trial.

Recently it has been demonstrated that Ox-LDL can induce members of peroxisome proliferator activated receptor (PPAR) family which has the ability to induce Ox-LDL

receptor CD36 (Tontonoz et al. 1998). On the other hand, fibrates that are PPARα ligands have the ability to inhibit progressive formation of atherosclerotic lesions. It seems activators of PPARα can inhibit inflammatory responses (Staels et al. 1998).

There is currently little evidence from controlled trials to suggest that lipid lowering slow the progression of renal disease. It is difficult to demonstrate a beneficial effect of lipid lowering in conditions such as nephrotic syndrome (Thomas et al. 1993). However, two collaborative studies, SOLAR and ALERT, are in progress with fluvastatin, to study the impact of early and late intervention with lipid-lowering agents in controlling both acute and chronic graft dysfunction in renal transplantation patients (Varghese, 1999).

1.4 AIMS OF THE STUDY

Although many studies using animal models of diet induced hyperlipidaemia support the hypothesis that lipid abnormalities contribute to renal injury, there are notable exceptions where hyperlipidaemic models do not cause such injury. The Watanabe heritable hyperlipidaemic rabbit (WHHL) model characterised by a deficiency of LDL receptors and hypercholesterolaemia develop atherosclerosis but not renal lesions (Keane et al. 1991). Another instance of a model where hyperlipidaemia does not lead to the development of glomerular injury is the Nagase analbuminaemic rat. These rats lack circulating albumin and is associated with hypercholesterolaemia, but there is no evidence of renal disease in these animals (Zatz et al. 1989).

In human, familial hypercholesterolaemia is not usually associated with renal failure (Kasiske et al. 1985). The occurrence of renal disease in patients with primary

hyperlipidaemia is low except for a few specific instances (McIntyre, 1988). Hyperlipidaemia in some patients do not usually cause glomerular injury, but other patients with renal disease, without hyperlipidaemia, often suffer glomerulosclerosis and atherosclerosis (Tracy, 1992; Wheeler et al. 1990b). Atherosclerosis regresses with reduction of serum cholesterol (Wissler, 1978), but human renal disease does not. In other words, the plasma level of cholesterol is not coincident with glomerulosclerosis. On the other hand, Scoble et al showed that renal function may be relatively better in the kidney with atherosclerotic renal artery stenosis, than in the contralateral kidney without renal artery stenosis. This was thought to be due to the protection of the stenosed kidney from hypertensive damage. Alternatively, we could consider that some circulating atherosclerotic factor(s) cause functional damage in the contralateral kidney, suggesting that there is a process causing renal dysfunction in patients with atherosclerotic disease independent of renal artery narrowing (Scoble et al. 1998; Farmer et al. 1999). These experimental and clinical evidences suggest that hyperlipidaemia alone probably does not cause kidney damage and that involvement of additional factors such as hypertension, immune-mediated glomerular and inflammation are necessary for the progression of lipid-induced renal dysfunction.

Studies done in many laboratories over the last ten years have shown an association between markers of inflammation and atherosclerosis with an exacerbation of the inflammatory process during acute myocardial ischemia and reperfusion (Mehta et al. 1998). It raises the possibility that atherosclerosis is an inflammatory process (Ross, 1999). Observational cohort studies in SLE and other chronic inflammatory diseases have led to the description of accelerated atherosclerosis as an important cause of

mortality and morbidity in these conditions. These changes may be associated with acute phase responses mediated by cytokine-induced changes in lipoprotein homeostasis (Urowitz and Gladman, 2000). For example, Lp(a) is considered to be an important lipoprotein associated with cardiovascular diseases because of its thrombogenic and atherogenic properties. Lp(a) is also an important acute phase protein (Murai et al. 1986) and it is increased in chronic inflammatory diseases (Urowitz and Gladman, 2000).

Chronic renal disease is also considered to be an inflammatory disease because many markers of inflammation have been identified with this condition and this inflammatory state is probably augmented by components of renal replacement therapy such as the use of bio-incompatible materials for dialysis therapy (Balow and Austin, 3rd, 2000; Perez et al. 2000; Kaysen, 1999; Litalien et al. 1999; Panzer and Stahl, 1999). It is indicated that in human glomerular disease the severity of proteinuria is correlated with tubulointerstitial infiltrates mainly comprising CD4 positive helper T lymphocytes and monocytes (Kuroiwa et al. 2000). Focal recruitment of lymphocytes and monocytes is one of the earliest detectable cellular response in the formation of lesions in atherosclerosis (Schena, 1999; Grcevska and Polenakovic, 1993). This localised accumulation of inflammatory cells and their mediators may have specific influence in modifying interactions with lipoproteins.

Therefore, it is logical to extend the lipid nephrotoxicity hypothesis to include the influence of inflammation. Systemic or local inflammation could be an additional factor which influences both intra and extracellular cholesterol homeostasis through the regulation of lipoprotein receptors. In this respect the regulation of lipoprotein receptor

centrally in the hepatic tissue and peripherally in the vascular bed is important. In the context of the progression of renal disease, the mesangial cell is thought to play an important role because of its similarities with vascular SMC. Therefore studies were designed specifically to unravel mechanisms involved in the induction and regulation of lipoprotein receptors in HMC under the influence of inflammatory cytokines.

CHAPTER 2. GENERAL METHODS

2.1. CELL CULTURE

An established stable human mesangial cell line (HMCL) was used (kindly donated by Dr.J.D.Sraer, Hopital Tenon, Paris). HMC were immortalised by transfection with T-SV40 and H-ras oncogene. It retains many morphological and physiological features of normal human mesangial cells (table 2.1)(Sraer et al. 1996).

Table 2.1. Comparison between parental human mesangial cells and the immortalised human mesangial cell line (Clone C2M12)

	Parental cells (HMC)	Transfected mesangial cells (HMCL)
Morphology	+	+
Stellate shape	+	+
Cytoplasmic projections	+	+
Microvillis	+	+
Antigenic markers	+	+
Alpha actin	+	+
T-SV 40	-	+
Extracellular matrix components	+	+
Collagen I	+	+
Collagen IV	+	+
Laminin	+	+
Fibronectin	+	+
Biosynthesis		
IL-6	+	+
Renin	+	+
t-PA	+	+
u-PA	-	+
PAI1	+	+

2.1.1. Materials

1) RPMI1640 (Gibco BRL , Paisley, UK)

2) Foetal calf serum (Gibco BRL , Paisley, UK)

3) Glutamine (Sigma, Dorset, UK)

4) Penicillin (Sigma, Dorset, UK)

5) Streptomycin (Sigma, Dorset, UK)

6) Amphotericin (Sigma, Dorset, UK)

7) ITS (insulin-human transferrin-sodium selenite) (Sigma, Dorset, UK)

8) Trypsin, ethylene diamine tetra acetic acid (EDTA)(0.025% and 0.01% respectively) (Gibco BRL, Paisley, UK)

9) 75 cm^2 culture flask (Falcon, UK)

2.1.2. Procedures

HMCL was cultured in medium containing RPMI1640, 5% foetal calf serum (FCS), 2 mmol/L glutamine, 10^5 unit/L penicillin, 0.1 g/L streptomycin, 2.5×10^3 g/L amphotericin, 5×10^{-3} g/L insulin, 5×10^{-3} g/L human transferrin, and 5×10^{-6} g/L sodium selenite in T75 cm^2 flask. Medium was changed completely every 3-4 days. The cells were subcultured at 7-8 day intervals. When confluency was reached, growth medium was removed and the cells washed with PBS. Trypsin, ethylene diamine tetraacetic acid (EDTA)(0.025% and 0.01% respectively) was then added and the cells incubated for 2-3 minutes at 37°C. Cell detachment was assessed by phase-contrast microscopy and enhanced by vigorous agitation. The enzymatic action of trypsin was arrested by adding FCS containing growth medium and the detached cells pelletted by centrifugation at 1000 g for 10 minutes. The cell pellet was then resuspended in fresh growth medium and replated in culture flask in1:3 split ratio.

2.2. CELL PROLIFERATION ASSAY

HMCL were plated in 96-well plates at a density of 6000 cells/well or subcultured at 20,000 cell/well into 24-well culture plates (Falcon, Oxford, UK) in standard growth medium in RPMI containing 5% FCS and cultured until nearly confluent. Experiments design and procedures see chapter 3 & 4.

2.3. PREPARATION OF LIPOPROTEIN

Plasma was collected from a healthy human volunteer and LDL was isolated by sequential ultracentrifugation as previously described (Fernando et al. 1993).

2.3.1. Materials

1) 0.2 M disodium EDTA (pH 7.4) (Merck Ltd. Dorset UK)

2) 2.5% sodium azide (Sigma Ltd. Dorset UK)

3) Benzyl penicillin 600 Unit/ml

4) Streptomycin sulphate 100 mg/ml

5) 2000 Unit/ml Kallikrein inactivator Aprotinin (Trasylol, Bayer UK Ltd, Newbury, UK)

6) 0.3 M Sodium chloride (NaCl, Merck Ltd, Poole UK)

7) Sodium bromide (NaBr, Merck Ltd. Poole UK)

8) Visking tubing

9) 0.15 M phosphate buffered saline (PBS pH 7.4)

2.3.2. Procedures

4 ml of solution 1), 1 ml of 2), 0.125 ml of 3), 0.25 ml of 4), 0.125 ml of 5) and 7 ml of 6) were mixed together and 1 ml added to universal tubes.

180 ml of blood was collected following an overnight fast by venipuncture from normolipidaemic healthy volunteers and 20 ml placed in universal containers pre-filled with 1 ml of preservative solution prepared as described above. The plasma was separated by centrifugation at 3000 g for 10 minutes. LDL (density range 1.019-1.063 g/ml) was isolated by ultracentrifugation in a Beckman L8-55 or L8-80 M ultracentrifuge fitted with a 50.3 Ti rotor, using NaBr for density adjustment. The plasma was adjusted to a density of 1.019 g/ml using formula 1 to calculate the amount of NaBr to be added and centrifuged at 40,000 rpm for 20 h at 4 °C to remove chylomicrons (CM), VLDL and IDL.

Formula 1.

$$X = \frac{v_i(d_f - d_i)}{1-(Vxd_f)}$$

X = g NaBr to be used for density adjustment
V_i = The initial volume
d_i = Initial density of plasma
d_f = Final density required
V = Partial specific volume of NaBr =0.235

The tubes were removed from the rotor, placed on ice, the tube caps removed and the supernatant containing the VLDL and IDL aspirated using a 19 gauge needle attached to a 10 ml syringe and discarded. The infranatants were pooled mixed well, and adjusted to a density of 1.063 g/ml by adding NaBr using the formula given above. The tubes were re-capped and centrifuged at a temperature of 4 °C for a further 20 h at 40,000 rpm to obtain LDL. The LDL could be visualised as an orange layer at the top of the tube. LDL was harvested using a syringe as described above, concentrated and purified by centrifugation for a further 20 h at the speed and temperature given above. The concentrated LDL was

harvested, placed within a dialysis membrane which had been softened by boiling in distilled water, and dialysed in 5 L of PBS containing 1mM EDTA for 24 h, changing dialysate 2 times (total volume of PBS used =15 L). 24 h prior to the commencement of an oxidation experiment a portion of the LDL was redialysed as described above omitting the EDTA. LDL protein concentration was measured using the Lowry assay as modified by Markwell et al (Markwell et al 1978).

2.4. LDL MODIFICATION

LDL was acetylated using the method described by Innerarity et al (Fernando et al. 1993; Innerarity et al. 1986).

2.4.1. Materials

1) Acetic anhydride (Sigma Ltd. Dorset UK)
2) Saturated sodium acetate solution (Sigma Ltd. Dorset UK)

2.4.2. Procedures

Ice cold LDL (2-5 mg/ml) was diluted 1:2 with saturated sodium acetate solution. The mixture was stirred continuously at 4 ^{0}C and 2-8 aliquots of acetic anhydride (1.5 µl per mg LDL) was added to the mixture over for 90 min. The acetylated LDL was then dialysed extensively for 18 h against PBS containing 0.01% EDTA pH 7.4. Ac-LDL was passed through a 0.2 µm filter before use, and not used beyond 10 days.

2.5. MODIFIED LOWRY ASSAY FOR LIPOPROTEIN AND CELL MEMBRANE PROTEIN ESTIMATION

Lipoprotein concentration was estimated using modified Lowry assay (Markwell et al. 1978).

2.5.1. Materials

1) 100 μg/ml stock solution of BSA (Sigma Ltd. Dorset UK)

2) Reagent A: consisting of 2% Na_2CO_3, 0.4% NaOH, 0.16% Sodium tartrate and 1% SDS (Sigma Ltd. Dorset UK)

3) Reagent B containing 4% $CuSO_45H_2O$ (Sigma Ltd. Dorset UK)

4) A 1 N Folin-Ciocalteu solution (F-C) (Sigma Ltd. Dorset UK)

2.5.2. Procedures

1) Dilute the BSA stock in d H_2O to obtain a standard curve as follows: 0, 5, 10, 20, 40, 60, 80, 100 μg/ml.

2) Add 3 ml of reagent C (sol A: sol B at 100:1) to 1 ml of sample containing 10-100 μg of protein and incubate at room temp for 10 min

3) Place 0.3 ml of 1 N F-C reagent vortex mix and incubate for 45 min at room temperature

4) Read absorbance at 660 nm and calculate sample protein content using the BSA standard curve

2.6. IODINATION OF LDL

LDL was iodinated using the enzymobead method. The enzymobead reagent consists of an immobilised preparation of lactoperoxidase and glucose oxidase, which are carefully blended to provide optimal enzymatic activity as a system. The solid phase support for this dual enzyme system consists of hydrophilic spheres several microns in diameter. On addition of glucose, Na ^{125}I, and protein to an enzymobead suspension, the immobilised glucose oxidase generates a small steady stream of hydrogen peroxide. The lactoperoxidase in turn, catalyses the peroxide oxidation of labelled Iodide to labelled Iodine, which reacts with the protein to produce the radioiodinated protein.

2.6.1. Materials

1) Enzymobead reagent (50 µl)

2) 1% Beta-D (+)-Glucose

3) Disposable tubes

4) 1.0 mCi $Na^{125}I$ (5-25 µl)

5) 0.2M Phosphate Buffer (50 µl)

6) Protein Sample 50-250 µg (10-25 µl)

7) Sephadex G-25 PD-10 Columns

9) Micropipette

10) Retort Stand

2.6.2. Procedures

The class III cabinet and surrounding area was monitored using a mini-monitor and readings recorded. The enzymobead reagent, phosphate buffer and protein sample were placed in a LP4/3 tube with ^{125}I and the total radioactivity used recorded. 50 µl of 1% Beta-D (+)-Glucose was then added to the reaction mixture and iodination was allowed to take place for 5-15 min. After the addition of cold protein the iodination reaction was quenched by applying the reaction mixture onto a PD-10 column. 0.5 ml fractions were then collected using TRIZMA (pH 7.0) as the eluting buffer. All tubes were capped and radioactive content measured. Fractions containing radio iodinated proteins were placed in a lead pot and labelled. All solid waste was sealed in Jencon plastic bags and consigned to the waste containment area. Personal and work area monitoring was carried out and readings recorded.

Newly prepared LDL and acetylated LDL were radiolabeled with ^{125}-Iodine (Amersham, Buckinghamshire, UK) by the Biorad Enzymobead method. The specific activity of ^{125} I-LDL was between 100-200 cpm/ng. The extent of lipid peroxidation of the LDL was estimated as the concentration of thiobarbituric acid reactive substances (TBARS) as described previously (Fernando et al. 1993) and the results expressed as nmol of malondialdehyde per mg LDL (nmol MDA/mg LDL). The level of TBARS in the

iodinated LDL used in the study was less than 0.1 nmol MDA/mg LDL. Agarose gel electrophoretic mobility of ^{125}I-LDL was not different to that of freshly isolated native LDL. 100 μM EDTA and 20 μM BHT was added immediately to the ^{125}I-LDL to prevent oxidation and dialysed for 18 h in 5L of PBS containing EDTA and BHT at the concentrations given above.

2.7. BINDING OF ^{125}I-LDL to HMCL at 4 ^{0}C

Lipoprotein binding experiments were carried out using the methods first described by Brown and Goldstein et al and modified by Innerarity et al. At 4 ^{0}C, lipoproteins bind to LDL receptors, but the lipoprotein-receptor complexes are not internalised. Binding reaches equilibrium in 3-6 h and we have determined that ^{125}I-LDL binding to HMC reaches equilibrium by 4 h at 4 ^{0}C.

2.7.1. Materials

1) Experimental medium: RPMI pH 7.4 containing 0.2% fatty acid free bovine serum albumin (FAF-BSA) and 1 mmol/l $CaCl_2$
2) Buffer A: PBS pH 7.4 containing 0.2 % BSA and 1 mmol/l $CaCl_2$
3) Buffer B: PBS pH 7.4 containing 1 mmol/l $CaCl_2$
4) ^{125}I-Lipoprotein
5) Unlabelled lipoprotein
6) 0.1mol/l NaOH

2.7.2. Procedures

Cells grown to confluence in 12 well clusters were washed twice with 1 ml of RPMI and cooled to < 4 ^{0}C by placing them in a cold room for 30 min on ice. The RPMI was then removed and replaced with 1 ml of experimental medium containing iodinated lipoproteins at a range of concentration between 0 and 100 μg/ml in duplicate for 4 h at 4 ^{0}C. After an incubation period of 4 h with gentle rocking, the

experimental medium was removed and the cells washed using 1 ml of buffer A and buffer B (PBS) in the following sequence:

1) Three rapid washes with buffer A
2) Two 10 min incubations in buffer A with gentle rocking
3) A 5 min incubation with solution B
4) A final rapid wash with solution B

After washing the cells were dissolved by incubation at room temperature for at least 1 h in 1 ml of 0.5 mol/l NaOH. The whole sample was assayed in a γ-counter to determine the ^{125}I radioactivity associated with cells, after which an aliquot was used to determine the content of cellular protein. The specific activity of ^{125}I-LDL ranged between 80-500 CPM/ng LDL protein. The results were expressed as ng of ^{125}I-LDL bound per mg cell protein and the means and SEM of 3 representative experiments is shown in the result section.

Non-specific binding was estimated by measuring ^{125}I-lipoprotein binding in the presence of a 50 fold excess of the corresponding non-iodinated lipoprotein.

2.8. SCATCHARD PLOTS AND CALCULATION OF DISSOCIATION CONSTANTS AND RECEPTOR CONCENTRATIONS

Analysis of ^{125}I-LDL or ^{125}I-AcLDL binding experiments at 4°C was carried out using the EBDA/LIGAND program. Saturation binding curves were analysed using the EBDA/LIGAND program originally written by Munson and Rodbard (Munson and Rodbard, 1980) and modified for microcomputers by McPherson (Mcpherson, 1983), to obtain values for the dissociation constant *Kd* and the total receptor concentration B_{max}. The raw data was first analysed by EBDA to obtain a graphical representation of the data in the form of a Scatchard and Hill plots. This programme also provides initial estimates for *Kd* and B_{max} and produces a file for use in the non-

linear curve-fitting program, LIGAND. Final estimates for *Kd* and B_{max} and a graph of the Scatchard plot was obtained using the LIGAND program.

2.9. HISTOLOGY

Foam cell formation was estimated by intracellular lipid droplets accumulation using oil Red O staining (Ong and Moorhead, 1994).

2.9.1. Materials

1) 5% Formal saline: 8.5 g NaCl, 50 ml formalin (40% H.CHO, Merck Ltd, Lutterworth, Leics, UK), 950 ml distilled H_20
2) Oil Red O (saturated solution): 0.2 g Oil Red O (Sigma, Dorset, UK) 100 ml Iso-Propanol (Merck Ltd, Lutterworth, Leics, UK)
3) Oil Red O working solution: Stock solution : distilled H_2O, 3:2 mix and stand for 10 min and filter through Whatman No.1 filter paper and use within 1 h
4) Carazzi's Haematoxylin: 500 g Potassium or ammonium Alum, 1 g haematoxylin, 0.2 g potassium iodate, 800 ml distilled H_20, 200 ml glycerine (all from Sigma, Dorset, UK). Dissolve the alumin the water and then add the haematoxylin. When this has completely dissolved add the potassium iodate to ripen. Finally add the glycerine when the solution has turned purple.
5) PBS
6) 1,2-Propanediol (Sigma, Dorset, UK)

2.9.2. Procedures

Mesangial cells were grown on tissue culture treated chamber slides and incubated with different treatments for various times. Following the incubation period the chamber slides were washed 3 times with PBS and fixed for 30 min with 5% formal saline and subjected to the following staining procedure.

1) 2 washes with distilled H_2O.
2) Incubated with 1,2-Propanediol for 2 min.
3) Stained with Oil red O working solution for 30 min at room temperature.

4) Rinsed 3 times with distilled H_2O.

5) Carazzi's haematoxylin for 1-2 min.

6) Washed in tap H_20 for 5 min to blue.

7) Drained and mounted in glycerine jelly on glass slides.

Bright-field micrographs were taken using a Leica Orthomat microscope using a magnification of ×40 or ×100 using an Kodak Ektachrome tungsten 60 film.

2.10. CELL LABELLING FOR FLOW CYTOMETRY

The use of DiI-labelled LDL or Ac-LDL as fluorescent probes to detect lipid protein receptor expression is an established method in studies of lipoprotein-receptor interactions and atherosclerosis (Ando et al. 1997; Via et al. 1989).

2.10.1. Materials

1) Ac-LDL or LDL labelled with 1,1'-dioctadecy-3,3,3',3',-tetramethylindocarbocyanine (DiI-Ac-LDL) was obtained from Biogenesis (Poole, UK).
2) 0.5% trypsin-EDTA (Sigma, Dorset, UK)
3) 10% formalin solution, neutral buffered (Sigma, Dorset, UK)
4) Flow cytometer (Coulter, EPICS XL-MCL)

2.10.2. Procedures

HMCL were labelled by 10 μg/ml of DiI-LDL or DiI-Ac-LDL in the presence or absence of 100-fold excess of unlabelled LDL or AC-LDL at 37°C. After 3-5 hours, the cells were detached from the plates by incubation with 0.5% trypsin-EDTA and fixed in 5% formalin solution in PBS.

The fixed cells were washed three times in PBS and analysed by fluorescence-activated cell sorter analysis (FACS) using a flow cytometer (Coulter, EPICS XL-MCL). Forward

angle and 90° light scatter gates were established to exclude dead cells and cell debris from analysis. Fluorescence signals from the accumulated DiI in the cells were collected at 555 to 600 nm by a photomultiplier, converted to digital format and processed for storage and display in one-parameter, log scale frequency histograms. Five thousand cells were analysed in each sample. The data were evaluated by two parameters of the histograms: the percentages of DiI-labelled positive cells and mean fluorescence intensity (MFI). Autofluorescence signals from unlabelled cells were used as negative controls in each experiment. The percentage of DiI-labelled positive cells was calculated by counting labelled cells that exceeded the upper limit of the autofluorescence of unlabelled cells. The MFI of DiI-labelled cells was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells. The average of the duplicate determination was used for statistical analyses.

2.11. WESTERN BLOT ANALYSIS

Sodium dedecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has become the most important method for the analysis of protein (Laemmli, 1970). In this technique, the sample to be fractionated was denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gave the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on a polyacrylamide gel and high voltage was applied, causing the protein components to migrate toward the positive electrode. The protein was transferred to a nitrocellulose membrane, then detected using antisera or specific antibodies. The method

of electrophoretic transfer of proteins from SDS gels to nitrocellulose and radiographic detection with antibody was defined as Western blotting (Towbin et al. 1989; Towbin et al. 1979; Burnette, 1981).

2.11.1. Materials

1) 5xCell lysis buffer (Promega, Hants, UK)
2) Protease inhibitors set: one tablet contains that antipain dihydrochloride, aprotinin, bestatin, chymostatin, E64, EDTA, leupeptin, pefabloc SC, pepstatin, and phosphoramidon. Dissolve one tablet with 10 ml PBS (10X stock solution). (Boehringer Mannheim, E. Sussex).
3) 1xCell lysis buffer: mix 5xCell lysis buffer, protease inhibitor stock solution and PBS in ratio 2:1:7.
4) 10% SDS solution (Bio-Rad, Herts, UK)
5) Sample Buffer (Laemmli): 62.5 mmol/l Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue. (Bio-Rad, Herts, UK)
6) 30% Acrylamide/Bis (29/1) solution (Bio-Rad, Herts, UK)
7) 10% APS: dissolve 0.1 g APS in 1ml dH_2O.
8) TEMED
9) 1xTGS buffer: 25 mmol/l Tris, pH 8.3, 192 mmol/l glycine, 0.1% SDS. (Bio-Rad, Herts, UK)
10) Blot Transfer Buffer: 1xTGS buffer containing 20% methanol (Sigma, Dorset, UK)
11) Coomassie brilliant blue R-250 staining solution (Sigma, Dorset, UK)
12) 10xPBS: 10 mmol/l sodium phosphate, 150 mmol/l NaCl pH 7.4 (Bio-Rad, Herts, UK)

13) 1x PBST (Phosphate buffered saline/1% Tween-20): 1% Tween-20 in phosphate buffered saline

14) 20%DMSO/PBST wash buffer: 20% Dimethyl sulfoxide (DMSO) (Sigma, Dorset, UK) in PBST

15) Antibody dilution buffer: 1% BSA (Sigma, Dorset, UK) in PBST

16) Mouse anti human LDLr monoclonal antibody (Amersham, Little Chalfont, Buck, UK)

17) HRP-linked Goat anti mouse Ig G (Bio-Rad, Herts, UK)

18) Bio-Rad Opti-4CN detection Kit (Bio-Rad, Herts, UK)

19) Nitrocelluose membrane (Bio-Rad, Herts, UK)

20) Bio-Rad mini Protein II apparatus (Bio-Rad, Herts, UK)

2.11.2. Procedures

1) Samples preparations:

Incubate HMCL with 900µl of 1x Cell lysis buffer in T75 flask for 15 minutes, scratch all cells down using a Policeman and collect cell lysates in microtubes. Keep the tubes in ice and vortex tubes for 15 seconds. Centrifuge the tubes for 30 second at 13,000 in room temperature in a micro centrifuge. Collect and keep supernatant at –70°C.

2) Electrophoresis:

The concentration of protein from each sample was measured. The same amount of total protein from HMCL lysates per sample were denatured with 2 volume of sample buffer at 95°C for 10 minutes, then subjected electrophoresis on a 5% stacking and 8% separating

SDS polyacrylamide gel. Electrophoresis was performed in a Bio-Rad mini Protein II apparatus in 1xTGS buffer at 200 Volts (constant) for 45 min.

Preparation of 8% separating SDS polyacrylamide gel (10ml): Mix 4.6 ml of ddH_2O, 2.5 ml of 1.5M Tris-HCl (pH8.8), 0.1ml of 10% SDS, 2.7 ml of 30% acrylamide/Bis, 0.1 ml of 10% APS and 0.006 ml of TEMED.

Preparation of 5% stacking SDS polyacrylamide gel (5ml): mix 3.4 ml of ddH_2O, 0.63 ml of 1.0M Tris-HCl (pH6.8), 0.05 ml of 10% SDS, 0.83 ml of 30% acrylamide/Bis, 0.05 ml of 10% APS and 0.005 ml of TEMED.

3) Electrophoretic transfer

   The samples were transferred to nitro-cellulose membrane at 100 V, 350 mA for 1 hours in blot transfer buffer in a Bio-Rad mini Trans-Blot Transfer Cell.

4) Blotting

In general, antigens immobilised on membranes were detected with antibodies in a three-step process. First, the primary antibody, an IgG directed against the antigen in question, was added to bind potential antigenic sites. In the second step, a secondary antibody-enzyme conjugate which recognises general features of all IgGs (anti-IgG), was added to find locations where the primary antibody bound. The enzyme (AP or HRP) conjugated to the secondary antibody catalyses a colorimetric reaction in the third step, when the appropriate substrate was added, resulting in deposition of coloured substrate on the membrane at the reaction site. This colour provides a visual indication of potential primary antibody recognition.

The membrane was washed twice in 1xPBST, then blocked with 3% blocker reagent for1 hour in room temperature, then followed two 5 minus wash in PBST. The membrane incubated with mouse anti human LDLr monoclonal antibody (1 μg/ml) for 1 hour in antibody dilution buffer followed by two washes in PBST. A goat anti-mouse antibody (Bio-Rad) was diluted in antibody dilution buffer then added to the membrane for 1 hours followed by two washes in PBST. Finally, amplification and colorimetric detection procedures were completed using Bio-Rad Opti-4CN detection Kit (Bio-Rad laboratories).

2.12. RNA ISOLATION

Total RNA was isolated from cultured HMCL by the guanidinium method (Chirgwin et al. 1979). In this method, Guanidine thiocyanate, in association with β-mecaptoethanol and N-lauroyl sarcosine powerfully inhibited RNase, also act to disrupt nucleoprotein complex, allowing RNA to be released into solution. Intact RNA was purified away from contaminants by phenol:chloroform extraction. RNA selectively partitions into the aqueous phase, free from DNA and protein and was easily concentrated by precipitation with isopropanol (Chirgwin et al. 1979; Perry et al. 1972) .

2.12.1. Materials

1) RNase-Free Water: ddH_2O was treated by 0.1% DEPC (Sigma, Dorset, UK) at 37 °C overnight, then autoclaved.

2) 0.75 M sodium citrate (pH7.0): 11.029g of citrate•3 Na (Sigma, Dorset, UK) was dissolved in dH_2O and filled up to 50 ml. Add 0.1 ml of DEPC, stir for 2 hours, then was autoclaved.

3) 10% Sarcosyl: 10 g of N--lauroylsarosine sodium salt (Sigma, Dorset, UK) was dissolved in dH_2O and filled up to 100 ml. Add 0.2 ml of DEPC and stir for about 2 hours, then was autoclaved.

4) Denature solution: Mix 250 g of guanidinium thiocyanate (GTC) (Sigma, Dorset, UK) (final concentration 4 M) with 17.6 ml of 0.75M sodium citrate (pH7.0) (final concentration 25 mmol/l), 26.4 ml of 10% sarcosyl (final concentration 0.5%) and 293 ml of RNase-free water. The mixture was stirred at 65 °C until GTC was dissolved. Before use, add 2-mercaptethanol (Sigma, Dorset, UK) 0.36 ml per 50 ml solution.

5) 2M Sodium acetate (pH 4.0): 27.216g sodium acetate (Sigma, Dorset, UK) was dissolved in dH_2O. Adjust pH using glacial acetic acid. Fill up to 100 ml, then was filtered.

6) 0.5 M EDTA: Add 186.1g of disodium ethylene diamine tetraacetate.$2H_2O$ (Sigma, Dorset, UK) to 800 ml of dH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (about 20g of NaOH pellets). Dispense into aliquots and sterilise by autoclaving.

7) 3M Sodium acetate (pH 5.2): Dissolve 408.1g of sodium acetate.$3H_2O$. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 litre. Dispense into aliquots and sterilise by autoclaving.

8) Phenol:Chloroform:Isoamyl Alcohol (125:24:1, pH 4.7) (Sigma, Dorset, UK)

9) Isopropanol (Sigma, Dorset, UK)

10) Ice-cold ethanol, 75%, RNase-free (Sigma, Dorset, UK)

2.12.2. Procedures

1) Creating a Ribonuclease-Free Environment

Ribonucleases are difficult to inactivate. Therefore, care should be taken to avoid inadvertently introducing RNase activity into the isolation procedure. The following precautions will help to prevent accidental contamination of samples. The more common sources of RNase contamination are the user's hands, and bacteria and moulds that may be present on airborne dust particles. Therefore, gloves were worn at all times and proper microbiological sterile techniques used when handling the reagents. We used sterile disposable plasticware for handling RNA. These materials are generally RNase-free and thus do not require pre-treatment to inactivate RNase. Nondisposable glassware and plasticware was treated before use to ensure that it is RNase-free. Glassware was baked at 200°C overnight. Plasticware was thoroughly rinsed with 0.1N NaOH, 1mM EDTA and then with nuclease-free water. RNase-free materials were used for weighing chemicals. Solutions were be treated by the addition of diethyl pyrocarbonate (DEPC) to 0.1% overnight at room temperature, and then autoclaved for 30 minutes to remove any traces of DEPC. Tris buffers were prepared by using a container of Tris designated only for RNA isolations and DEPC-treated water that had been autoclaved.

2) Total RNA purification

HMCL were cultured in 75-cm2 flask. Before RNA extraction, cultured cells were pelletted and washed once in ice-cold phosphate-buffered saline. The cell pellet was lysed in ice-cold denature solution using 600 µl per 1 flask (about $5x10^6$). Cell lysates were sheared six times through a 21-gauge needle. 0.1 volume (60 µl per flask) of 2 M sodium acetate (pH 4.0) was added to the lysate and mixed thoroughly by inverting the tube 4-5

times. 1 volume of phenol:chloroform:isoamylalcohol was added. The mixture were vortexed after each addition and for at least 10 seconds after the final step. The emulsion was incubated on ice for 15 minutes and then centrifuged at 12,000 rpm for 20 minutes at 4 °C. The aqueous phase is carefully transferred to a fresh RNase free tube, taking care not to touch the interface, added to an equal volume of isopropanol, mixed and precipitated at -20 °C for at least 2 hours. The crude RNA pellet was recovered by centrifugation at 12,000 rpm for 20 minutes at 4°C and washed by resuspending in 1 ml of 75% ice-cold ethanol. The RNA was recovered by centrifugation at 12,000 rpm for 10 minutes at 4 °C. The pellet was dried in air and resuspended in 150 µl RNase free water. 10 µl of 3M sodium acetate (pH 5.2) was added, mix thoroughly by inverting the tube 4-5 times. 1 volume (150 µl) of phenol:chloroform:isoamylalcohol was added. The mixture were vortexed for at least 10 seconds, then centrifuged at 12,000 rpm for 10 minutes at 4 °C. The aqueous phase was carefully transferred to a fresh RNase free tube, then added to an equal volume of 2 volume of ethanol (100%), mixed and precipitated at -20 °C at least 2 hours. The concentration of RNA was determined by measuring the absorbance at 260 nm.

2.13. DNA PROBE LABELLING

The cDNA probes were labelled by nick translation. The nick translation system has been designed for the efficient incorporation of labelled deoxynucleotide triphosphates into duplex DNA. Free 3' hydroxyl ends nicks are created within the unlabelled DNA by DNase I. At the same time, the 5' to 3' exonuclease activity of this enzyme removes the nucleotide from the 5'phosphoryl terminus of the nick. The new nucleotide labelled by ^{32}P was incorporated at the position where the original nucleotide was excised, and the nick is

thus shifted along one nucleotide at a time in a 3' direction. This 3' shift of the nick results in the sequential addition to radioactively labelled nucleotides to the DNA while the pre-existing nucleotides are removed (Sambrook et al. 1989).

The oligonucleotide was labelled using DNA5'end-labelling system. T4 polynucleotide kinase catalyses the transfer of the terminal (γ-^{32}P) phosphate of ATP to the 5'-hydroxyl terminus of a DNA molecule

2.13.1. Materials

1) Nucleotide solution (300 μM): dATP, dTTP and dGTP (Promega, E. Hants, UK)
2) DNA polymerase I/DNase I Mix (Promega, E. Hants, UK)
3) Nick Translation 10x buffer: 500mM Tris-HCl, pH 7.2,100 mmol/l $MgSO_4$, 1mmol/l DTT. (Promega, E. Hants, UK)
4) Stop solution: 0.25 M EDTA, 0.5 M sodium phosphate (Promega, E. Hants, UK)
5) [α-^{32}P] dCTP (3000Ci/mmole and 10mCi/ml) (Amersham, Essex, UK)
6) T4 polynucleotide kinase (Promega, E. Hants, UK)
7) T4 10xbuffer (Promega, E. Hants, UK)
8) γ-^{32}P ATP (3000Ci/mmole, 10 mCi/ml) (Amersham, Essex, UK).
9) 0.5 M EDTA (see section 2.11.1)
10) Sephadex G-50 columns (Pharmacia, Herts, UK)

2.13.2. Procedures

1) Nick translation: Mixed 1μg of cDNA, 10μl of nucleotide mix (dATP, dTTP and dGTP), 5 μl of DNA polymerase I/DNase I mix, 5 μl of nick translation 10X buffer,

7µl of [α-^{32}P] dCTP and dH_2O to a final volume of 50 µl. Incubate the mixture at 15°C for 60 minutes. Add 5 µl of stop solution to stop the reaction. The labelled probes were separated from unincorporated nucleotides by sephadex G-50 columns.

2) 5'end labelling of DNA: Mix 29 µl of oligonucleotides (up to 10 pmoles of 5'-ends), 15 µl of γ-^{32}P ATP (3000Ci/mmole, 10 mCi/ml), 1µl of T4 polynucleotide kinase (8-10U/µl) and 5µl of T4 10xbuffer. Adjust the volume to 50 µl. Incubate at 37°C for 10 minutes. Stop the reaction by adding 2 µl of 0.5 M EDTA. The labelled probes were separated from unincorporated nucleotides by sephadex G-50 columns (Pharmacia, Herts, UK).

2.14. RNA ELECTROPHORESIS AND NORTHERN BLOT

Northern Blot is a method for the analysis of RNA yield information on the size, abundance, splice point, in which total or poly(A) mRNA is run on denaturing agarose gel and detected by hybridisation of a labelled probe on a membrane. The method used in this thesis is based on the protocol described by Sambrook (Sambrook et al. 1989).

2.14.1. Materials

1) 1M MOPS solution: 104.6g of MOPS (Sigma, Dorset, UK) was dissolved in 400 ml dH_2O, adjust pH to 7.0 with NaOH, fill up to 500 ml, then was autoclaved.
2) 10X MOPS buffer: Mix 100 ml of 1M MOPS solution (final concentration 200 mmol/l) with 8.3 ml of 3 mol/l sodium acetate (pH7.0) (Sigma, Dorset, UK) (final

concentration 50 mmol/l), 10 ml of 0.5 mol/l EDTA (final concentration 10 mmol/l) and 381.7 ml of dH_2O.

3) Sample buffer**:** Mix 587 µl of deionised formamide (Sigma, Dorset, UK), 182 µl of formaldehyde (Sigma, Dorset, UK), 138 µl of 10xMOPS buffer and 53 µl of DEPC-treated water in a total volume 960 µl.

4) 50x Denhardt's reagent: Dissolve 5g Ficoll (Type 400), 5g Polyvinylpyrrolidone and 5g Bovine serum albumin (Fraction V) in up to 500 ml dH_2O. All reagents obtained from Sigma Ltd (Dorset, UK). Filter through a filter. Dispense into 25-ml aliquots and store at -20 °C.

5) 20X SSC: dissolve 175.3g NaCl (Sigma, Dorset, UK) and 88.2 g of sodium citrate in 800 ml of dH_2O. Adjust pH to 7.0 with a few drops of a 10 N solution of NaOH (Sigma, Dorset, UK). Adjust volume to 1 litre. Sterilise by autoclaving.

6) 20X SSPE: dissolve 174 g of NaCl, 27.6 g of $NaH_2PO_4.H_2O$ (Sigma, Dorset, UK) and 7.4 g of EDTA (Sigma, Dorset, UK) in 800 ml of dH_2O. Adjust pH to 7.4 with NaOH (about 6.5 ml of a 10N solution). Adjust volume to 1 litre. Sterilised by autoclaving.

7) Prehybridisation buffer: 50% formamide, 5×SSPE, 5× Denhardt's, 100 µg/ml hsDNA

8) Hybridisation buffer: 50% formamide, 5×SSPE, 1× Denhardt's, 100 µg/ml hsDNA.

9) Nylon membranes (positive charge) (Boehringer Mannheim, East Sussex, UK).

10) Agarose gel (Gibco, UK)

11) Hybridisation oven.

2.14.2. Procedures

To make 1% agarose gel, weigh_1.8 g agarose and mix it with 152.1 ml of DEPC-treated water, 18 ml of 10xMOPS buffer. Melt the gel in microwave oven, then add 9.9 ml of formaldehyde (37%) to total volume 180 ml in a fume hood. The agarose solution was mixed well and then poured into an RNase-free 15x10 cm^2 gel tray. The gel was allowed to sit for 1 hour before use.

20 μg of total RNA were denatured with sample buffer. 1 volume of RNA sample was mixed with 4 volumes of sample buffer and heated to 65 °C for 15 minutes and then put onto the ice for denaturing the RNA before it was loaded. The denatured RNA was subjected to electrophoresis in 1X MOPS buffer in constant 50 voltage at room temperature for 4 hours.

After electrophoresis, the gel was soaked with 20x SSC for 45 minutes with gentle shaking. The RNA was transferred to nylon membranes by capillary blotting with 20×SSC for 18 hours. After RNA was transferred, the membranes were baked for 30 minutes at 120 °C for fixing the RNA to the membranes and used in hybridisation directly or stored dry between two filter paper in plastic at room temperature.

Prehybridisation were performed in prehybridisation buffer for 2 hours at 42 °C. The blots were hybridised in hybridisation buffer overnight at 42 °C. The filters were washed two 5 minutes in 2×SSC with 0.1% SDS at room temperature, followed by two 15 minutes high stringency wash in 0.1×SSC, 0.1% SDS at 68 °C. The filters were exposed to Kodak X-film at -70°C.

2.15. RT-PCR

The thermostable polymerases used in the basic PCR process require a DNA template. In order to apply PCR methodology to the study of RNA, the RNA sample must first be revere transcribed to cDNA to provide the necessary DNA template for the therostable polymerase . This process is called reverse transcription (RT), hence the name RT-PCR (Becker-Andre and Hahlbrock, 1989).

2.15.1. Materials

All reagents were obtained from Perkin-Elmer (PE Applied Biosystems Ltd, Warrington, Cheshire, UK).

1) 10xPCR buffer II: 500 mmol/l KCl, 100 mmol/l Tris/HCl
2) 25 mmol/l $MgCl_2$ solution
3) DNTPs: 10mmol/l deoxyribonucleoside triphosphates
4) Random Hexamers 50 μmol/l
5) RNase inhibitor (20Unit/μl)
6) M-MLV reverse transcriptase (50Unit/μl)
7) *Taq* DNA polymerase (5 Unit/μl)

2.15.2. Procedures

Total RNA (500 ng) was used as a template for RT-PCR. The RT reaction was set up in a 20 μl mixture containing 50 mmol/l KCl, 10 mmol/l Tris/HCl, 5 mmol/l $MgCl_2$, 1 mmol/l of each dNTPs, 2.5 μmol/l randomhexamers, 20 U RNase inhibitor (RNAsin), and 50 U of M-MLV reverse transcriptase. Incubations were performed in a DNA Thermal Cycler

(Perkin-Elmer 9600) for 10 minutes at room temperature, followed by 30 minutes at 42 °C and 5 minutes at 99 °C. After cDNA synthesis by RT, the incubation mixture was split into two 10 µl aliquots for separate amplification of cDNA using specific primers. For PCR, the final concentrations of the PCR reaction mixture were 50 mmol/l KCl, 10 mmol/l Tris/HCl, 2 mmol/l $MgCl_2$, 200 µmol/l dNTPs, 0.125-0.25 µmol/l of primers, 1.25 U *Taq* DNA polymerase. After incubation for 145 seconds at 95°C, 28-36 cycles were performed for 30 seconds at 95 °C, 30 seconds at 55 -65°C and 60 seconds at 72 °C. Twenty microlitres of each PCR reaction were subjected to electrophoresis in a 2% agarose gel.

2.16. SOUTHERN BLOT ANALYSIS

The PCR product was transferring to a nylon membrane from agarose gel then subjected to Southern blot analysis using a specific internal oligo nucleotide probe which located in between two PCR primers. The method for Southern Blotting used in this thesis was based on the protocol described by Sambrook (Sambrook et al. 1989).

2.16.1. Materials

1) Gel denature solution: 1.5M NaCl, 0.5 M NaOH
2) Gel neutralisation solution: 1M Tris (pH 8.0), 1.5M NaCl
3) 50x Denhardt's reagent: see section 2.13.1
4) 20X SSC : see section 2.13.1
5) 20X SSPE: see section 2.13.1
6) Prehybridisation buffer: 50% formamide, 5×Denhardt's solution, 5×SSPE, 100 µg/ml hsDNA, 50 mmol/l sodium phosphate (pH 6.8).

7) Hybridisation buffer: 50% formamide, 5×SSPE, 100 μg/ml hsDNA, 50 mmol/l sodium phosphate (pH 6.8)
8) Nylon membranes (positive charge) (Boehringer Mannheim, East Sussex, UK)
9) Agarose gel (Gibco, UK)
10) UV crosslink (Stratagene, UK)
11) Hybridisation oven.

2.16.2. Procedures

2.16.2.1DNA electrophoresis

After PCR is completed, 20 μl of PCR product was loaded with 4 μl of DNA loading buffer on 2% agarose gel. The fragment of DNA was separated by electrophoresis. When electrophoresis finished, DNA was then photographed in agarose gels stained with ethidium bromide by illumination with UV light.

Transfer of DNA onto filters after electrophoresis, the gel was transferred to a container. The DNA on the gel was denatured by soaking the gel for 45 minutes in several volumes of gel denature solution with constant gently agitation. The gel was then rinsed briefly in deionised water and neutralised by soaking gel for 1 hours in several volumes of a solution of gel neutralisation at room temperature with constant, gentle agitation.

The gel was removed from neutralisation solution and placed inverted on the support. The DNA in the gel was transferred to the Nylon membranes in 20 x SSC by capillary action using soft tissues to enhance capillary action. After the DNA was transferred, the membranes was dry in room temperature for 1 hours, then DNA was fixed by UV crosslinking for 3 minutes with UV 254 nm (Stratagene). The membrane used in

hybridisation directly or stored dry between two filter paper in plastic at room temperature.

2.16.2.2. Southern hybridisation

Membranes prehybridised in prehybridisation buffer at 42 °C for 4 hours, and hybridised in hybridisation buffer overnight at 42 °C with at least 1×10^6 cpm/ml of the labelled probe. The membranes were washed twice for 15 minutes at room temperature with 5×SSC, then washed for 10 minutes at 42 °C with 5×SSC. The blots were exposed to X-ray films (Kodak) for 4 hours. Relative radiation density (the ratio of Scr to GAPDH) was calculated for each sample to adjust for the differences in RNA mass between templates and was used for the quantitative comparisons.

2.17. CLONING IN PLASMID VECTORS

A DNA fragment can be cloned into a plasmid vector depends on several factors for different applications such as protein expression and reporter gene studies. All methods used in this thesis were based on the protocols described by Sambrook (Sambrook et al. 1989).

2.17.1. Transformation for plasmid DNA

2.17.1.1. Materials

1) High efficiency competent cells HB101 or JM109 (Promega, E. Hants, UK)
2) 2M Mg^{2+} stock: Dissolve 101.5g $MgCl_2.6H_2O$ and 123.3g $MgSO_4.7H_2O$ in 500 ml distilled water and filter sterilise through a 0.2 μm filter unit.
3) Ampicillin stock solution (50mg/ml) (Sigma, Dorset, UK)

4) SOC medium: Add 2.0g bacto-Trypton, 0.5g bacto-yeast extract. 1ml of IM NaCl, 0.25ml of 1M KCl in 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose stock, each to a final concentration 20mM. The pH should be 7.0.
5) LB medium: dissolve 20 g LB broth base (Sigma, Dorset, UK) in 1 L of deionised water. Sterilised by autoclaving. Add ampicillin to a final concentration of 50 μg/ml before use.
6) LB agar antibiotic plate: Dissolve 35 g LB agar (Sigma, Dorset, UK) in 1 L of deionised water. Sterilised by autoclaving. Add ampicillin to the LB agar (final concentration 50μg/ml) when the temperature drop to 60 °C, then make LB agar plates. LB agar plates were stored in fridge.

2.17.1.2. Procedures

Remove the tube of frozen competent cells from -70 °C and place in an ice-bath for 5 minutes. Gently mix the cells and remove 100 μl per transformation into a sterile pre-chilled polypropylene tube. Add 1-50 ng of plasmid DNA to the 100 μl of competent cells and place the tube on ice immediately for 10 minutes. Heat shocks the cells for 45 seconds in a 42 °C water bath, then immediately place the tubes on ice for 2 minutes. Add 900 μl of room temperature SOC medium and incubate for 60 minutes at 37 °C with shaking at 225 rpm. Plate 100-200 μl of the transformation mix onto antibiotic plate (ampicillin 50 μg/ml) and incubate overnight for selection of positive clone.

Pick up a positive clone into a 5 ml of LB medium with 50 μg/ml ampicillin and shake it at 225 rpm in 37 °C for overnight. Overnight culture in LB was subjected to Mini-preparation for identification.

2.17.2. Mini-preparation of plasmid DNA

2.17.2.1. Materials

1) Solution I: 50 mmol/l glucose (Sigma, Dorset, UK), 25 mmol/l Tris.Cl (pH8.0), 10 mmol/l EDTA.

2) Solution II: 0.2 N NaOH, 1% SDS. It was made up from stock solutions of 10 N NaOH and 20% SDS.

3) Solution III: Mix 60 ml of 5 M KOAC, 11.5 ml of HOAC and 28.5 ml of dH_2O

2.17.2.2. Procedures

Grow 5 ml overnight culture in LB containing 50μg/ml ampicillin. Take 1.5 ml of culture in a 1.5 ml tubes and centrifuge the cells at 12,000 rpm for 30 seconds at 4°C. Remove and discard the supernatant. Resuspend the cells in 100 μl of solution I, shake vigorously. Add 200 μl of freshly prepared Solution II and mix carefully and thoroughly by inversion. Incubate sample for 5 minutes in room temperature. Add 150 μl of ice-cold Solution III and shake gently 10 seconds then put in the tube in ice for 5 minutes. Centrifuge the tube at 12000 rpm for 5 minutes. Transfer the supernatant to a fresh tube and add 1 volume phenol. Mix and spin at 12,000 rpm for 2 minutes at 4 °C. Transfer the upper phase to a new tube and add 1 volumes of isopropanol. Mix and keep the tube in -20 °C for 20 minutes. Centrifuge at 12,000 x g for 5 minutes at 4 °C. Remove the supernatant and wash the pellet with ice-cold 70% ethanol. Centrifuge at 12,000 rpm for 5 minutes. Dry the

pellet under vacuum. Dissolve the pellet in 50 µl H_2O or TE buffer with 50 µg/ml Ribonuclease A.

2.17.3. Identification of plasmid DNA

The plasmid DNA was digested by specific restriction enzyme then subjected to electrophoresis in 1% agarose gel.

2.17.4. Maxi-preparation of plasmid DNA

2.17.4.1. Materials: same to the mini-preparation

2.17.4.2. Procedures

Grow a 500 ml overnight culture in LB containing 50µg/ml ampicillin. Centrifuge the cells at 5,000 x g for 15 minutes at 4°C. Remove and discard the supernatant. Resuspend the cells in 18 ml of solution I. Add 40 ml of prepared Solution II and mix carefully and thoroughly by inversion (do not vortex), and incubate sample for 5 minutes in room temperature. Add 20 ml of ice-cold Solution III and mix thoroughly and put the tube in ice for 10 minutes. Centrifuge the tube at 12,000 x g for 15 minutes. Transfer the supernatant to a fresh tube, avoiding the white precipitate. Transfer the upper, aqueous phase to a fresh tube and add 0.6 volumes of isopropanol. Incubate the tube in room temperature for 10 minutes Centrifuge at 12,000 x g for 15 minutes at room temperature. Remove the supernatant and wash the pellet with 70% ethanol. Centrifuge at 12,000 x g for 5 minutes. Dry the pellet under vacuum. Dissolve the pellet in 3 ml H_2O or TE buffer (100-500µl).
The DNA was purified using CsCl gradient ultracentrifugation

2.17.5. Purification of plasmid DNA

2.17.5.1. Materials

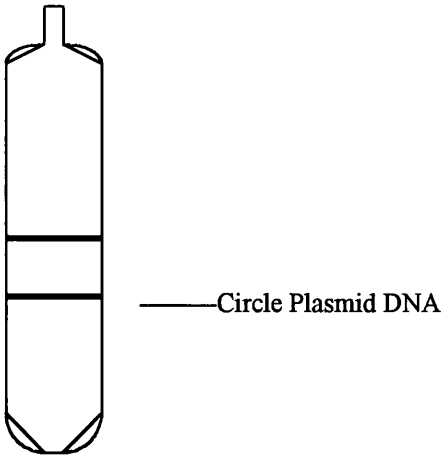
1) TE buffer pH8.0

2) CsCl (Sigma, Dorset, UK)

3) Beckman ultracentrifuge and rotor NVT65.2

2.17.5.2. Procedures

Dissolve the DNA from 500 ml culture in 4 ml TE (pH 8.0). Add 4.35 g CsCl to DNA solution and mix gently until all of the salt is dissolved. Fill DNA into a Beckman quick seal ultracentrifuge tube (5ml). Add 200-300 μl of EB (10 mg/ml) to the ultracentrifuge tube and mix well. Fill the remainder of the tube with light paraffin oil. Centrifuge at the sample at 45,000 rpm for 25 hours at 20 °C using Beckman rotor NVT65.2. Two bands of DNA were visible in ordinary light. The upper band consists of linear bacterial DNA and nicked circularplasmid DNA; the lower band consists of closed circular plasmid. Remove the cap from the tube. Collect the lower band of DNA in a new tube through a 17 gauge needle inserted into the side of the tube.



2.17.6. Protruding 5'-termianl dephosphorylation of vector DNA

The fragment and vector DNA should be digested with restriction enzymes that will generate compatible ends for cloning. The vector also was treat with calf intestinal phosphatase (CIAP) to remove 5′ phosphate groups, thus preventing reclosure of the vector on itself without an insert.

2.17.6.1. Materials

1) Calf intestinal alkaline phosphatase (CIAP) (Promega, E. Hants, UK)
2) 10X CIAP buffer (Promega, E. Hants, UK)
3) 0.5 M EDTA
4) Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Sigma, Dorset, UK)
5) 7.5 M ammonium acetate (Sigma, Dorset, UK)
6) 100% and 70% ethanol

2.17.6.2. Procedures

Plasmid DNA was incubated with specific enzyme in appropriate restriction enzyme buffer for overnight. Check reaction completion by electrophoresis of a sample on 1% agarose mini-gel. Subsequent to digestion, the DNA was treated with CIAP as follows. For protruding 5'-terminal dephosphorylation. To calculate the pmole of ends of linear doubles-stranded DNA using formula: (μg DNA + kb size of DNA)×3.08. Add CIAP at 0.01 U/pmole of ends; 10 μl of 10X CIAP buffer to digested DNA and adjust final volume 100 μl with dH_2O. Incubate for 30 minutes at 37°C. Add another 0.01 U CIAP/pmole of ends and incubate an additional 30 minutes at 37°C. To stop the reaction, add 2.0 μl of 0.5 M EDTA and heat for 20 minutes at 65°C. Add 1 volume of phenol:chloroform:isoamyl alcohol. Vortex for 1 minutes and centrifuge at 12,000 rpm for 2 minutes. Transfer the

upper aqueous phase to a fresh tube and add 1 volume of chloroform isoamyl alcohol. Vortex for 1 minutes and centrifuge at 12,000 rpm for 2 minutes. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5 mol/l ammonium acetate. Add 2 volume of 100% ethanol and leave at -70°C for at least 30 minutes. Centrifuge at 12,000 rpm for 15 minutes. Carefully pour off the supernatant, wash the pellet with 1 ml of 70% ethanol, dry the sample and resuspend the pellet in 10 µl dH_2O or TE. Determine the exact DNA concentration by absorption spectroscopy at 260nm.

2.17.7. Preparation of Insert for Cloning

2.17.7.1. Materials

7) Specific restriction enzyme (Sigma. Dorset, UK)
8) Agarose (Gibco, UK)
9) QIAquick Gel Extraction Kit (QIAGEN, West Sussex, UK).

2.17.7.2. Procedures

To ensure capture of the correct insert DNA, plasmid DNA which contains target DNA fragment was digested for overnight and the desired restriction fragment can be purified by electrophoresis on agarose gel and then recovered from the gel by QIAquick Gel Extraction Kit.

2.17. 8. Ligation

2.17.8.1. Materials

10) T4 DNA ligase (Promega, E. Hants, UK)
11) 10x T4 DNA ligase buffer (Promega, E. Hants, UK)

12) Vectors (see section 2.16.6)

13) Insert DNA (see section 2.16.7)

14) High efficiency competent cells JM109 (Promega, E. Hants, UK)

2.17.8.2. Procedures

Test various vector: insert DNA ratios in order to find the optimum ratio for a particular vector and insert using following formula:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

Vector DNA (50ng) and insert were incubated with T4 DNA ligase and buffer for 3 hours at 22 °C for sticky ends. Following the ligation reaction, remove a 2 µl aliquot for the transformation step. The ligation product was transformed into competent cells of JM109 as described in section 2.16.1. The positive clones were identified using them methods as described in section 2.16.2-4.

2.18. TRANSIENT EXPRESSION ASSAY

Gene reporter systems have contributed greatly to the study of eukaryotic gene expression and regulation. Although reporter genes have played a significant role in numerous applications both in vitro and in vivo (Alam and Cook, 1990), they are most frequently used as indicators of transcriptional activity in cells (Rosenthal, 1987). Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transferred into cells. Following transfer, the cells are assayed for the presence of the reporter protein. An ideal reporter gene is not endogenously expressed in the cell type of interest. In this

thesis, we used firefly luciferase & β-galactosidase as reporter proteins which are very sensitive, quantitative, rapid, reproducible and easy to be detected.

2.18.1. Materials

1) Reporter genes
2) Plasmid of psv-β-galactosidase
3) Report lysis buffer (Promega, E. Hants, UK)
4) Luciferase assay buffer (Promega, E. Hants, UK)
5) β-galactosidase assay buffer(Promega, E. Hants, UK)
6) Gene Pulser (Bio-Rad, Herts, UK)
7) Luminometer 1250 (LKB, UK)
8) Spectrophotometer (Perkin Elmer, UK)

2.18.2. Procedures

HMCL in 6×10^7/ml were transfected with 100 μg of supercoiled reporter genes and 100 μg control plasmid of psv-β-galactosidase by electroporation. Standard electroporation conditions were 340 volts and 125 uF with a Gene Pulser (Bio-Rad, Herts, UK). After electroporation, the cells were placed into 6 well plates with growth medium. Approximately 24 hours after replating, cells were washed with PBS and the medium was replaced with RPMI 1640 or RPMI 1640 plus treatments for various times. The cells then were washed twice with PBS and lysed by cell lysis buffer (250 μl/well). Rock the plate slowly several times to ensure complete coverage of the cells. Incubate at room temperature for 15 minutes, rocking the dish halfway through the incubation period. Scrape all areas of the dish then tilt the plate. Take care to scrape down all visible cell

debris. Transfer the cell lysate to a microcentrifuge tube with a pipette and place the tube on ice. Vortex the tube for 10-15 seconds, then centrifuge at top speed in a microcentrifuge for 15 seconds at room temperature. Transfer the supernatant to a fresh tube and the tubes were kept in –70C°.

The luciferase activity driven by the targeting promoter is assayed by measurement of light production upon addition of luciferin and ATP using a luminometer. The assay was performed by adding 20 µl sample to 100 µl of luciferase assay buffer. Generally, these assays are very rapid and sensitive. The reaction is monitored in a luminometer for 1 minutes. The light intensity of the reaction is nearly constant for about 20 seconds and then decays slowly, with a half-life of about 5 minutes.

The β-galactosidase activity in the supernatants was measured. The assay was performed by adding a sample to an equal volume of assay 2x buffer that contains the substrate O-nitrophenyl-β-D-galactopyranoside (ONPG). Samples were incubated for 30 min to 3 hours, during which time the β-galactosidase hydrolyses ONPG to O-nitrophenol (which is yellow) and galactose. The reaction then is terminated and the absorbance at 420nm is read with a spectrophotometer.

The luciferase activity in the supernatants was measured and normalised by comparison with β-galactosidase enzyme activity

2.19. ESTABLISHMENT OF THE CELL LINE BY STABLE TRANSFECTION

The study of eukaryotic gene regulation and expression has been advanced by technology which allows the introduction of nucleic acids into eukaryotic cells. Physical and chemical methods are used to introduce nucleic acids in process referred to as transfection. Transfection of mammalian cells with expression and/or selection vectors can result in suboptimal expression of the protein of interest. The stable cell line could be established by selecting transfected cells with the antibioticG418 (Goeddel, 1990).

2.19.1. Materials

1) Expressive vector
2) SuperFect transfection reagent (QIAGEN, West Sussex, UK)
3) Antibiotic G418 (Sigma, Dorset, UK)

2.19.2. Procedures

HMCL were transfected with linear expressive vector, which contains cDNA encoding targeting gene using SuperFect Reagent (QIAGEN, Crawley, West Sussex, UK). Two days after transfection, G418 was added to a final concentration (400 μg/ml) and resistant cells were selected over 2 weeks. Transfected cells expressing high levels of targeting gene were detected using RT-PCR (gene) and FACS (protein).

2.20. DATA ANALYSIS

In all experiments, groups of data were evaluated for significance by one way analysis of variance (*ANOVA)* using Minitab software. Data were considered significant if the p value ≤ 0.05.

CHAPTER 3. HUMAN MESANGIAL CELLS EXPRESS AN INDUCIBLE MACROPHAGE SCAVENGER RECEPTOR

3.1. INTRODUCTION

Scavenger receptors class A (Scr) are integral membrane proteins that bind and internalise a broad array of ligands, including acetylated and oxidised low-density lipoprotein (Goldstein et al. 1979; Steinbrecher et al. 1989; Parthasarathy et al. 1986). Although their normal physiological role remains uncertain, biochemical studies have demonstrated that both isoforms (Scr AI and Scr AII) of scavenger receptor are capable of binding and internalising acetylated LDL (Ac-LDL) and oxidised LDL (Ox-LDL) (Kodama et al. 1990; Freeman et al. 1991). Non-macrophage cells transfected with cDNAs encoding scavenger receptor accumulate cholesterol and develop foam cell characteristics when incubated with modified LDL, whereas mock-transfected cells do not (Freeman et al. 1991). These findings suggest that the expression of scavenger receptor protein might be both necessary and sufficient to account for foam cell formation in vivo. Conventionally, development of fatty streak lesion based solely on the presence of oxidatively modified LDL within the arterial wall (Steinberg et al. 1989). Within the vessel wall, the native LDL is oxidised either by endothelial cells, macrophages, smooth muscle cells (SMC), or mesangial cells contributing to subsequent recruitment of circulating monocytes through the generation of monocyte chemotactic protein–1 (MCP-1). These monocytes then undergo phenotypic modifications under the influence of Ox-LDL and cytokines and become tissue macrophages. Their return to plasma compartment is now inhibited by the Ox-LDL. Tissue macrophages take up Ox-LDL through the scavenger receptor pathway and become foam cells. "Lipoprotein toxicity" in the arteries is called atherosclerosis (Parthasarathy et al. 1986; Henriksen et al. 1981; Heinecke et al. 1986; Fernando et al. 1998). It is our contention that similar mechanisms are operational in the development of glomerulosclerosis and human mesangial cells play

an important role in this process. On the basis ability of scavenger receptor to internalise and degrade Ox-LDL, scavenger receptor also appears to provide a mechanism for the development of cholesterol-engorged mesangial cells.

Mesangial cells play a central role in the physiology and pathophysiology of the glomerulus and are thought to have macrophage-like properties. They are also capable of oxidising LDL in vitro. Previous studies characterising the expression of scavenger receptor on human mesangial cells have been conflicting. In vitro, Gröne reported that Ac-LDL and Ox-LDL were not taken up specifically, suggesting no active scavenger receptor in cultures of human mesangial cells (HMC) (Grone et al. 1992). Lee also reported that there was very low Ox-LDL binding and internalising activity in normal cultures of HMC compared to rat mesangial cells (Lee and Koh, 1994). However, Takemura demonstrated scavenger receptor on the membranes of mesangial cells of renal biopsy tissues from patients with several types of glomerular diseases and that the expression of scavenger receptor was increased in glomeruli with marked mesangial proliferation (Takemura et al. 1993). One explanation for these discrepancies is that growth conditions in culture led to alterations in scavenger receptor gene expression that would not occur in vivo. Therefore, it is our hypothesis that there may be inducible expression of scavenger receptor in HMC.

This study was designed to first explore the induction of scavenger receptor on HMC under influence of phorbol 12-myristate 13-acetate (PMA) and Angiotensin II (Ang II); secondly, to study the influence of the inflammatory cytokines on the induction of scavenger receptor. PMA, which activate PKC pathway, induces differentiation of human THP-1 monocytic cells into macrophages, and both scavenger receptor activity

and scavenger receptor mRNA appear to be up-regulated during this transformation (Moulton et al. 1992). Ang II is known to activate PKC in HMC and has been shown to possess atherogenic properties (Ruan and Arendshorst, 1996; Barnett et al. 1995). It increases the activity of the macrophage scavenger receptor, enhances macrophage lipid peroxidation both in vivo and in vitro, and also binds to LDL, forming modified LDL which is taken up by macrophage scavenger receptor at an enhanced rate (Keidar, 1998). Activator protein-1 (AP-1) and ets transcription factors are necessary for scavenger receptor induction by PMA (Wu et al. 1994). As HMC exhibit macrophage-like function, the present study was undertaken to determine whether PMA and Ang II could induce scavenger receptor expression in HMC and to investigate the role of AP-1/ets transcription factors in scavenger receptor expression.

Inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), and interleukin-1β (IL-1β) have been shown to have an important role in progressive glomerulosclerosis in various animal models and clinical studies. Elevated plasma levels of cytokines including TNF-α, IL-1β have been found in various kidney diseases (Fine et al. 1992; Boswell et al. 1988; Luger et al. 1987; Levy et al. 1993). Therefore, the second part of this study was undertaken to investigate whether a sub-mitogenic dose of cytokines, which could not induce HMC proliferation, but could induce scavenger receptor expression on human mesangial cells. An understanding of this mechanism is essential to see whether inflammatory cytokines have any major effect in influencing lipid mediated glomerular injury.

3.2. METHODS

3.2.1. Cell culture

An established stable human mesangial cell line (HMCL) was used in all experiments (kindly donated by Dr.J.D.Sraer, Hôpital Tenon, Paris). Experiments were carried out in serum free RPMI medium containing 0.2% bovine serum albumin (BSA, Sigma, Poole, Dorset, UK). All reagents for cell culture were obtained from Gibco BRL (Paisley, UK).

Primary human mesangial cells (HMC) were cultured from glomeruli isolated by differential sieving technique (sieve mesh sizes 250 and 106 μm) from normal cortical parts of kidneys from human donor kidneys unsuitable for transplantation. HMC were cultured in the same medium with HMCL as described in Chapter 2.1 except that 20% of FCS was used and these cells were used between passages 6 to 12.

3.2.2. Preparation of acetylated lipoprotein

Plasma was collected from healthy human volunteers. LDL was isolated by sequential ultracentrifugation (Fernando et al. 1993) and acetylated (Ac-LDL) by the method as described in chapter2 (Fig.3.1.). Ac-LDL labelled with 1,1'-dioctadecy-3,3,3',3',-tetramethylindocarbocyanine (DiI-Ac-LDL) was obtained from Biogenesis (Poole, UK).

3.2.3. Iodination of Ac-LDL

Ac-LDL was iodinated using the enzymobead method as previously described in chapter 2.

3.2.4 Characterisation of scavenger receptor in HMCL (binding study)

HMCL in twelve well cluster were incubated for 24 h in serum free medium and then incubated with ^{125}I-AcLDL at a range of concentrations between 0 and 100 μg/ml in duplicate for 4 h at 4°C. This temperature was selected for avoiding the internalisation of the bound ligand. Non-specific binding was estimated by measuring ^{125}I-AcLDL bound to HMCL in the presence of a 50 fold excess of unlabelled Ac-LDL. After the incubation period the cell monolayers were washed as described in Chapter 2.7 and radioactivity associated with cells measured by γ-counting. The results were expressed as ng of ^{125}I-AcLDLbound per mg cell protein. Specific binding was calculated by subtraction of non-specific binding from total binding values.

3.2.5. Cell labelling for flow cytometry

HMCL were cultured in serum free medium containing different concentrations of PMA and Ang II for various times before addition of 10 μg/ml of DiI-Ac-LDL in the presence or absence of 100-fold excess of unlabelled Ac-LDL at 37°C. After 3 hours, the cells were detached from the plates by incubation with 0.5% trypsin-EDTA and fixed in 5% formalin solution in PBS. Each experiment was carried out in duplicate.

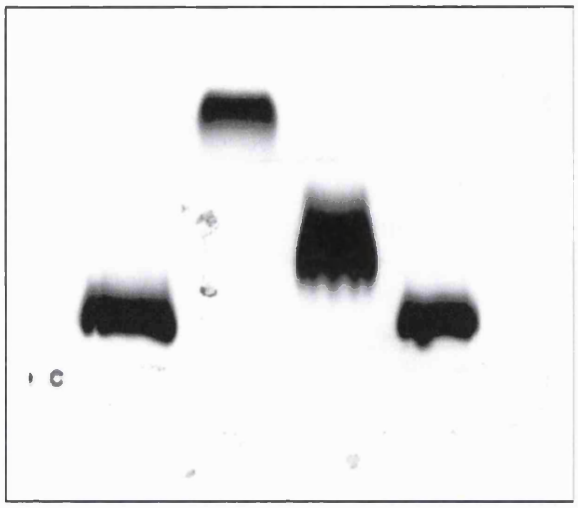


Fig 3.1. Agarose gel electrophoretic mobility of native LDL, Ac-LDL and Ox-LDL. Ac-LDL was prepared as described in chapter 2. 2μl of lipoproteins were loaded in BECKMAN Paragen LIPO Gel for electrophoresis. Lane 1& 4: freshly isolated native LDL. Lane 2: Ac-LDL. Lane 3, Ox-LDL.

3.2.6. Flow cytometry analysis

The fixed cells were washed three times in PBS and analysed by fluorescence-activated cell sorter analysis (FACS) using a flow cytometer (Coulter, EPICS XL-MCL) (see chapter 2).

3.2.7. Establishment of stable cell line expressing a high level of scavenger receptor

HMCL were co-transfected with expressive vector pXhSR1 which contains cDNA encoding scavenger receptor (kindly donated by Dr. Kodama, University of Tokyo) and pUC18Neo (kindly donated by Dr Wang, University of Cambridge) which contains cDNA encoding a G418 resistant gene, using SuperFect Reagent (QIAGEN, Crawley, West

Sussex, UK). The day before transfection, seed HMCL at density of 8×10^{5} in 25 cm^2 tissue culture flask. Incubate the cells at 37°C and 5% CO_2 in an incubator. Dilute 2.5 μg of linear pXhSR1 DNA and 2.5 μg of linear pUC18Neo in TE buffer (pH 7.4) with cell growth medium containing no serum, protein, or antibiotics to a total volume of 150 μl. Mix and spin down the solution for a few seconds to remove drops from the top of the tube. Add 30 μl of SuperFect transfection Reagent to the DNA solution. Mix by pipetting up and down 5 times, or vortexing for 10 seconds. Incubate the samples for 10 min at room temperature to allow complex formation. While complex formation takes place, gently aspirate the growth medium from the dish and wash cells once with 4 ml PBS. Add 1 ml of cell growth medium to reaction tube containing the transfection complexes, Mix by pipetting up and down twice, and immediately transfer the total volume to the cells in the flask. Incubate cells with the complexes for 2-3 hours at 37°C and 5% CO_2. Remove medium containing the remaining complexes from the cells by gentle aspiration and wash cells 3-4 times with 4 ml of PBS. Add fresh cell growth medium and incubate for 48 hours. Two days after transfection, G418 was added to a final concentration (400 μg/ml) and resistant cells were selected over 2 weeks. Transfected cells (HMCL-Scr) expressing high levels of scavenger receptor activity were detected using RT-PCR and FACS. HMCL-Scr was subjected to morphologic studies (Fig.3.2).

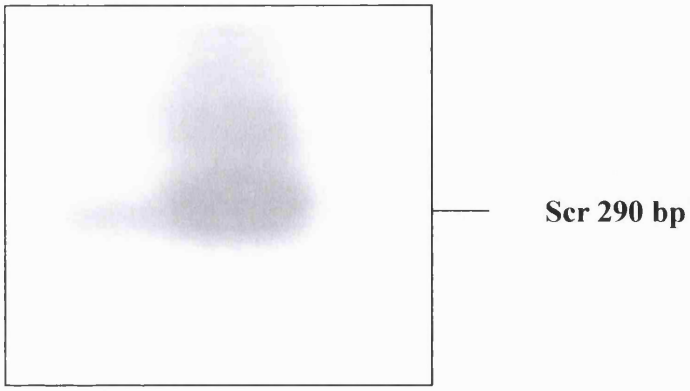


Fig 3.2. Scavenger receptor mRNA expression in normal and transfected HMCL. Total RNA from normal HMCL and transfected cell line by pXhSR1. RT-PCR followed by a southern blot was performed using specific primer and probe as described in the **Section 3.2.7 or 3.2.8**.

3.2.8. Morphological examination

HMCL or HMCL-Scr were plated in chamber slides for tissue culture (Nunc Inc., Naperville, IL) and incubated in serum free RPMI medium, or serum free medium in the presence of 16 nmol/l PMA or 1 μmol/l Ang II. After 72 hours incubation, the cells were further incubated with 50 μg/ml Ac-LDL for 48 hours at 37 °C. The cells were washed three times with PBS, fixed for 30 min with 5% formalin solution in PBS, stained with Oil Red O for 30 min and counter stained with hematoxylin for another 5 min. Finally, the cells were examined by light microscopy. For fluorescence microscopy, both HMCL and primary cultures of HMC treated with 16 nmol/l PMA for 72 hours were incubated for 14 hours with 1 μmol/l of colchicine to synchronise populations, then cultured for 5 hours with 10 μg/ml of DiI-Ac-LDL at 37°C. The cells were then washed in PBS and fixed

in 5% formalin solution in PBS. Slides were examined on a Leitz fluorescence microscope.

3.2.9. RT-PCR

Total RNA (500 ng) was used as a template for RT-PCR. Scavenger receptor 5' primer (nucleotide position 75-94): TCGCTCAATGACAGCTTTGC, 3' primer (nucleotide position 345-364): CCATGTTGCTCATGTGTTCC (Matsumoto et al. 1990). Glyceraldehyde phosphate dehydrogenase (GAPDH): 5' primer (nucleotide position 73-92) TCATAGACAAGATGGTGAAG, 3' primer (nucleotide position 303-327) TGACGGGATCTCGCTCCTGGAAGAT (Ando et al. 1996). Twenty microliters of each PCR reaction were subjected to electrophoresis in a 2% agarose gel.

3.2.10. Southern blot analysis and quantitative evaluation

Nucleic acids were transferred to a nylon membrane using the Southern analysis method as described in chapter 2. The nylon membranes were probed with [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham, Little Chalfont, Buckinghamshire, UK) labelled oligonucleotides. Scavenger receptor probe (nucleotide position 128-157): GTGCAGCTTTGAAGGACTTCAGTTTCTCTT (Matsumoto et al. 1990). GAPDH probe (nucleotide position 170-191): AATGAAGGGGTCGTTGATGGCA (Ando et al. 1996). The DNA probes were labelled using 5'-end labelling system.

3.2.11. Construction of wild type scavenger receptor promoter reporter gene with SV40 enhancer

3.2.11.1. Preparation of scavenger receptor promoter gene

To determine the molecular mechanisms responsible for PMA and cytokine-dependent scavenger receptor transcriptional activation in HMC, the scavenger receptor promoter-luciferase fusion gene pGL3SCR was constructed. This contains scavenger receptor promoter 5' upstream (-696 to + 46) from the scavenger receptor gene (Fig.3.3) (Wu et al. 1994). 5'upstream of scavenger receptor was a gift (Fxbal AI) from Dr. CK Glass.

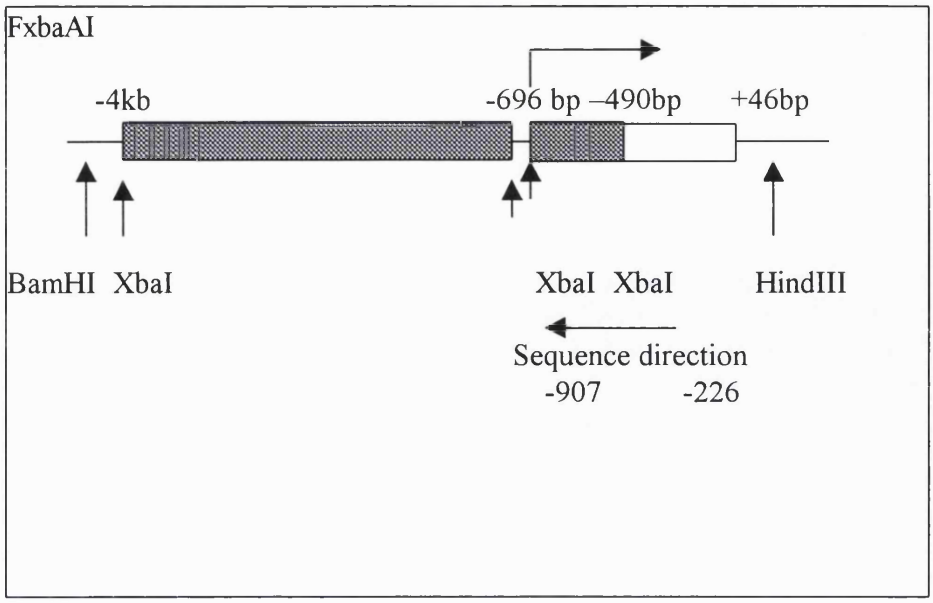
A antisense primer was designed to sequence the upstream region of scavenger receptor (solid bar). Sequence primer 5'CAGGGTGGAGTGCAGTGGTGTG 3', which locates in scavenger receptor promoter region underlined (Fig.3.3).

3.2.11.2. Preparation of pGL3 enhancer vector

pGL3 enhancer vector was digested by SacI /Bgl II to produce sticky end for ligation as described in chapter 3

3.2.11.3. Ligation of pGL3 enhancer and scavenger receptor promoter

pGL3 enhancer vector digested by Sac I/Bgl II and scavenger receptor promoter with Sac I/Bgl II adapters was ligated using standard method described in chapter 2. The ratio of pGL3 enhancer and scavenger receptor promoter of ligation is 3/1. The ligation product was transformed to JM109 cells, and then subjected to selection. The positive clones were selected for mini-preparation, identification and maxi-preparation of plasmid using the methods as described in chapter2. The plasmid pGL3SCR was used for transfection experiments.



-490*attaatgtat gttttagaag gcatagttac ttataaaaaa ggaaagatca ggctgggcac*
-430*ggttcgcgcc tgtaatccca gcactttggg aggccaaggc gagcggacca tgaggtcagg*
-370*ggatcaagac catcctgacc aacatggcga aaccctgtct ctactaaaat acaaaaaatt*
-310*agccgggtgt gatggcacac gcctgtagaa cctgggaggc agaggttgca gtgagctgag*
-250*at**cacaccac tgcactccac cctg**gtgaga cagcgagact ccatctccaa aaaaaaagga*

◄—*3′gtgtggtg acgtgaggtg ggac 5′ (sequencing primer)*

-190*aagctcaatc tgctgtaaat tatgtgcttg tttcaacaac ccttgtttct tttccttttc*
-130*acttctcttt ttttttaaa gcggcctaaa tggggtgaag agtgagttat ctgacaaatt*
-70 *tagattttgc aaacctgtgc attgatgaga gtgctattga aacacattaa gaaagatttt*
-10 *caacgcagga **atg**tgtcatt tcctttcttc atgtac**caga tgctgaaata ctatga**gata*

(PCR lower primer) ◄— 3′gtct acgactttat gatact 5′

+50 aagattttag gtttcaattg taaagagaga gaagtggata aatcagtgct gctttcttta
+110 ggacgaaag

Fig.3.3. Structure and sequence of scavenger receptor promoter. The sequence shown above represent the open bar region. Transcription start point ATG has shown in ***italic bold***. The sequence in solid bar region is unclear.

The sequence (antisence) of 5'upstream of scavenger receptor gene was performed by MWG-biotech and sequence show following:

-226
5′
TGCAACCTCTGCCTCCCAGGTTCTACAGGCGTGTGCCATCACACCCGGCTAATTTTTTGTATTTTAGT
AGAGACAGGGTTTCGCCATGTTGGTCAGGATGGTCTTGATCCCCTGACCTCATGGTCCGCTCGCCTTG
GC
CTCCCAAAGTgCTGGGATTACAGGCGCGAGCACCGTGCCCAGCCTGATCTTTCCTTTTTTATAAGTAA
TCTATGCCTTCTAAAACATACATTAAT **-490**

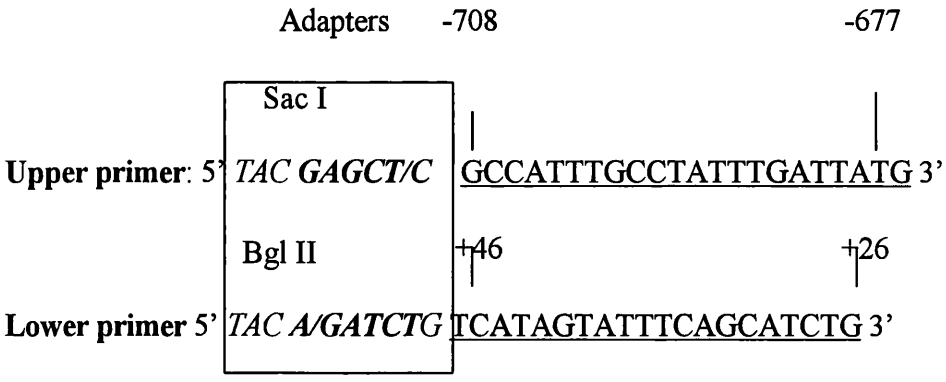
-491
TCTCATGATCTCCTCTTTCACGTTACTATAATGTTGAGTAAAGACTCCATGTTTGGCTAGGTGTGGTG
GCTCATGCCTGTAATCCCAGCACTTTGGGTGGCCGAGGTGGCTGGATSSACTTGAGGTCAGGAGCTCT
AGAGCGGCCGCTCTAGAATGAGGTCCATGTAAGATTATGCCCAGTGACCAAACAGAGATT

CATAATCAAATAGGCAAATGGCTTGAGAGAATTGATTCAAATGTCCTAGGACTCACTGTGGTACAGGT
AT

3′GTATTAGTTTATCCGTTTACCG 5′ (PCR UPPER PRIMER)

ACTGAACTCAAAAGAAGAGTTAACATTTTACACTCATTAAGAGGTATTTTTGTATGTCTAAAGTTACA
GCAGGTTGTTAGTCATAATATATAGTTAATACATTATGACTTATTTATTGTATGATATATATATGATA
AAGTTTCGAGATAAA 3′ **-907**

A pair of specific primers with an adapter was designed to subclone scavenger receptor promoter.



PCR reaction was performed using plasmid FxbaI as template. The PCR product was purified using Qiagen gel purification kit, then digested by Sac I and Bag II to get rid of extra sequence, and ligated to PGL3 enhancer vector.

3.2.12. Functional analysis of scavenger receptor promoter: the role of transcription factors AP-1/ets

The 5' upstream of scavenger receptor gene (-4.5kb to +46) was found to lack a conventional TATA box and initiated transcription from a cluster of closely spaced start sites. These sequence motifs include three near consensus transcription factor AP-1 binding sites (AP-1 motifs), two of which are juxtaposed with binding sites for members of the transcription factor ets family (ets motifs) (Moulton et al. 1992; Wu et al. 1994). To further examine the relative roles of the AP-1 and ets (AP-1/ets) motifs in mediating PMA and Ang II -dependent transcriptional activation, luciferase reporter genes were made in which either one or three copies of the AP-1/ets motifs, or the corresponding sequences containing mutations in either the AP-1 or ets, were introduced upstream of the TATA box of the rat prolactin gene promoter (Fig.3.4) (Wu et al. 1994).

3.2.13. Transient Expression Assay

HMCL at a density of 6×10^{7} cells /ml were transfected with 100 μg of supercoiled reporter genes and 100 μg control plasmid of psv-β-galactosidase (Internal standard) by electroporation at 340 volts and 125 uF with a Gene Pulser (Bio-Rad, Hemel Hempstead, Herefordshire, UK). After electroporation, the cells were placed in 6 well plates with growth medium. Approximately 24 hours after replating, cells were washed with PBS and the medium was replaced by fresh serum free RPMI medium in the presence or absence of different concentrations of PMA, Ang II and inflammatory cytokines (TNF-α and IL-1β) for various times. The cells were then washed twice with PBS and lysed using a lysis buffer (Promega). The luciferase and β-galactosidase enzyme activity in the supernatants assayed using Promega luciferase and β-galactosidase assay systems.

3.2.14. Effect of various signal transduction inhibitors on PMA and Ang II–mediated activity of scavenger receptor promoter

Using the transient expression assay system described above, the reporter gene PGL3SCR was transfected into HMCL. Transfected cells were incubated for 24 hours in serum free RPMI medium or serum free medium with 16 nmol/l of PMA or 1μg/ml of Ang II in the presence or absence of different signal transduction pathway inhibitors: 25 μmol/l W-7 (calmodulin antagonist), 25 μmol/l genistein (receptor tyrosine kinase inhibitor), 100 nmol/l calphostin C (PKC inhibitor), 1 nmol/l staurosporine (serine/threonine kinase inhibitor), 10 ng/ml pertussis toxin (G-protein inhibitor). Scavenger receptor promoter activity was estimated by measuring luciferase luminescence.

3.2.15. Data analysis

In all experiments, groups of data were evaluated for significance by one way analysis of variance (*ANOVA)* using Minitab software. Data were considered significant if the p value ≤ 0.05.

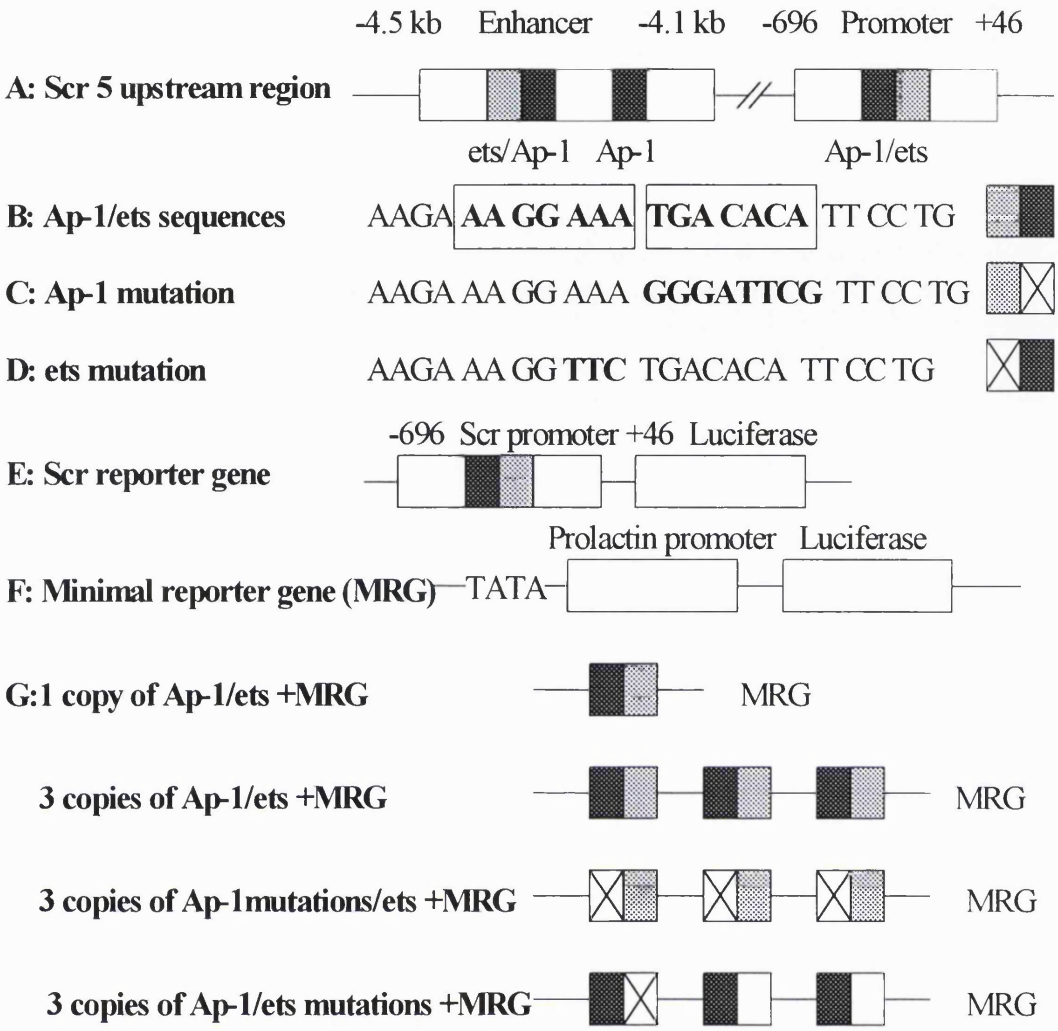


Fig.3.4. Scavenger receptor promoter and reporter gene constructs used in this study. 'A' represents the scavenger receptor 5' upstream region; 'B' represents the nucleotide sequences of the scavenger receptor promoter AP-1/ets motifs. 'C' and 'D' represent corresponding sequences containing mutations in the AP-1 or ets; 'E' represents reporter gene construct (pGL3SCR) containing the full scavenger receptor promoter (-696 to +46) and luciferase gene; 'F' represents a minimal reporter gene construct which contain upstream of the minimal prolactin gene promoter and luciferase gene; 'G' represents reporter gene constructs which contain minimal reporter gene plus one or three copies of AP-1 or ets motifs with or without mutations. The reporter genes shown above will be used in transfection experiments.

3.3. RESULTS

3.3.1 Estimation of values for the dissociation constant (Kd) for scavenger receptor in HMCL.

HMCL bound ^{125}I-Ac-LDL in a dose dependent manner at 4°C, but saturable at high concentrations (Fig.3.5). Analysis of ^{125}I-Ac-LDL binding experiments carried out at 4°C using the EBDA/LIGAND program showed that the kinetics of Ac-LDL binding to HMCL best fitted a single model with a *Kd* of $1.3x10^{-7}$ M and B_{max} is $1.1x10^{-9}$M. The Scatchard Plot is shown in Figure 3.6. There is a first estimation of *Kd* value of scavenger receptor in HMCL. It suggests that affinity of Ac-LDL to scavenger receptor in HMCL is lower than that in macrophages, which has a *Kd* of $8x10^{-9}$ (Rose, et al, 1973; Via, et al, 1989).

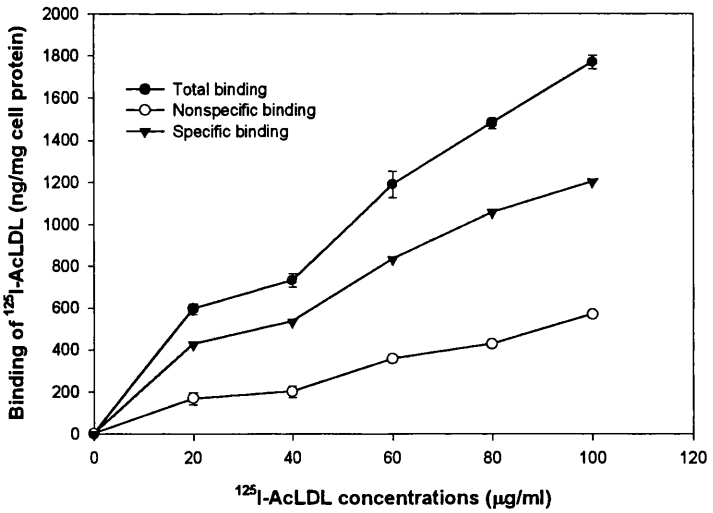


Fig.3.5. Binding of ^{125}I-AcLDL to HMCL at 4°C. Various concentrations of ^{125}I-AcLDL were incubated with HMCL for 4 hours at 4°C, in the presence or absence of a 50-fold excess of unlabelled AcLDL. Specific binding was calculated by subtracting non-specific binding (binding in the presence of excess unlabelled) from total binding values.

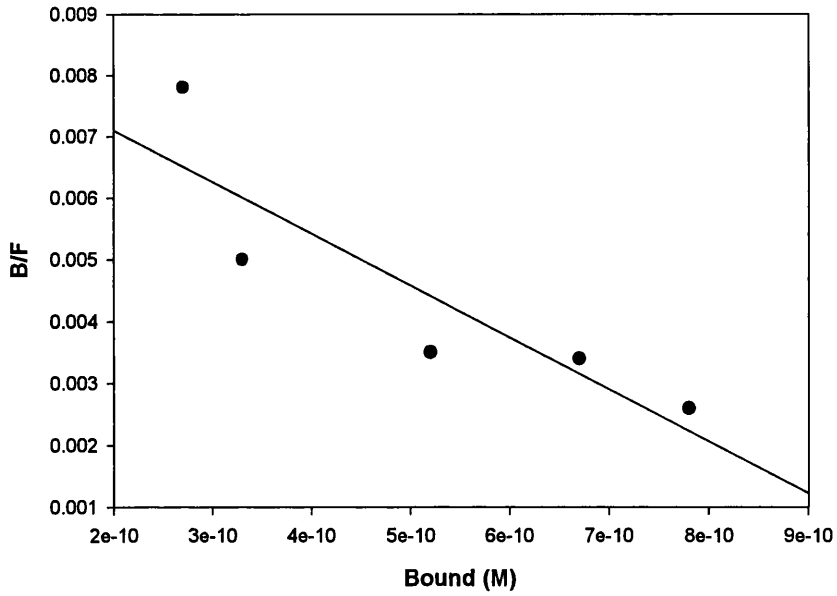


Fig.3.6. Scatchard Plot of transformed data of ^{125}I-AcLDL binding to HMCL assuming a one site model

Data was linearised using the LIGAND program. The calculated *Kd*=1.3x10^{-7} and B_{max}=1.1x10^{-9}

3.3.2. PMA and Ang II induced scavenger receptor expression in HMCL

3.3.2.1. Analysis of flow cytometry

We assessed the binding and internalisation of DiI-labelled Ac-LDL in HMCL treated with PMA and Ang II by FACS analysis. The parameters used to evaluate data were the percentage of scavenger receptor positive cells and the mean fluorescence intensity (MFI) of DiI labelled cells. The results showed that PMA and Ang II increased the percentage of DiI-labelled positive cells in a time dependent manner (Fig.3.7A). We also investigated if PMA and Ang II affected the density of scavenger receptor on HMCL. The data showed

that PMA and Ang II increased MFI in a time dependent manner (Fig.3.7B), indicating an increased intracellular level of DiI labelled Ac-LDL in HMCL. This suggests that PMA and Ang II increases scavenger receptor density on HMCL. The specificity of the analysis was confirmed by showing that excess amounts of unlabelled Ac-LDL inhibited the uptake of DiI-Ac-LDL (Fig.3.8). These results suggest that the enhanced uptake of Ac-LDL result from an increase in both the numbers of scavenger receptor positive cells and scavenger receptor density in HMCL.

3.3.2.2. Visualisation of Ac-LDL uptake and lipid droplets

Staining of HMCL with Oil Red O before (Fig. 3.9A) and after PMA (Fig. 3.9B) & Ang II (Fig.3.9C) stimulation showed that both PMA & Ang II increased the number of intracellular Oil Red O stained lipid droplets. The stable cell line HMCL-Scr showed a stronger Oil Red O staining (Fig 3.9D) which confirmed that scavenger receptor had functional relevance in mesangial cells cultured in the appropriate environment. Visual inspection of DiI-Ac-LDL labelled cells was performed in both HMCL and primary cultures of HMC by fluorescence microscopy. HMCL and primary cultures of HMC were treated by 16 nmol/l of PMA for 72 hours, and then treated with colchicine which arrests mitotic cells in the metaphase by blocking the spindle apparatus for the chromosome separation. The synchronised HMCL and primary cultures of HMC were used to evaluate DiI-Ac-LDL uptake. Result showed that the majority of cells displayed high fluorescence after stimulation by PMA. However, some PMA treated cells were low - or non-fluorescent even after synchronisation with colchicine (Fig.3.9.E&F). This data suggests that not all cells in this population express scavenger receptor to the same degree when stimulated by PMA, and that the non-homogeneous behaviour of HMCL with respect to DiI-Ac-LDL uptake, is not a function of the cell cycle, with temporary

suspension of the cells during certain phases of the cycle. It is also excluded that heterogeneity results from different functional specificity of the HMCL cell line because the same heterogeneity also observed in the primary culture of HMC.

3.3.2.3. Expression of scavenger receptor mRNA

RT-PCR followed by Southern blotting showed that HMCL had a low mRNA level for scavenger receptor under normal tissue culture conditions. The stable cell line HMCL-Scr that was transfected by scavenger receptor cDNA showed a high level of scavenger receptor mRNA (Fig.3.2). However, HMCL expressed an inducible form of scavenger receptor when stimulated by PMA and Ang II. PMA (16 nmol/l) induced scavenger receptor expression in a time dependent manner. Ang II (1 μmol/l) also induced scavenger receptor expression (Figs.3.10A &B). Both PMA at 1.6 nmol/l to 160 nmol/l and Ang II at 10 to 1000 nmol/l induced scavenger receptor mRNA expression in HMCL in a dose responsive manner (Figs.3.11A &B).

3.3.2.4. Activity of scavenger receptor promoter

To investigate whether enhanced scavenger receptor mRNA resulted from increased gene transcription in HMCL, we analysed the scavenger receptor promoter activity. The reporter gene (pGL3SCR) containing the full scavenger receptor promoter was transfected into HMCL. The transfected HMCL were stimulated by different concentrations of PMA and Ang II at various times, then promoter activity was measured. The results showed that PMA and Ang II induced scavenger receptor promoter activity in a time and dose dependent manner, which were consistent with the induction of scavenger receptor mRNA (Figs.3.12 A & B.).

3.3.2.5. The effect of Antiotensin II on HMCL proliferation

Mitogenesis assays involving ^{3}H-thymidine incorporation revealed that Angiotensin II at 1000 nmol/l significantly increased HMCL proliferation (Fig.3.13.), suggesting that Ang II at high concentration may have a proliferation-dependent influence on scavenger receptor induction.

3.3.2.6. The Effect of various signal transduction pathway inhibitors on scavenger receptor promoter activity

To determine the role of signal transduction pathways in the regulation of scavenger receptor gene expression in PMA or Ang II -stimulated HMCL, we evaluated the effects of various signal transduction inhibitors on scavenger receptor transcription using the luciferase system. At non-cytotoxic concentrations of inhibitors, we observed that the promoter activity of scavenger receptor in PMA or Ang II treated HMCL in the presence of PKC inhibitor (calphostin C) or calmodulin inhibitor (W-7) was significantly lower than in the absence of calphostin C or W-7, suggesting that both PKC and calmodulin pathways were involved in scavenger receptor upregulation. Additionally, a serine/threonine kinase inhibitor (staurosporine) significantly inhibited scavenger receptor promoter activity induced by PMA, suggesting that serine/threonine kinase pathway also involved in upregulation of scavenger receptor induced by PMA. (Table 3.1).

3.3.2.7. Functional analysis of the scavenger receptor promoter

The molecular mechanism of gene transcription was further investigated using several reporter gene constructs described in Fig.3.4. Functional analysis showed that the minimal prolactin promoter exhibited very little activity either before or after PMA and Ang II treatment (Fig.3.14). However, when either one or three copies of the AP-1/ets

motifs were introduced upstream of the minimal prolactin promoter, the promoter could respond to PMA stimulation in HMCL. Three copies of the AP-1/ets motifs increased basal activity by two to three folds in response to PMA stimulation. This suggested that AP-1/ets motifs are specific response elements to PMA stimulation (Fig.3.14). Mutation of either the AP-1 or the ets motifs decreased its ability to respond to PMA, suggesting that both AP-1 and ets motifs are necessary response elements for gene transcription induced by PMA (Fig.3.14). The reporter gene containing 3 copies of AP-1/ets responsive elements had a very small response to Ang II stimulation, suggesting that AP-1/ets motifs are not specific response elements for Ang II (Fig.3.14).

3.3.3. Inflammatory cytokines TNF-α and IL-1β induced scavenger receptor expression in HMCL

3.3.3.1. Analysis of flow cytometry

We assessed the binding and internalisation of DiI-labelled Ac-LDL in HMCL treated with TNF-α and IL-1β by FACS analysis using method as described before. The results showed that TNF-α and IL-1β increased MFI (Fig.3.15), indicating an increased intracellular level of DiI labelled Ac-LDL in HMCL.

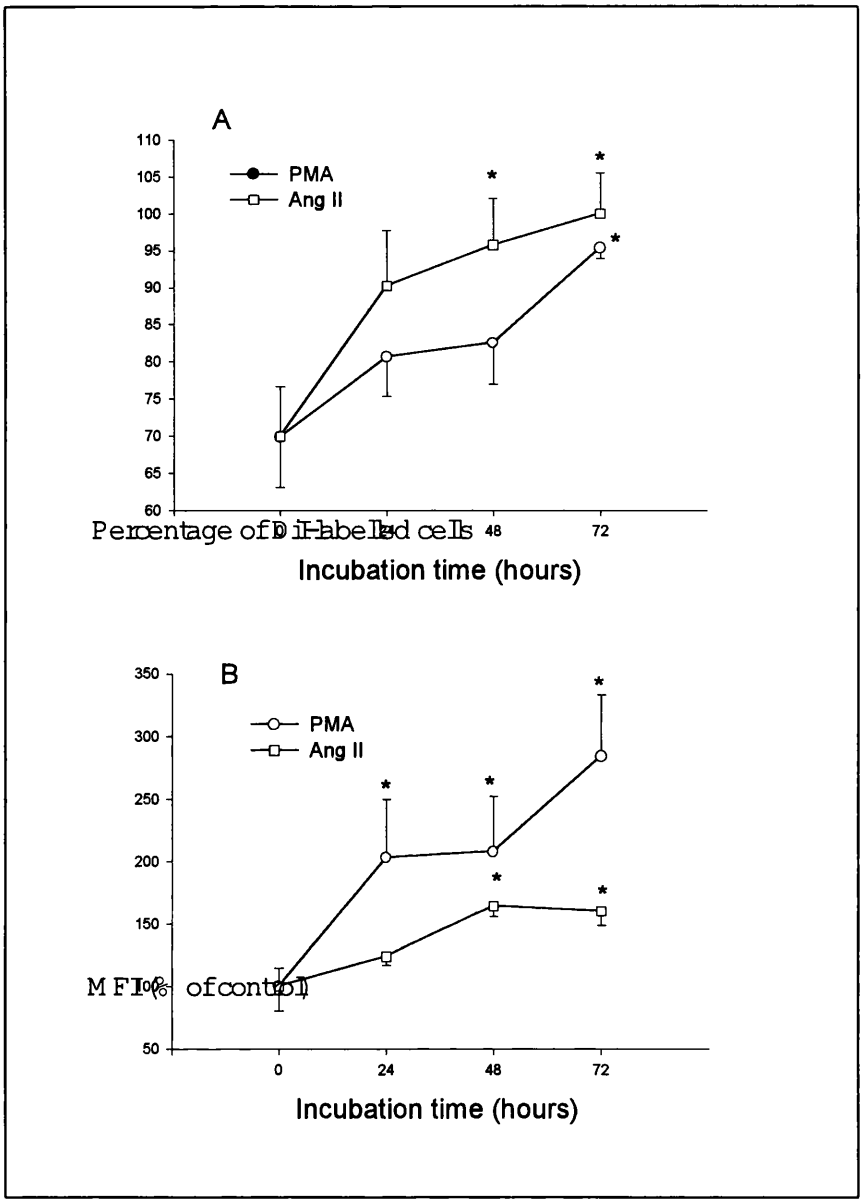


Fig.3.7. Analysis of the mean fluorescence intensity (MFI) and the percentage of DiI-labelled cells in the PMA & Ang II treated-HMCL HMCL were incubated in serum free medium with 16 nmol/l of PMA (●) or 1 μmol/l of Ang II (■)for 0, 24, 48 and 72 hours respectively, then the medium was replaced by fresh serum free medium containing 10 μg/ml DiI labelled Ac-LDL for 3 hours at 37°C. The cells were analysed by FACS. The percentage of DiI-labelled cells was calculated by counting labelled cells that exceeded the upper limit of the autofluorescence of unlabeled cells (A). MFI was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells (B). Results represent means±SD of duplicate wells from four experiments * $p<0.05$ vs control (time 0).

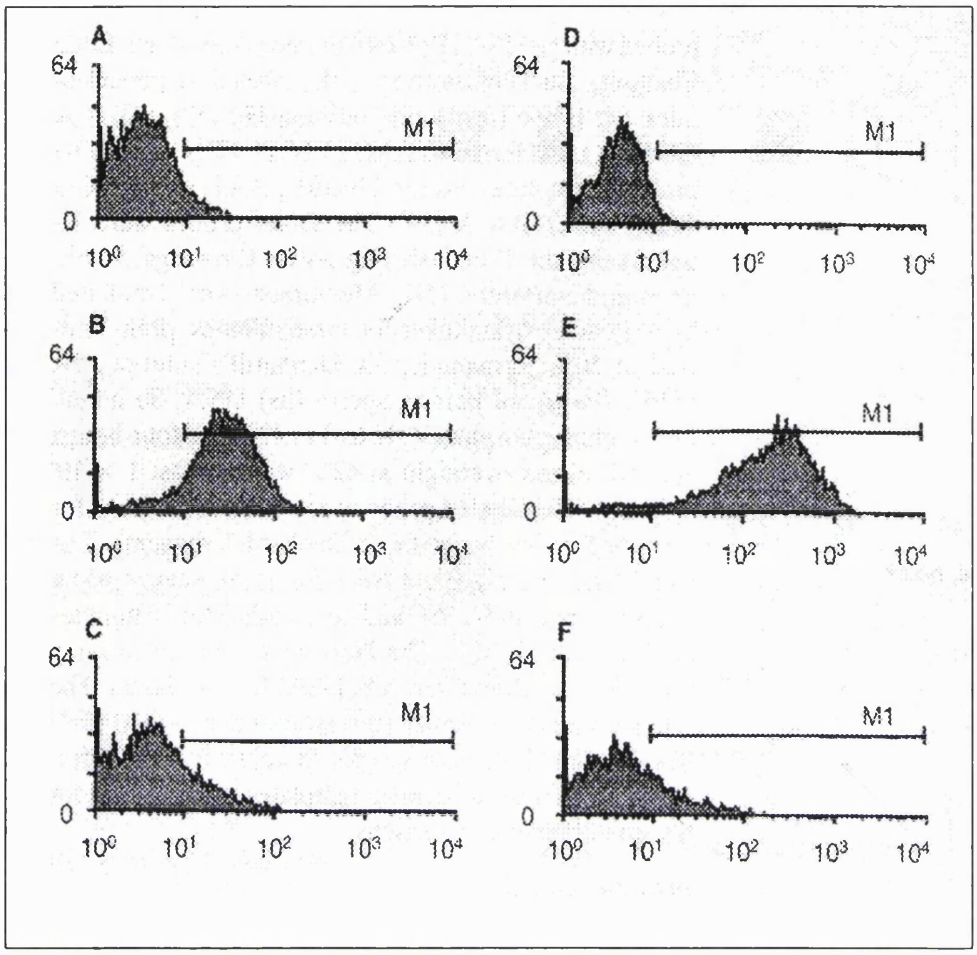


Fig.3.8. Specificity of the flow cytometric analysis of uptake of DiI-Ac-LDL in HMCL. HMCL were incubated in serum free medium (A, B, & C) or serum free medium with 16 nmol/l of PMA (D, E, & F) for 72 hours, then the medium was replaced by fresh serum free medium with: 10 μg/ml unlabelled Ac-LDL (A, D), or 10 μg/ml DiI labelled Ac-LDL (B, E) or 10 μg/ml DiI labelled Ac-LDL plus 1000 μg/ml of unlabelled Ac-LDL (C, F) for 3 hours at 37°C. Fluorescence intensity per cell is shown on the X-axis and the number of DiI labelled cell on the Y-axis. M range indicated in the histograms was arbitrarily determined in each experiment to calculate the percentage of labelled cells, based on autofluorescence of the unlabeled cells.

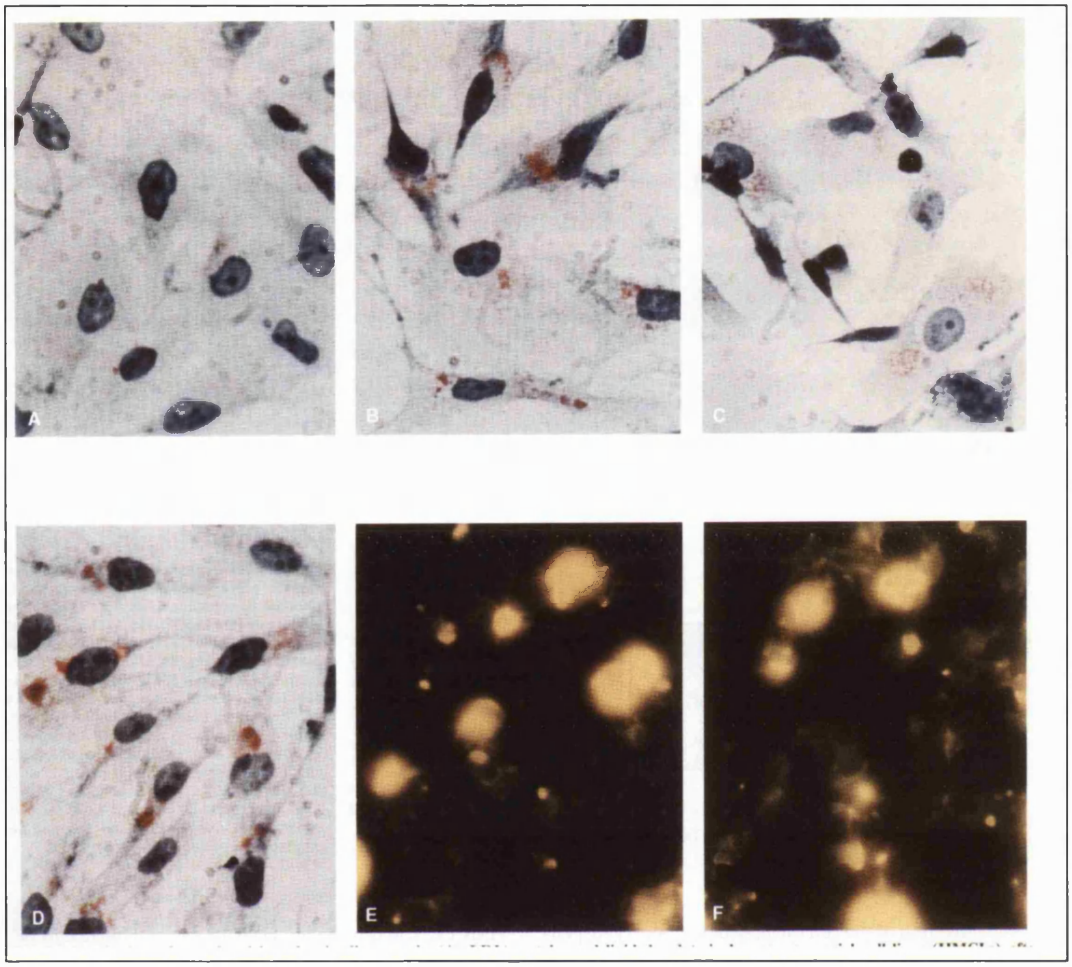


Fig.3.9. Visualisation of Ac-LDL uptake and lipid droplets in HMCL after PMA or Ang II treatment. HMCL were incubated for 72 hours in serum free medium in the absence (A) or presence (B) of 16 nmol/l PMA, or 1 μmol/l of Ang II (C). The cells (A, B & C) and cultured HMCL-Scr (D) were further incubated with 50 μg/ml Ac-LDL for 48 hours at 37 °C, then examined for lipid inclusions by Oil Red O staining. For fluorescence microscope examination, HMCL (E) and primary cultures of HMC (F) were incubated with 16 nmol/l PMA for 3 days, then treated with 1 μmol/l of colchicine for 14 hours and finally labelled with 10 μg/ml DiI-Ac-LDL for 5 hours at 37°C. The cells were fixed and examined for fluorescence. The results are typical of those observed in four experiments.

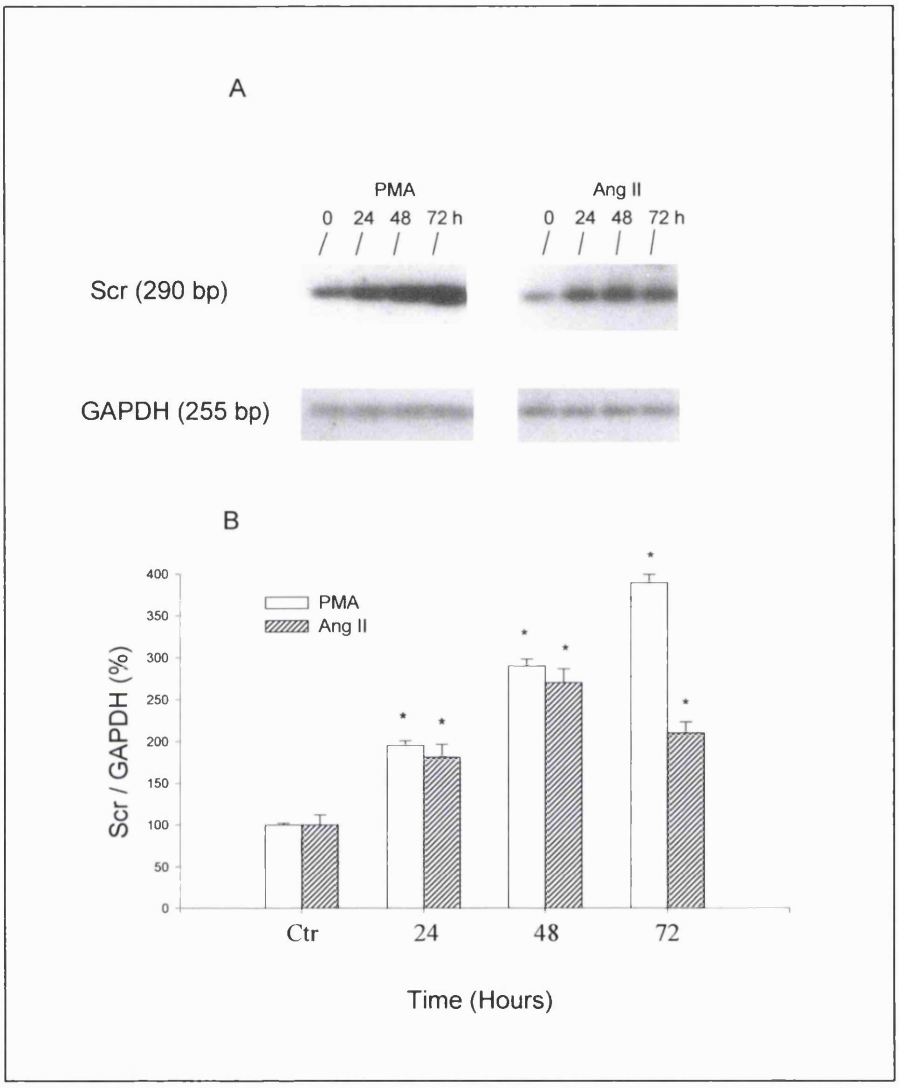


Fig.3.10. Time dependent expression of scavenger receptor mRNA in response to PMA and Ang II. HMCL were incubated in serum free medium containing 16 nmol/l of PMA or 1 μmol/l of Ang II for different periods of time (0, 24, 48, 72h). (A): Scavenger receptor mRNA expression was examined using RT-PCR followed by Southern blotting as described in methods. (B): The histogram represent means±SD of the densitometric scans of the scavenger receptor mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of time 0. *$p < 0.001$ vs control.

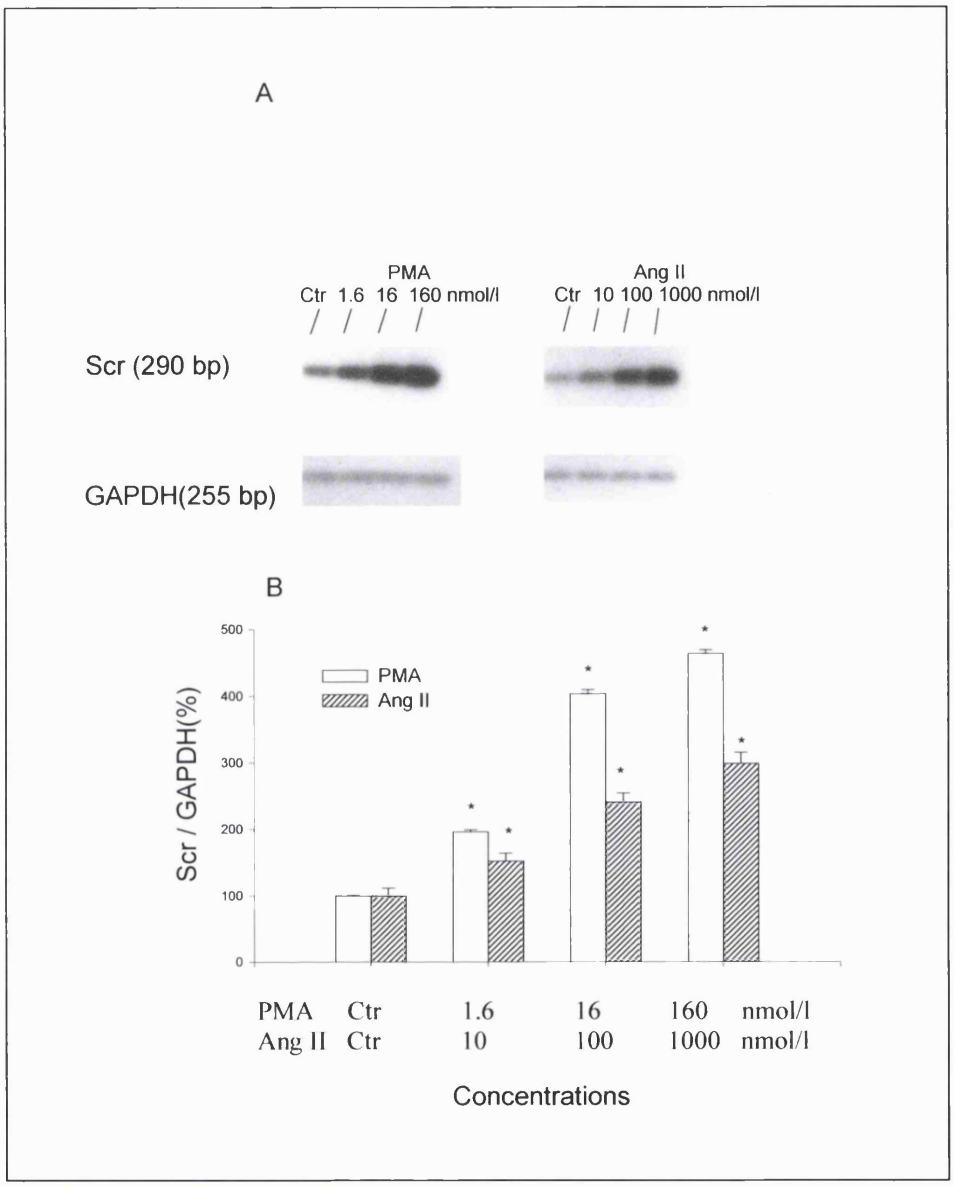


Fig.3.11. The effect of different concentrations of PMA and Ang II on scavenger receptor mRNA expression. HMCL were incubated in serum free medium (control) or serum free medium containing various concentrations of PMA (1.6, 16 & 160 nmol/l) for 72 hours or Ang II (10, 100, 1000 nmol/l) for 48 hours. (A): Scavenger receptor mRNA expression was examined using RT-PCR followed by Southern blotting as described in methods. (B): The histogram represent means±SD of the densitometric scans of the scavenger receptor mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of control. * $p<0.001$ vs control.

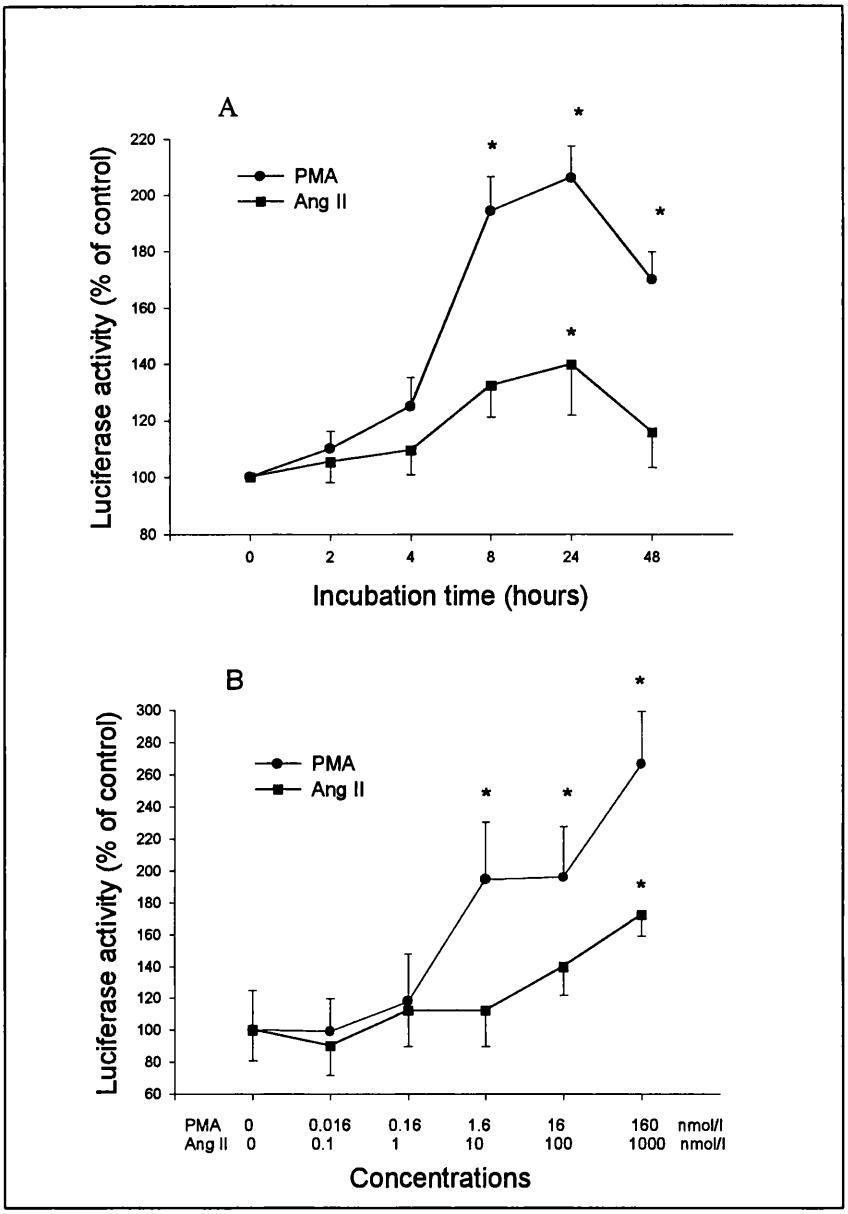


Fig.3.12. The response of scavenger receptor promoter to PMA HMCL were transfected with PGL3SCR construct, as a internal standard, using a psv-β-galactosidase plasmid. The transfected cells were cultured in a standard RPMI medium containing 5% FCS. After 24 hours, the cells were incubated in serum free medium with 16 nmol/l of PMA (●) or 1 μmol/l of Ang II (■) for different times (0, 2, 4, 8, 24, 48) (time 0 as a control) (A); or the cells were incubated in serum free medium (control) or serum free medium containing various concentrations of PMA (0.16, 1.6, 16 & 160 nmol/l) (●) or Ang II (0.1, 1, 10, 100, 1000 nmol/l) (■) for 24 hours (B). Luciferase activity was measured and normalised by comparison with β-galactosidase activity. Results represent the mean±SD of duplicate determinations from six experiments, which were normalised to their respective control (100%). * $p<0.001$ vs control.

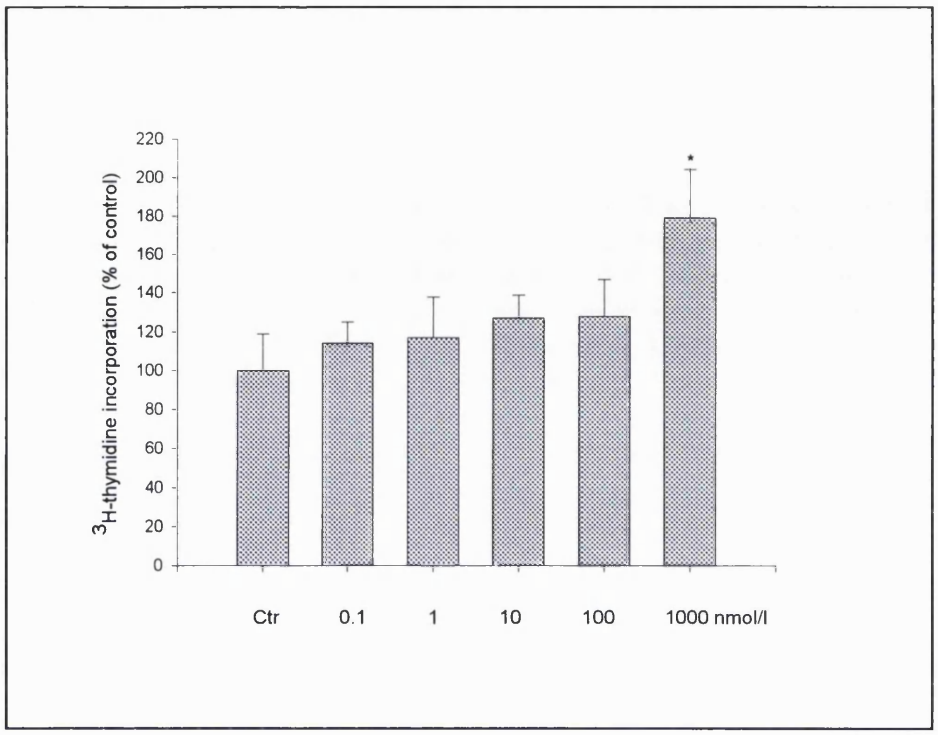


Fig.3.13 The effect of Angiotensin II on HMCl proliferation. Quiescent HMCL were incubated for 24 hours in serum free medium (control), or serum free medium with different concentrations of Angiotensin. The cells were harvested after labelling for 18 hours with 3H-thymindine.Radioactivity associated with DNA of proliferating cells was measured in a scintillation counter. *$p<0.001$ vs control.

Table 3.1. The effect of PMA & Ang II on scavenger receptor promoter activity in the presence of various signal transduction pathway inhibitors

Treatment	Relative scavenger receptor promoter -luciferase activity		
	Control	PMA	AngII
No inhibitor	100±6.32	195.92±20.07	172.20±13
Calmodulin inhibitor (W-7)	100±15.71	134.66±18.92**	119.68±16.81*
Tyrosine kinase inhibitor (Genistein)	100±10.86	253.04±44.29	226.44±61.57
PKC inhibitor (Calphostin C)	100±18.39	148.53±30.50*	124.65±21.52*
Serine/threonine kinase inhibitor (Staurosporine)	100±6.91	164.76±16.69*	158.86 ±18.71
G-protein inhibitor (Pertussis toxin)	100±15.13	190.88±41.36	176.86±49.61

*p<0.05. ** p<0.001

Transfected HMCL were treated with serum free medium (control) or serum free medium containing 16 nmol/l of PMA or 1 μmol/l of Ang II in the presence or absence of different signal transduction pathway inhibitors: w-7, 25 μmol/l; genistein, 25 μmol/l; calphostin C, 100 nmol/l; staurosporine, 1 nmol/l; and pertussis toxin, 10 ng/ml. The relative luciferase activity of scavenger receptor promoter is presented as percentages of experiments with serum free (control value). Results represent the mean±SD of duplicate wells from four experiments, then normalised to their control values (100%).

*p< 0.05 vs no inhibitor.

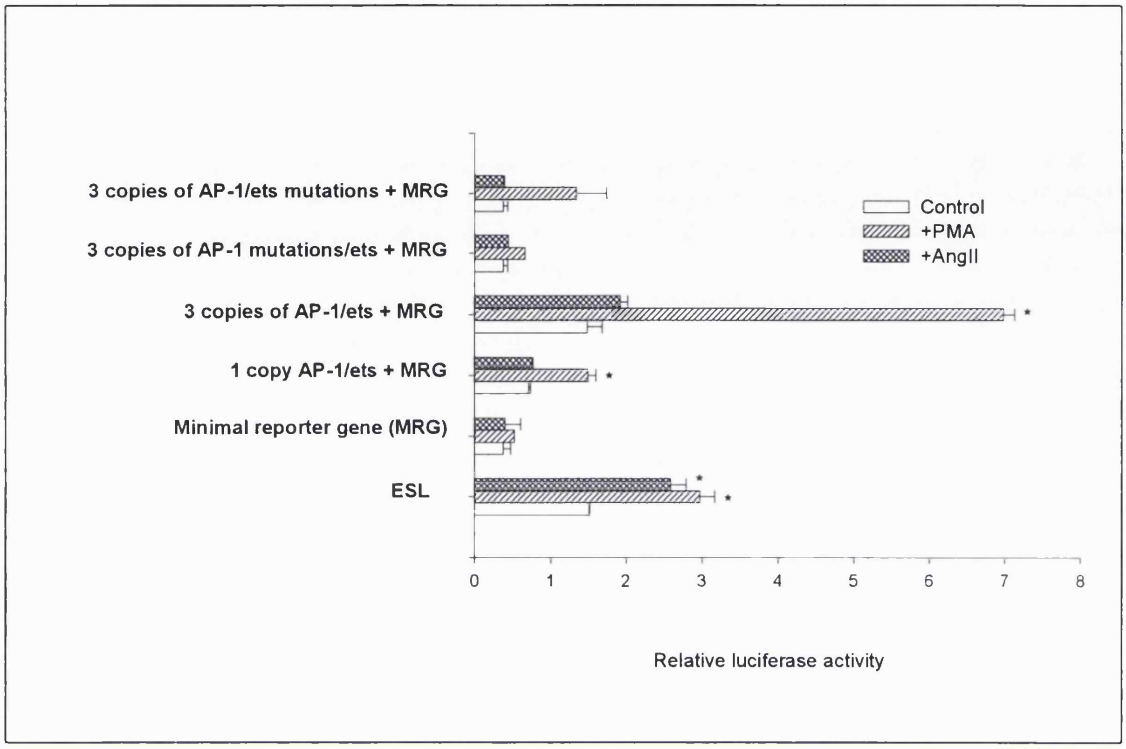


Fig.3. 14. Functional analysis of the scavenger receptor promoter and AP-1/ets motifs in the context of the minimal prolactin gene promoter. The reporter gene constructs shown in Fig.3.4. were transfected into HMCL. Transfected cell were incubated in serum free RPMI medium in the absence (control) or presence of 16 nmol/l PMA or in the presence of 1000 nmol/l Ang II for 24 hours and the promoter activity was determined. Results represent the mean of duplicate determinations from six experiments. *$p<0.001$ vs respective control.

3.3.3.2. Expression of scavenger receptor mRNA

RT-PCR followed by Southern blotting showed that TNF-α and IL-1β induced scavenger receptor mRNA expression in HMCL in a time dependent manner (Fig 3.16).

3.3.3.3. Activity of scavenger receptor promoter

We also analysed the scavenger receptor promoter activity under influence of inflammatory cytokines. The reporter gene (pGL3SCR) containing the full scavenger receptor promoter was transfected into HMCL. The transfected HMCL were stimulated by 50 ng/ml of TNF-α or 5 ng/ml of IL-1β at 24 hours, then promoter activity was measured. The results showed that TNF-α and IL-1β induced scavenger receptor promoter activity, which were consistent with the induction of scavenger receptor mRNA (Figs.3.17).

3.3.3.4. Functional analysis of the scavenger receptor promoter

We also examined the role of transcription factor AP-1/ets on the scavenger receptor transcription induced by inflammatory cytokines. The series of reporter genes, which have been used in PMA and Ang II research, have been transfected into cytokine stimulated-HMCL. The results showed that one or three copies of AP-1/ets did not response either TNF-α or IL-1β stimulation suggesting that AP-1/ets are not specific response elements for TNF-α or IL-1β stimulation (Fig. 3.18).

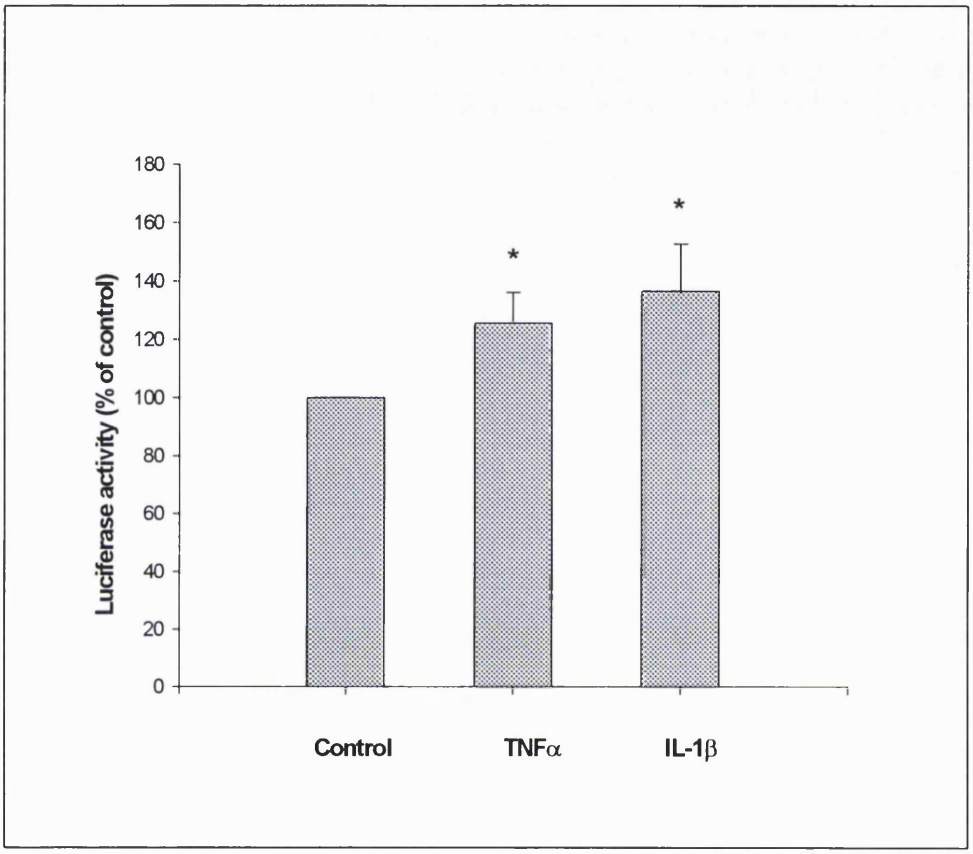
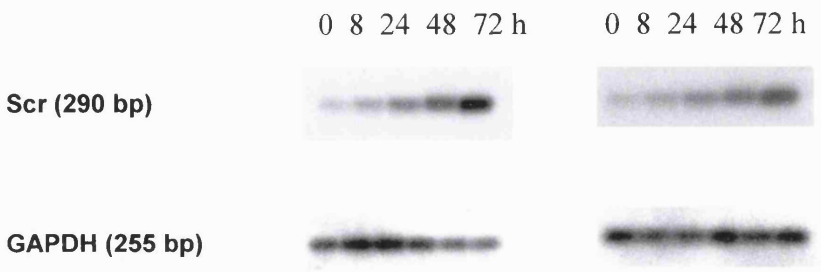


Fig.3.15. Analysis of the mean fluorescence intensity (MFI) of DiI-labelled cells in TNF-α or IL-1β treated-HMCL HMCL were incubated in serum free medium with 50 ng/ml of TNF-α or 5 ng/ml of IL-1β for 72 hours respectively, then the medium was replaced by fresh serum free medium containing 10 μg/ml DiI labelled Ac-LDL for 3 hours at 37°C. The cells were analysed by FACS. MFI was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells. Results represent means±SD of duplicate wells from four experiments * $p<0.05$ vs. control (time 0).



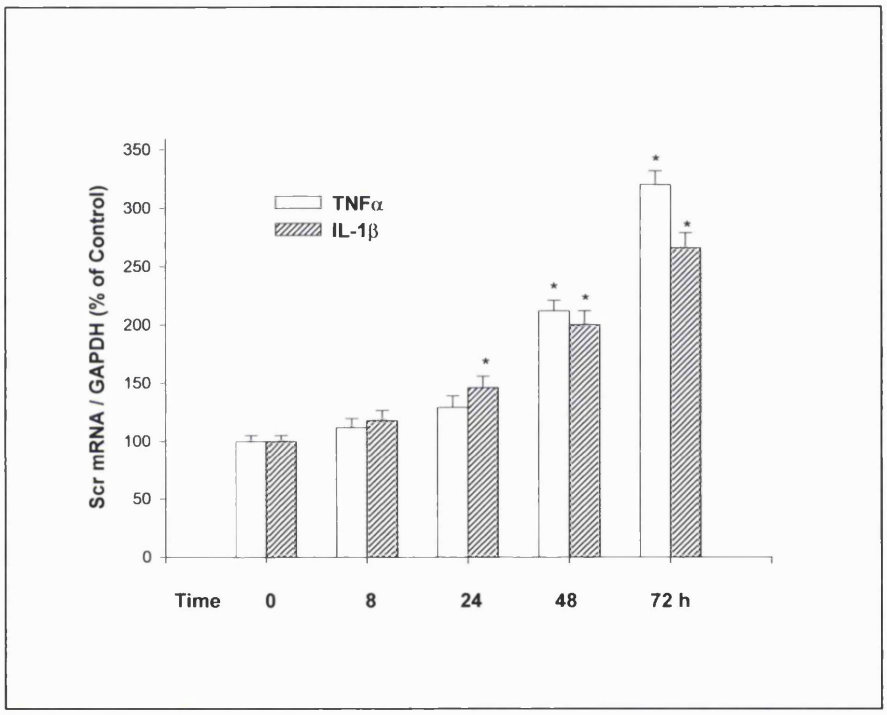


Fig.3.16. Time dependent expression of scavenger receptor mRNA in response to TNF-α and IL-1β. HMCL were incubated in serum free medium containing 50 ng/ml of TNF-α or 5 ng/ml of IL-1β for different periods of time (0, 8, 24, 48, 72h). (A): Scavenger receptor mRNA expression was examined using RT-PCR followed by Southern blotting as described in methods. (B): The histogram represent means±SD of the densitometric scans of the scavenger receptor mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of time 0. * $p<0.001$ vs time 0.

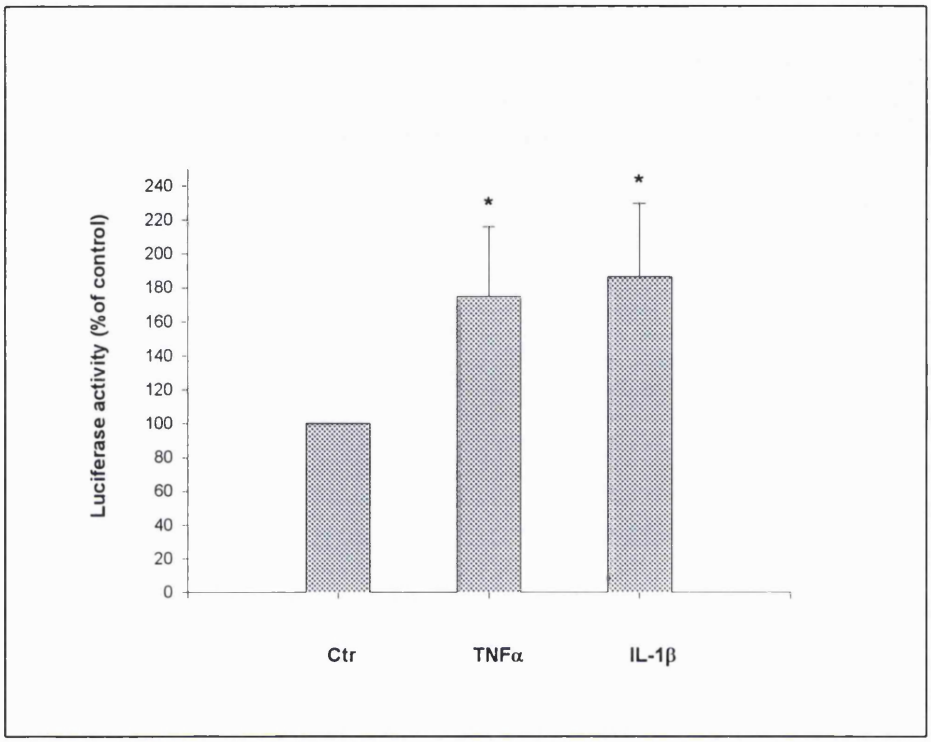


Fig.3.17. The response of scavenger receptor promoter to TNF-α and IL-1β stimulation. HMCL were transfected with PGL3SCR construct, as a internal standard, using a psv-β-galactosidase plasmid. The transfected cells were cultured in a standard RPMI medium containing 5% FCS. After 24 hours, the cells were incubated in serum free medium with 50 ng/ml of TNF-α or 5 ng/ml of IL-1β for 24 hours. Luciferase activity was measured and normalised by comparison with β-galactosidase activity. Results represent the mean±SD of duplicate determinations from six experiments, which were normalised to their respective control (100%). * $p<0.001$ vs. control.

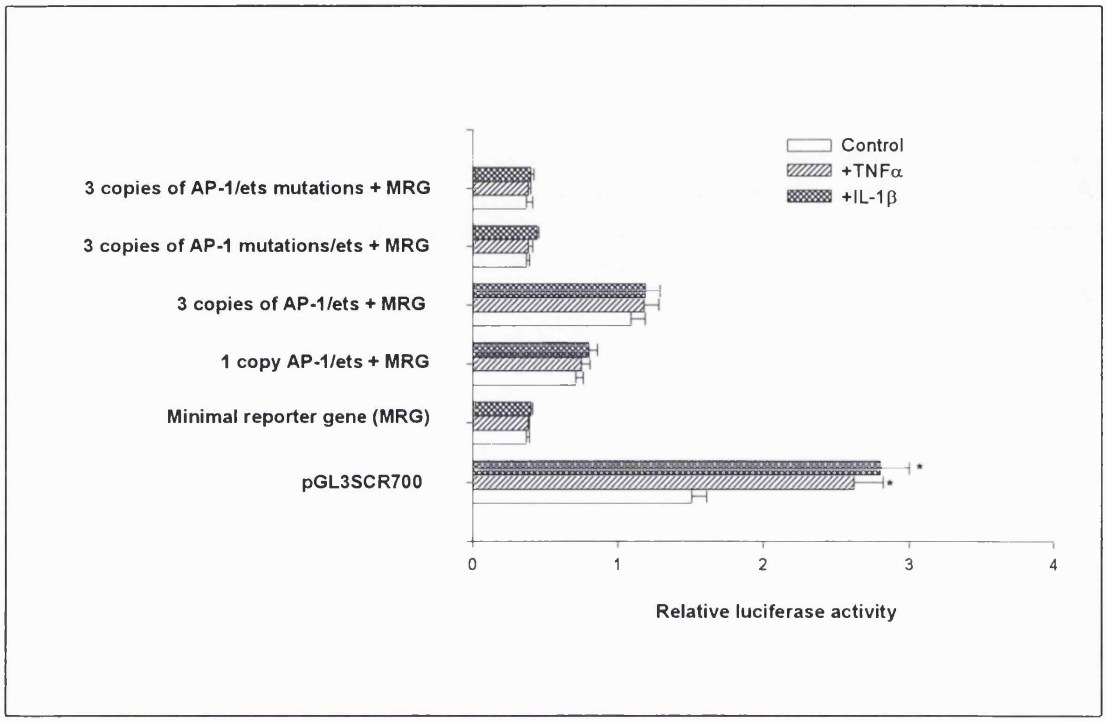


Fig.3. 18. Functional analysis of the scavenger receptor promoter and AP-1/ets motifs in the context of the minimal prolactin gene promoter. The reporter gene constructs shown in Fig.3.4. were transfected into HMCL. Transfected cell were incubated in serum free RPMI medium in the absence (control) or presence of 50 ng/ml of TNF-α or 5 ng/ml of IL-1β for 24 hours and the promoter activity was determined. Results represent the mean of duplicate determinations from six experiments. *$p<0.001$ vs respective control.

3.4. DISCUSSION

Scavenger receptor mediates the uptake of modified low-density lipoproteins by macrophages. The accumulation of lipids via this process is thought to lead to foam cell formation in atherosclerotic plaques and in glomerulosclerosis (Diamond and Karnovsky, 1988; Keane et al. 1988; Moorhead et al. 1982). In vitro studies have shown that a variety of agents including phorbol esters, macrophage colony-stimulating factor (M-CSF), endotoxin, transforming growth factor beta (TGF-β), interferon-γ (IFN-γ), retinoic acid, platelet secretory products, prostacyclin agonists and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are capable of stimulating scavenger receptor activity in different cell types excluding mesangial cells.

The present experiments showed that PMA, Ang II, and inflammatory cytokines TNF-α & IL-1β increased scavenger receptor protein activity by increasing both the number of scavenger receptor positive cells and the density of scavenger receptor per cell in HMCL. We also analysed the mRNA expression of scavenger receptor and showed that there was an inducible expression of scavenger receptor mRNA in cultures of HMCL. These results imply that there is limited expression of scavenger receptor under physiological conditions, but when HMC were stimulated by inflammatory cytokines, they expressed inducible scavenger receptor. This may explain why HMC which have not previously been shown to express scavenger receptor in normal culture, can express scavenger receptor in vivo (Takemura et al. 1993; Grone et al. 1992). Ang II has been shown to have an important role in progressive glomerulosclerosis in various animal models and clinical studies (Harris and Martinez-Maldonado, 1995). We therefore suggest that Ang II could contribute to atherogenesis in vivo at least in part through induction of scavenger receptor.

Previous studies of scavenger receptor regulation under effects of inflammatory cytokines have been conflicting. TNF-α has been shown to suppress macrophage scavenger receptor function (Van Lenten and Fogelman, 1992). Hsu evaluated the mechanism by which TNF-α inhibited macrophage scavenger receptor surface expression and binding of Ac-LDL by transcriptional and post-transcriptional mechanisms but principally by destabilization of macrophage scavenger receptor mRNA (Hsu et al. 1996). Binding of Ac LDL to PMA-differentiated THP-1 macrophages was suppressed by TNF-α in a dose-dependent manner. Inhibition of Ac-LDL binding was paralleled by a reduction of macrophage scavenger receptor protein as detected by the Western blot. TNF-α partially decreased macrophage scavenger receptor mRNA steady state levels in PMA-differentiated THP-1 macrophages, a result that was confirmed by reverse transcription-polymerase chain reaction. PMA increased the luciferase activity driven by the macrophage scavenger receptor promoter in the transfected cells, whereas TNF-α partially reduced luciferase activity. However, macrophage scavenger receptor mRNA half-life was dramatically reduced in cells treated with TNF-α relative to untreated cells. Reduction in macrophage scavenger receptor message in response to TNF-α was dependent on new protein synthesis because it was blocked by cycloheximide. These results indicate that TNF-α regulates macrophage scavenger receptor expression in PMA-differentiated THP-1 macrophages by transcriptional and post-transcriptional mechanisms but principally by destabilization of macrophage scavenger receptor mRNA. Roselaar reported that lipopolysaccharide (LPS) down regulates scavenger receptor activity in cultured macrophages through release of TNF-α (Roselaar and Daugherty, 1997). However, Li reported that TNF-α increased scavenger receptor mRNA levels, protein expression, and Ac-LDL degradative activity in cultured rabbit aortic smooth muscle cells. Cytokines, such

as TNF-α may stimulate some of the phenotypic changes that characterize the alteration in gene expression of intimal smooth muscle cells in rabbit atherosclerotic lesions (Li et al. 1995). The induction of scavenger receptor expression in intimal smooth muscle cells in vivo could be a useful marker of smooth muscle cell activation during atherogenesis and may contribute to foam cell formation by this cell type following balloon injury and/or hypercholesterolaemia.

IFN-γ increased scavenger receptor mRNA levels, protein expression, and Ac-LDL degradative activity in cultured rabbit aortic smooth muscle cells (Li et al. 1995). IFN-γ, may stimulate some of the phenotypic changes that characterize the alteration in gene expression of intimal smooth muscle cells in rabbit atherosclerotic lesions (Li et al. 1995). There is decreased number of lipid-rich foam cells in T cell-rich areas. (Geng et al. 1995). The mRNAs for interleukin-2 (IL-2) and IFN-γ, two major products of activated T cells, were detected by RT-PCR in all plaques tested. This indicates that activation of T lymphocytes occurs in atherosclerotic plaques. Since interferon-gamma down regulates scavenger receptor expression, these observations suggest a potential mechanism for local regulation of macrophage scavenger receptor expression in the atherosclerotic plaque (Geng et al. 1995). However, Horvai reported that IFN-γ inhibits transcription of the macrophage scavenger receptor gene by antagonising the Ras-dependent activities of AP-1 and cooperating ets domain transcription factors, apparently as a result of competition between AP-1/ets factors and activated signal transducer and activator 1 of transcription (STAT1) for limiting amounts of CBP (a binding protein of cAMP response element-binding protein) and p300 (an adenovirus E1A-associated protein). Consistent with this model, STAT1 alpha interacts directly with CBP in cells, and microinjection of anti-CBP and anti-p300 antibodies blocks transcriptional response to IFN-γ. Cell lacking STAT1 fail

to inhibit AP-1/ets activity, and over expression of CBP both potentiates IFN-γ-dependent transcription and relieves AP-1/ets repression. Thus, CBP and p300 integrate both positive and negative effects of IFN-γ on gene expression by serving as essential co-activators of STAT1 alpha, modulating gene-specific responses to simultaneous activation of two or more signal transduction pathways (Horvai et al. 1997).

Granulocyte macrophage colony-stimulating factor (GM-CSF) can down regulate both types AI and AII scavenger receptor in human monocyte-derived macrophages, which might have implications for foam cell formation (van der Kooij et al. 1996). Treatment with GM-CSF resulted in a significant twofold to threefold decrease in the number of binding sites for Ac-LDL and Ox-LDL on the surface of macrophages without affecting the affinity of the receptor for these ligands. The mRNA levels of both types AI and AII scavenger receptor were reduced in macrophages differentiated in the presence of GM-CSF. However, de Villiers demonstrated that M-CSF markedly and selectively increased scavenger receptor synthesis in murine macrophages; post-translationally the receptor appeared more stable and shifted to a predominantly surface distribution. Functionally M-CSF enhanced modified lipoprotein uptake and increased divalent cation-independent adhesion in vitro. These results suggest a plausible mechanism whereby M-CSF production in the atheromatous plaque microenvironment could promote the recruitment and retention of mononuclear phagocytes and subsequent foam cell formation. In addition, the Th1 cytokine (IFN-γ) and Th2 cytokine (interleukin-4) had differential effects on macrophage scavenger receptor glycosylation in vitro suggesting a further possible regulatory role by these cytokines on macrophage scavenger receptor function (de Villiers et al. 1994)

Nishimura reported that secretion of TGF-β1 leads to autonomous suppression of scavenger receptor activity in a monocyte-macrophage cell line (THP-1) (Nishimura et al. 1998). However, Gong reported that rabbit SMC express types I and II scavenger receptors that are up-regulated by TGF-β1, but not in human SMC (Gong and Pitas, 1995). This suggests that the regulation of scavenger receptor also depend on cell types, probably the culture condition as well.

Liao reported that macrophages derived from peripheral monocytes and PMA-differentiated THP-1 monocytic cells showed significantly reduced uptake and /or binding of the scavenger receptor ligand, acetylated LDL when treated with IL-6. This effect was paralleled by a reduction in the expression of scavenger receptor protein and mRNA. Analysis of scavenger receptor promoter activity in THP-1 cells transfected with scavenger receptor reporter gene construct demonstrated decreased activity of the scavenger receptor promoter in IL-6-treated THP-1 macrophages. Electrophoretic mobility gel shift assay also showed a reduction in the binding of a transcription factor to the scavenger receptor promoter AP-1/ets elements in IL-6 treated cells (Liao et al. 1999).

Gong reported that rabbit SMC express types AI and AII scavenger receptors that are up-regulated by platelet secretion products. Platelet-derived growth factor (PDGF-BB) at 10 ng/ml increased scavenger receptor activity in rabbit SMC but not in human SMC. Epidermal growth factor (EGF) or insulin-like growth factor I (IGF-I) alone had little effect on SMC scavenger receptor activity. The growth factors had synergistic effects on scavenger receptor activity and on types I and II scavenger receptor mRNA expression. In rabbit SMC, PDGF-BB, EGF, and TGF-β1 together stimulated scavenger receptor activity 12-fold. In human SMC, EGF and TGF-β1, together with either IGF-I or PDGF-

BB, stimulated receptor activity approximately 7-fold. Growth factor-mediated induction of scavenger receptor activity in rabbit and human SMC was blocked by the tyrosine kinase inhibitor, whereas the induction of scavenger receptor activity in rabbit but not human SMC was blocked by the protein kinase C inhibitor. Studies using neutralizing antibodies demonstrated that TGF-β1 is the predominant factor in vitro preparations of platelet secretory products which regulates scavenger receptor activity (Gong and Pitas, 1995). The growth factors that act synergistically in regulating scavenger receptor activity in vitro are all present in atherosclerotic lesions, where they are produced by macrophages, endothelial cells, SMC, and platelets. The data suggest that these growth factors may regulate scavenger receptor activity in SMC in vivo and contribute to foam cell formation (Gong and Pitas, 1995).

The treatment with 10 nmol/l $1,25(OH)_2D_3$ for 72 h inhibited the degradation of acetyl LDL by THP-1 macrophages in a dose-dependent manner, suggesting that $1,25(OH)_2D_3$ inhibits scavenging function in macrophages. Scatchard analysis revealed that $1,25(OH)_2D_3$ decreased the number of scavenger receptors without changing the affinity for acetyl LDL. The treatment with $1,25(OH)_2D_3$ for 72 h dramatically decreased the mRNA levels after the acquisition of macrophage phenotypes (Suematsu et al. 1995). The influence of $1,25(OH)_2D_3$ inhibiting the expression of scavenger receptor is interesting because a deficiency of this hormone in chronic renal failure may have influence in inducing scavenger receptor in renal cells.

As discussed here, various cytokines have different influence on the expression and regulation of scavenger receptor on number of cell types. These discrepancies probably mean either that different cell types or cell culture conditions have different

regulation of scavenger receptor. We demonstrated for the first time that TNF-α & IL-1β induced scavenger receptor expression in HMCL during long-term incubation. The inhibitory effect of TNF-α reported previously on scavenger receptor in culture was achieved in the condition when macrophages were pre-stimulated by PMA. We considered that this phenomenon was a result of competition between AP-1/ets factors and activated transcription factor involved in TNF-α stimulation for limiting amounts of co-activators. As we showed in this thesis that AP-1/ets transcription factors were involved in scavenger receptor expression induced by PMA rather than TNF-α. When cells were incubated with TNF-α after PMA stimulation. The transcription factors involved in TNF-α stimulation could bind limiting amount of co-activators of AP-1/ets, therefore, reducing AP-1/ets activity and scavenger receptor expression.

Our results showed that human mesangial cells express an inducible scavenger receptor. The inducible expression was also linked to phenotypic changes in the population of HMCL. We observed that there was some degree of heterogeneity of scavenger receptor expression within the HMCL population. A proportion of the cell population was negative for receptor as judged by cell sorting analysis and fluorescence microscopy, regardless of the length of exposure to PMA. This may explain several aspects of the phenotypic conversion of HMC, including cell proliferation, transformation from smooth muscle cells to phagocytes, and foam cell formation. Synchronised HMCL and primary cultures of HMC after colchicine arrest also showed a non-homogeneous staining pattern with DiI-Ac-LDL, suggesting that the non-homogeneous behaviour of

HMCL with respect to DiI-Ac-LDL uptake did not result from different functional specificity within the cell line used, nor was it a function of the cell cycle.

The mechanism of scavenger receptor inducible expression in HMC induced by PMA is unclear. Both PMA and Ang II are activators of PKC pathway. The experiments using signal transduction pathway inhibitors showed that the PKC and calmodulin pathways were involved in the induction of scavenger receptor by PMA and Ang II during the first 24 hours of culture. PMA induced down-regulation of PKC has been examined in various cell types in long term incubation. Different cell lines displayed a range of sensitivities to tetradecanoyl phorbol acetate (TPA)-induced down-regulation of PKC, suggesting that there could be cell-type-specific differences in the pattern of down-regulation (Adams and Gullick, 1989). In the present HMCL experiments, we could not exclude PMA induced down-regulation of PKC. Differentiation of cells is a very complex event involving a number of pathways, activation of the PKC pathway may be an early event and other signal transduction pathways may be involved during long-term differentiation.

Additionally, we focused on the molecular mechanism of scavenger receptor up-regulation by PMA, Ang II and inflammatory cytokines. A reporter gene containing scavenger receptor promoter (-696 to + 46) was transfected into HMCL. The results showed that PMA, Ang II, TNF-α & IL-1β could increase scavenger receptor promoter activity significantly. This result suggests that PMA, Ang II, TNF-α & IL-1β increases scavenger receptor expression by enhancing scavenger receptor gene transcription. The scavenger receptor 5' upstream (-4.1kb to +46) of genomic DNA contains several copies of binding motifs of AP-1/ets transcription factors. To further examine the relative roles of the AP-1/ets motifs in mediating PMA, Ang II, TNF-α & IL-1β dependent

transcriptional activation, a minimal prolactin gene promoter was used, which exhibited very little activity either before or after PMA, Ang II, TNF-α & IL-1β treatment. Luciferase reporter constructs were made in which either one or three copies of the AP-1/ets motifs, or the corresponding sequences containing mutations in either the AP-1 or ets, were introduced upstream of a minimal promoter derived from the rat prolactin gene. Using this conventional method, Wu reported that combinatorial interaction between AP-1 and ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene in the THP-1 cell line. AP-1/ets activity could be induced by and were necessary for PMA induced monocyte differentiation (Sraer et al. 1996). Our results showed that there was a similar regulatory effect of AP-1/ets in HMCL. AP-1/ets motifs were specific response elements to PMA stimulation in HMCL. Mutation of either the AP-1 or the ets motifs decreased its ability to respond to PMA, suggesting that AP-1/ets motifs are necessary response elements for gene expression induced by PMA. These results imply that PMA induces scavenger receptor promoter activity by increasing binding of AP-1/ets transcription factors to the scavenger receptor promoter region and that AP-1/ets transcription factors are necessary for scavenger receptor expression induced by PMA in HMCL.

AP-1 proteins regulate the expression of a diverse set of immediate/early genes that exhibit very rapid and transient transcriptional responses to signals transmitted from cell surface receptors (Angel and Karin, 1991). These immediate responses reflect post-translational modifications of AP-1 proteins, which have been particularly well studied in the case of c-jun (Pulverer et al. 1991; Smeal et al. 1991). They may increase transcriptional activity independent of new protein synthesis (Pulverer et al. 1991). In contrast to immediate/early genes, Moulton reported that TPA maximally stimulated

macrophage scavenger receptor gene transcription between 8-16 hours after TPA treatment. This induction was dependent on new protein synthesis and could be blocked by staurosporine (Moulton et al. 1992). Consistent with these observations, we found that transcriptional activation of the scavenger receptor gene in response to PMA did not become maximal until 72 hours following PMA treatment in HMCL. This suggests that PMA induce scavenger receptor expression in HMCL by increasing transcription and translation of AP-1/ets protein, rather than by post-translational modification of AP-1/ets protein.

The reporter gene containing three copies of AP-1/ets responsive elements had a very small response to Ang II, TNF-α & IL-1β stimulation suggesting that AP-1/ets motifs are not specific response elements for Ang II, TNF-α & IL-1β. This probably implies that transcription factors are inducer specific and different transcription factors are involved in inducing scavenger receptor. Future work will be directed toward identification of the specific response elements for Ang II, TNF-α & IL-1β in scavenger receptor promoter region.

In summary, during inflammation, HMC may express an inducible scavenger receptor, through which cells can acquire lipids and can be converted to foam cells in developing glomerulosclerosis. The induction of scavenger receptor expression in HMC could be a useful marker of HMC activation during the development of glomerulosclerosis. Different transcription factors are involved in scavenger receptor induction by different stimulators. Identification of transcription factors and their binding sites may become useful targets for therapeutic manipulation.

CHAPTER 4. FUNCTIONAL TRANSFORMATION OF LDL RECEPTOR UNDER THE INFLUENCE OF INFLAMMATORY CYTOKINES IN CULTURED HUMAN MESANGIAL CELLS

4.1. INTRODUCTION

Recent studies have linked systemic infection and inflammation with atherosclerosis (Ross, 1999; Nieto, 1998). Furthermore, local inflammation occurs at the site of plaque formation. Liuzzo reported that the acute-phase reactants C-reactive protein and serum amyloid A protein were elevated in most of their patients with diagnosis of unstable angina including those who subsequently proved to have had a myocardial infarction. Patients with elevated levels of acute-phase reactants, which reflect the stimulation of hepatic production by circulating inflammatory mediators such as cytokines, had a less favourable clinical course than those with normal levels of acute-phase reactant (Liuzzo et al. 1999). Elevated plasma level of cytokines including TNF-α, TGF-β, PDGF and IL-1 have also been found in various kidney diseases, suggesting that inflammatory cytokines may contribute to disease progression (Fine et al. 1992; Boswell et al. 1988; Luger et al. 1987).

Brown and Goldstein observed that LDL receptor activity is under tight metabolic control from intracellular cholesterol concentration through a feedback system (Brown and Goldstein, 1986; Goldstein and Brown, 1977). The key molecules of feedback regulation are sterol regulatory element binding proteins (SREBPs) and SREBP cleavage-activating protein (SCAP) (Hua et al. 1996; Sakai et al. 1998). This system maintains a constant level of cholesterol in hepatocytes and other cells by controlling both the rate of cholesterol up-take from LDL and the rate of cholesterol synthesis (Goldstein and Brown, 1985). Therefore, native LDL is ineffective in generating lipid rich foam cells that found in fatty streaks. The ineffectiveness of native LDL led to the speculation that oxidised LDL may account for atherogenicity through its interaction with scavenger receptors and foam cell formation.

As we discussed in chapter 3, HMCL express an inducible scavenger receptor under the influence of inflammatory cytokines, which contributes to glomerulosclerosis through its interaction with modified-LDL. However, LDL receptor is a major receptor in HMC. We propose that inflammatory cytokines may break LDL receptor feedback regulation resulting in the LDL receptor itself acquiring scavenger functions. If this were true, in any acute or chronic inflammatory state, inflammatory cytokines could confer a scavenger function on the LDL receptor. The affected cells would then accumulate unmodified native LDL particles in an unregulated way to form foam cells and fatty streaks.

Following experiments are designed to test the above hypothesis. To elucidate the logic of the experimental design, it is thought that a discussion about LDL receptor gene and transcriptional regulation would be appropriate.

4.1.1. LDL receptor feedback regulation

The expression of LDL receptor could be regulated by many factors, such as intracellular levels of cholesterol, oxysterols, and of several growth factors (Hoogerbrugge et al. 1996; Streicher et al. 1996; Srivastava et al. 1995; Srivastava et al. 1993). Cholesterol is the main regulator of LDL receptor. Intracellular cholesterol regulates LDL receptor gene transcription through the LDL receptor promoter. This requires a well-defined sterol regulatory site SRE-1 and an adjacent binding site for the universal transcription factor Sp1. In sterol-depleted cells, a two-step cleavage process releases the NH2-terminal segments of SRE binding proteins (SREBP-1 and SERBP-2), which then enter the nucleus, bind to the LDL receptor promoter region, and activate gene transcription (Yokoyama et al. 1993). The process begins when a protease cuts

SREBP-2 at site 1, which is at or near an arginine in the lumenal loop, thereby separating the two membrane-spanning segments. This allows a second protease to cut the protein at site 2, in the middle of the first transmembrane segment, releasing the N-terminal domain into the cytosol. The site 1 protease, which is strictly regulated by sterols, is active in sterol-depleted cells and turned off when sterol accumulates. The site 2 protease is not regulated directly by sterols, but can act only after the site 1 protease.

SREBP cleavage-activating protein (SCAP) has recently been identified in hamster and human as a key molecule (Hua et al. 1996) in the regulation of cholesterol metabolism by stimulating cleavage of SREBP-1 and –2. It has multiple membrane-spanning regions, five of which resemble the sterol-sensing domain of HMG-CoA reductase, an endoplasmic reticulum enzyme whose degradation is accelerated by sterols. A mutation at codon 443 of SCAP enhances the cleavage-stimulating ability of SCAP and renders LDL receptor in Chinese hamster ovary (CHO) cells resistant to inhibition by sterols suggesting that SCAP may be a key factor in the feedback regulation of LDL receptor (Hua et al. 1996). Therefore, SCAP appears to act as a "cholesterol sensor" in animal cells (Hua et al. 1996; Sakai et al. 1998) (Fig.4.1.).

4.1.2. Effects of hormones and cytokines on LDL receptor regulation

It has long been known that some hormones can influence cellular uptake of LDL and more recent studies have confirmed increases in receptor synthesis and mRNA content. For instance both LDL receptor protein and mRNA have been shown to be increased by stimulators of steroid hormone production such as ACTH and chorionic gonadotropin in their target cells (Faust et al. 1977; Golos et al. 1986). The hepatic LDL receptor protein

content of WHHL rabbits and the receptor mRNA content of human placenta (Furuhashi et al. 1989) are increased during pregnancy. Thyroid hormone also increased hepatic LDL receptor (Ness and Lopez, 1995). Hypothyroidism leads to a reduction in hepatic receptor content of rat liver (Scarabottolo et al. 1986) and thyroxine can stimulate LDL uptake in isolated rat hepatocytes, an effect which is opposed by dexamethasone (Salter et al. 1988). LDL uptake was increased by insulin (Chait et al. 1978) in fibroblasts. A similar effect of insulin is seen in Hep G-2 cells and rat hepatocytes (Salter et al. 1988).

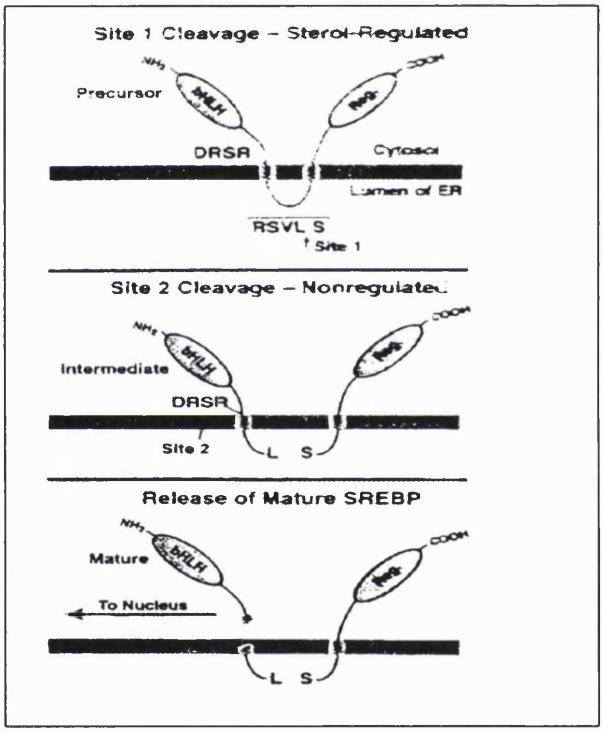


Fig4.1. Model for two-site proteolytic cleavage of membrane-bound SREBPs. bHLH, basic-helix-loop-helix-leucine zipper domain of SREBP; Reg, regulatory domain of SREBP.

Mitogens platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) increased LDL receptor synthesis and mRNA expression in cultured fibroblasts (Chait et al. 1980). Hsu showed that basic fibroblast growth factor (bFGF) increased LDL binding, uptake, and degradation in arterial smooth muscle cells in a dose-dependent

manner by activation of tyrosine kinase pathway. This increase was paralleled by an increase in LDL receptor mRNA steady state levels (Hsu et al. 1994). Oncostatin M (OM), a 28 kilodalton glycoprotein cytokine, is structurally and functionally related to interleukin-6 and leukemia-inhibitory factor. Liu reported that OM strongly up-regulated LDL receptors in human liver cells by a tyrosine kinase-mediated mechanism. IL-1 and OM also induced transcription factor Egr-1. The Egr-1, tyrosine kinase, and LDL receptor responses were inhibited at similar concentrations of genistein, suggesting that induction of Egr-1 and up-regulation of LDL receptors depended on activation of tyrosine kinase by OM (Liu et al. 1993).

In summary, LDL receptor regulation could be affected by cholesterol, cytokines or growth factors. However, cholesterol is a major element. LDL receptor regulation may have different patterns depending on cell types and cell culture condition.

4.1.3. Effects of drug on LDL receptor regulation

HMG-CoA reductase inhibitors (statin) are a class of powerful LDL lowering drugs. These drugs act primarily in the liver to inhibit cholesterol synthesis and thereby lower intracellular cholesterol concentration and increase LDL receptor. Simvastatin increased the LDL receptor activity of human mononuclear cells from the polygenic hypercholesterolaemic subjects by 70% while lowering their plasma cholesterol by 26%, but reducing the fat intake from 38% to 20% of energy and cholesterol from 239 to 96 mg/day had no effect on the receptor despite a 10% reduction in plasma cholesterol. Up-regulation of the LDL receptor may therefore have been involved in the lowering of plasma cholesterol by simvastatin but not by the reduction in dietary fat and cholesterol (Roach et al. 1993). Calcium channel blockers (CCBs) and antagonists of calmodulin

action (Filipovic and Buddecke, 1986a; Eckardt et al. 1988) increase the LDL-receptor protein and mRNA content of cultured fibroblasts. A detailed description of the influence of CCBs is given in chapter 5. Cyclosporin, an immunosuppressant which has been shown to inhibit the 27-hydroxylation of sterols, in high concentration was found to inhibit the esterification of cholesterol and to increase the cellular level of free cholesterol resulting in suppression of LDL receptor activity (Winegar et al. 1996). Phorbol esters, which activate protein kinase C, have little effect on fibroblasts (Filipovic and Buddecke, 1986b) but markedly increase the LDL receptor mRNA content of monocytic leukaemia cells (Auwerx et al. 1989), which also show a reduction of LDL uptake with the prostaglandins PGE, and PGE (Krone et al. 1988). Many of these effects can be explained by changes in the requirement of the cells for sterols to support a stimulation of growth.

4.1.4. Expression of LDL receptor in mesangial cells

LDL receptor regulation may play an important role in the progression of various renal diseases. Therefore the characteristics of LDL binding and uptake were examined in cultured both rat or human mesangial cells (Grone et al. 1992; Wheeler et al. 1991). Both rat and human mesangial cell expressed LDL receptor and bound and transported LDL in a manner consistent with specific receptor mediation. Furthermore, exposure of mesangial cells to LDL enhanced intracellular cholesteryl esterification and decreased de novo cholesterol synthesis. Mesangial cells expressed LDL receptor mRNA and the expression of which was down regulated after pre-loading of cells with LDL. These results are consistent with regulation of cholesterol uptake and metabolism by a specific LDL receptor pathway.

In the present study, we investigated the molecular mechanisms by which inflammatory cytokines regulated LDL receptor expression in the presence of a maximally suppressive concentration of native LDL. An understanding of this mechanism is essential to see whether cytokines have any major effect in influencing lipid mediated glomerular injury and modifying the progression of glomerulosclerosis.

4.2. METHODS

4.2.1. Cell culture

An established stable human mesangial cell line (HMCL) was used (kindly donated by Dr.J.D.Sraer, Hopital Tenon, Paris). Experiments were carried out in serum free RPMI medium containing 0.2% bovine serum albumin (BSA) with anti-oxidant ethylenediaminetetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) at final concentrations of 100 μmol/l and 20 μmol/l respectively (Sigma, Poole, Dorset, UK). All reagents for cell culture were obtained from Gibco BRL (Paisley, UK). Recombinant human tumour necrosis factor alpha (TNF-α, $2.0\text{-}5.0\times10^7$ Unit/mg), transforming growth factor β (TGF-β, 2×10^7 Unit/mg), platelet-derived growth factor (PDGF, $0.33\text{-}1.0\times10^6$ Unit/mg), and interleukin-1β (IL-1β, $1.0\text{-}3.3\times10^8$ Unit/mg) were obtained from R&D System (Europe Ltd, Abingdon, UK).

4.2.2. Cell proliferation assay

HMCL were plated in 96-well plates (Falcon, Oxford, UK) at a density of 6000/well in RPMI containing 5% FCS and cultured until nearly confluent. The cells were synchronised to the quiescent state by incubation in serum free RPMI medium for 48 hours, then each well was washed with PBS. Cells were incubated for 24 hours in serum free RPMI medium with or without different cytokines. The cells were labelled for 18 hours in 1 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK), then washed

with PBS, trypsinised for 30 minutes then harvested. Radioactivity associated with the DNA of proliferating cells was measured in a scintillation counter. Tests were performed in quadruplicate for each experiment. For cell counts, HMCL were subcultured at 20,000 cell/well into 24-well culture plates (Falcon, Oxford, UK) in standard growth medium. At confluency, the cells were synchronised to the quiescent state as described above. After 48 hours, viable cells/well (assessed by trypan blue staining) were counted using a haemocytometer (time 0 hour). Then cells were incubated for 24 hours in serum free RPMI medium or RPMI plus different cytokines and cells were counted.

4.2.3. Preparation of Lipoprotein

Plasma was collected from a healthy human volunteer and LDL was isolated by sequential ultracentrifugation as previously described in chapter 2.

4.2.4 Morphological examination

HMCL were plated in chamber slides for tissue culture (Nunc Inc., Naperville, IL) and incubated in serum free RPMI medium with native LDL or cytokines plus native LDL in the absence or presence of polyinosinic acid (PolyI) or heparin. After 24 hours incubation, the cells were washed three times with PBS, fixed for 30 min with 5% formalin solution in PBS, stained with Oil Red O for 30 min and counter stained with hematoxylin for another 5 min. Finally, the cells were examined by light microscopy.

4.2.5. Iodination of LDL

LDL was iodinated using the enzymobead method as previously described in chapter 2.

4.2.6. Binding of ^{125}I-LDL to HMCL at 4 ^{0}C

Analysis of ^{125}I-LDL (10-100 μg/ml) binding experiments carried out at 4°C using the EBDA/LIGAND program as described in Chapter 2.7 & 2.8.

4.2.7. Western Blot analysis

Western blot analysis was carried out using methods as previously described in chapter 2. Mouse anti-human LDL receptor monoclonal antibody (1 μg/ml) and a goat anti-mouse HRP labelled antibody were used. Finally, amplification and colorimetric detection procedures were completed using Bio-Rad Opti-4CN detection Kit (Bio-Rad laboratories, Herts., UK).

4.2.8. Cell labelling and flow cytometry analysis. HMCL were cultured in serum free medium alone or with cytokines or cytokines plus native LDL for 24 hours before addition of 10 μg/ml of DiI-LDL in the presence or absence of 100-fold excess of unlabelled LDL at 37°C. After 5 hours, the cells were fixed, washed, and analysed by fluorescence-activated cell sorter analysis (FACS) using a flow cytometer as described in Chapter 2. The data were evaluated by mean fluorescence intensity (MFI). Autofluorescence signals from unlabelled cells were used as negative controls in each experiment. The MFI of DiI-labelled cells was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells. The average of the duplicate determinations was used for statistical analyses. Each experiment was carried out in duplicate.

4.2.9. Northern Blot Analysis

Total RNA was isolated and electrophoresed (Fig.4.2.) as described in chapter 2.

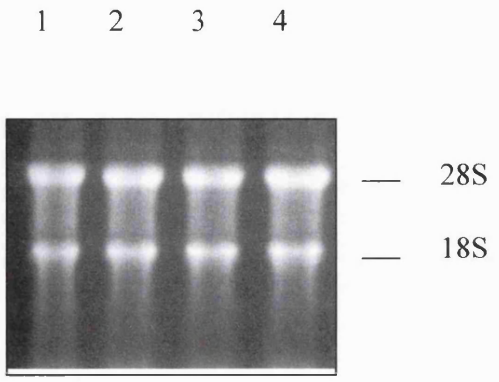


Fig 4.2. RNA electrophoresis

For hybridisation, a fragment of the LDL receptor cDNA, as a control, glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was used (Fig.4.3.). The LDL receptor cDNA probe (2.8 kb) was prepared by digesting the plasmid pLDLR3 (American Type Culture Collection, Rockville, MD, USA) with the restriction endonucleases Hind III and Sma I. The GAPDH cDNA probe (0.8 kb) was prepared by digesting plasmid pHcGAP (American Type Culture Collection, Rockville, MD, USA) with the restriction endonucleases Pst I and Xba I. The cDNA probes were labelled with ^{32}P-dCTP (3000 Ci/mmol, Amersham, Buckinghamshire, UK) by nick translation as previously described in chapter 2. Hybridisations were performed using a standard method as described in chapter 2.

4.2.10. RT-PCR

Total RNA (500 ng) was used as a template for RT-PCR to examine SCAP mRNA expression using specific primers. SCAP 5' primer (nucleotide position 2574-2595); GCCCTCCGCCGCCTTCCCTCTT, 3' primer (nucleotide position 2933-2953); GCTGCTCCGCCCCACCACGAT (Hua et al. 1996). GAPDH: 5' primer (nucleotide position 73-92) TCATAGACAAGATGGTGAAG, 3' primer (nucleotide position 303-

327) TGACGGGATCTCGCTCCTGGAAGAT (Ando et al. 1996). RT-PCR were performed as described in Chapter 2

4.2.11. Southern blot analysis and quantitative evaluation

Above PCR products were transferred to a nylon membrane using the Southern analysis method as described in Chapter 2. The nylon membranes were probed with [γ-^{32}P]ATP labelled oligonucleotides. SCAP probe (nucleotide position 2654-2676): GGGCTGAGTGGGCTGTGAGGA (Hua et al. 1996); GAPDH probe (nucleotide position 170-191): AATGAAGGGGTCGTTGATGGCA (Ando et al. 1996). The DNA probes were labelled using 5'-end labelling system as described in chapter 2.

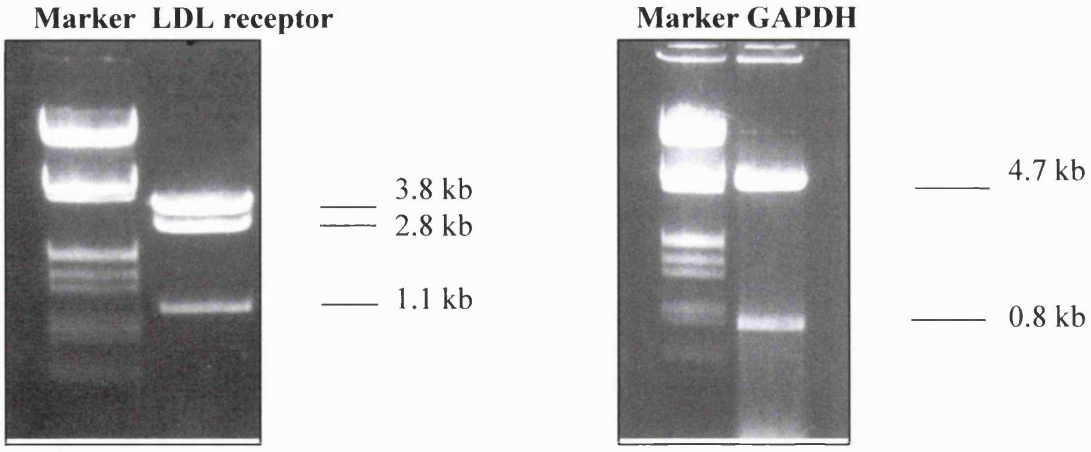


Fig 4.3. LDL receptor and GAPDH probe preparation. The LDL receptor cDNA probe (2.8 kb) was prepared by digesting the plasmid pLDLR3 with the restriction endonucleases Hind III and Sma I. The GAPDH cDNA probe (0.8 kb) was prepared by digesting plasmid pHcGAP with the restriction endonucleases Pst I and Xba I. The probes were purified from the agarose gel . Marker: λ DNA HindIII/EcoRI digestion.

4.2.12. LDL receptor promoter-report gene constructs

The LDL receptor promoter-luciferase fusion gene pGL3LDLR6500 was constructed using standard molecular clone techniques as described in chapter 2. The LDL receptor promoter was subcloned into the unique HindIII site of pGL3Luciferase vector, a recombinant plasmid that contains a SV40 enhancer (Promega, Southampton, UK). LDLR6500 (a gift from Dr. D. Russell, University of Texas) contains up to 6500 base pairs of 5' flanking DNA from the LDL receptor gene which contains three imperfect direct repeats of 16 bp and two TATA-like sequences of 7 bp each (Sudhof et al. 1987).

4.2.12.1. pGL3 enhancer Luciferase Reporter Vectors

The pGL3 enhancer Luciferase Reporter Vectors provides a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression (Promega). The pGL3 enhancer Vectors (Fig.4.4.) contain a high copy number prokaryotic origin of replication for maintenance in E. coli, an ampicillin-resistance gene for selection, and a filamentous phage origin of replication (f1 ori) for single-stranded DNA (ssDNA) production. Restriction sites for insertions of DNA fragments are located upstream and downstream of the luciferase gene. Two of the upstream sites (Xho I and Bgl II) yield cohesive ends compatible with the downstream sites (Sal I and BamH I, respectively), allowing the interchange of the DNA insert for rapid analysis of positional effects. The pGL3-Enhancer Vector contains an SV40 enhancer located downstream of luc+ and the poly(A) signal. This aids in the verification of functional promoter elements because the presence of an enhancer will often result in transcription of luc+ at higher levels.

4.2.12.2. Transformation for pGL3 enhancer Vectors

Because the Luciferase Reporter Vectors are supplied as modified DNA, E. coli hosts may be either restriction+ or restriction-. JM109 bacterial cells is preferred because this prevents undesirable recombination between the insert and the host chromosomal DNA. A strain that has an F′ episome is required for ssDNA production.

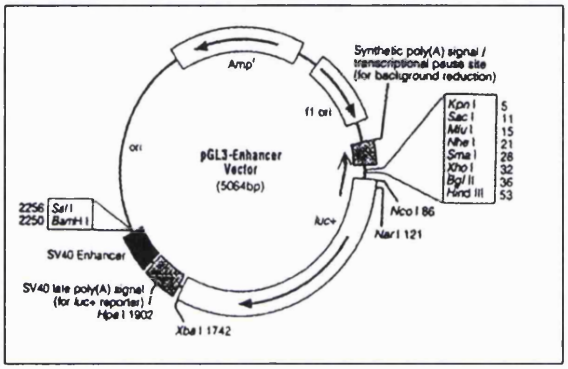


Fig 4.4. The map of pGL3-Enhancer Vector:

Promoter	(none)
Enhancer	2005-2249
SV40 late poly (A) signal	1772-1993
Luciferase gene (luc+)	88-1737
Upstream poly (A) signal	4904-5057
Multiple cloning site	1-58
RV primer3 binding site	5006-5025
RV primer4 binding site	2326-2307
GL primer2 binding site	111-89
Beta-lactamase gene (Amp[r])	4186-3329
f1 origin	4319-473
ColE1-derived plasmid replication origin	2564

4.2.12.3. Mini-preparation and Identification of pGL3 enhancer plasmid

The pGL3 enhancer DNA was digested by restriction enzymes Hinc II (position 1392,1902,2258) and Hind III (position 53), then subjected to electrophoresis in 1% agarose gel.

4.2.12.4. Maxi-preparation and purification of pGL3 enhancer plasmid DNA

The positive clone of pGL3 enhancer was subjected to Maxi-preparation and purification as described in chapter 2.

4.2.12.5. Protruding 5'-terminal dephosphorylation

The fragment and vector DNA was digested with restriction enzymes that generated compatible ends for cloning. The vector was also treated with calf intestinal phosphatase (CIAP) to remove 5′ phosphate groups, thus preventing reclosure of the vector on itself without an insert. 5 μg of pGL3 enhancer DNA was incubated with Hind III in appropriate restriction enzyme buffer at 37°C for overnight. Reaction completion was checked by electrophoresis of a sample on 1% agarose gel. Subsequent to digestion, the DNA was treated with CIAP as described in chapter 2.

4.2.12.6. Preparation of Insert (LDLR6500) for Cloning

To ensure capture of the correct insert DNA, plasmid pLDLRCAT 6500 was digested by Hind III at 37 °C for overnight and the desired restriction fragment (LDL receptor promoter 6500 bp) can be purified by electrophoresis on agarose gel (Fig.4.5.). A 6.5 kb fragment of DNA is LDL receptor promoter up to –6500bp, and then recovered from the gel by Gel Extraction system (QIAGEN, West Sussex, UK).

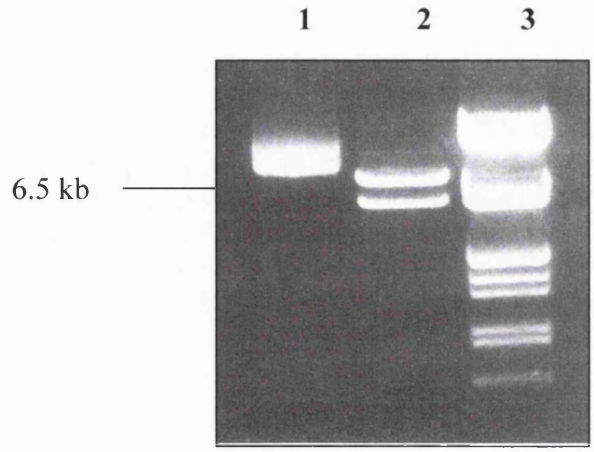


Fig 4.5. Preparation of LDL receptor promoter. Lane 1: pLDLCAT6500 2 μg; Lane 2: pLDLRCAT6500 2 μg Hind III digestion; Lane 3: λDNA Hind III/EcoRI digestion.

4.2.12.7. Ligation

We used a 1/5-8 ratio of insert/vector in the ligation of pGL3 enhancer and LDL receptor6500. pGL3 enhancer and LDLR6500 were incubated with T4 DNA ligase and buffer for 3 hours at 22 °C. Following the ligation reaction, the plasmid DNA was transformed into JM109competent cells using the method described in chapter 2. 12 positive clones were checked using mini-preparation of plasmid DNA and 3 of them (C1, C6, C7) were successful ligation. The C7 was selected as a reporter gene and used in transient expression assay (Fig.4.6.).

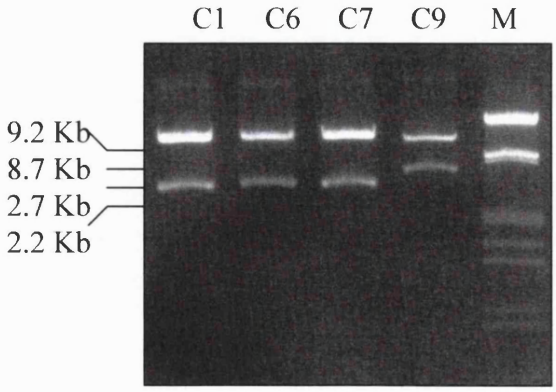


Fig 4.6. Identification of positive clones. Positive clones were digested by BamH I. It should produce 9.2kb &2.2 bands according to the map. Marker: λDNA Hind III/EcoRI digestion.

4.2.13. Transient Expression Assay

HMCL in 6×10^7/ml were transfected with 100 μg of supercoiled pGL3LDLR6500 (C7) and 100 μg control plasmid of psv-β-galactosidase (Internal standard) by electroporation at 340 volts and 125 uF with a Gene Pulser (Bio-Rad, Herts, UK). After electroporation, the cells were placed into 6 well plates with growth medium. Approximately 24 hours after replating, cells were washed with PBS and the medium was replaced with RPMI medium with or without different cytokines in the presence or absence of high concentration of LDL for 24 hours. The cells then were washed twice with PBS and lysed using cell lysis buffer (Promega, Southampton, UK). The luciferase activity in the supernatants was measured and normalised by comparison with β-galactosidase activity using the Promega luciferase and β-galactosidase assay systems.

4.2.14. The effect of various signal transduction inhibitors on cytokines-mediated activity of LDL promoter

Using a transient expression assay system, as described above, the electroporated HMCL were incubated for 24 hours in serum free RPMI medium or serum free medium with different cytokines in the presence or absence of different signal transduction pathway inhibitors: 25 μmol/L W-7 (calmodulin antagonist), 25 μmol/L genistein (receptor tyrosine kinase inhibitor), 100 nmol/L calphostin C (PKC inhibitor), 1 nmol/L staurosporine (serine/threonine kinase inhibitor), 10 ng/ml pertussis toxin (G-protein inhibitor).The activities of luciferase driven by LDL receptor promoter were measured.

4.3. RESULTS

4.3.1 Estimation of values for the dissociation constant (Kd) for LDL receptor in HMCL.

HMCL bound ^{125}I-LDL in a dose dependent manner at 4°C (Fig.4.7). The binding was saturable with a high affinity. Analysis of ^{125}I-LDL binding experiments carried out at 4°C using the EBDA/LIGAND program showed that the kinetics of LDL binding to HMCL best fitted a single model with a *Kd* of $1.1x10^{-8}$ M and B_{max} is $4.7x10^{-10}$M. Scatchard Plot is shown in Figure 4.8. It suggests that affinity of LDL to LDL receptor in HMCL is similar to primary culture of human mesangial cells with a *Kd* of $8.7x10^{-8}$ M (Grone, E.F., 1992).

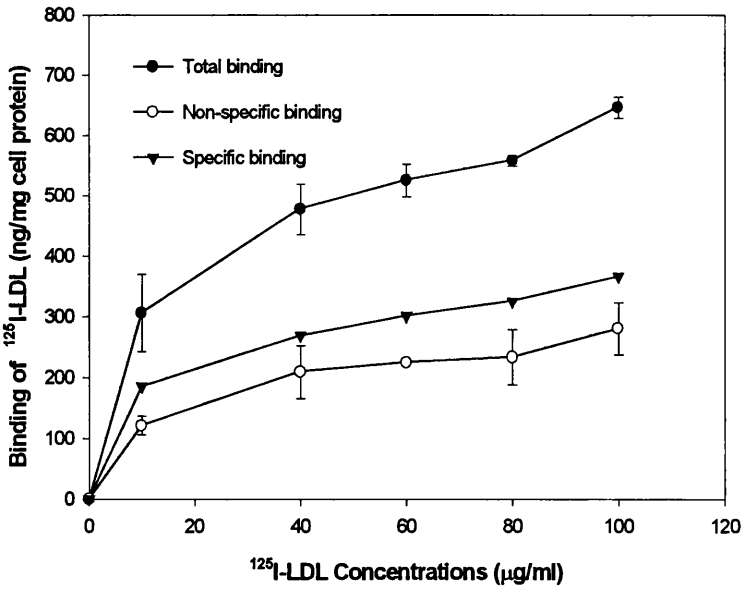


Fig.4.7. Binding of ^{125}I-LDL to HMCL at 4°C. The indicated concentrations of ^{125}I-LDL were incubated with HMCL for 4 hours at 4°C, in the presence or absence of a 50-fold excess of unlabelled LDL. Specific binding was calculated by subtracting non-specific binding (binding in the presence of excess unlabelled) from total binding values.

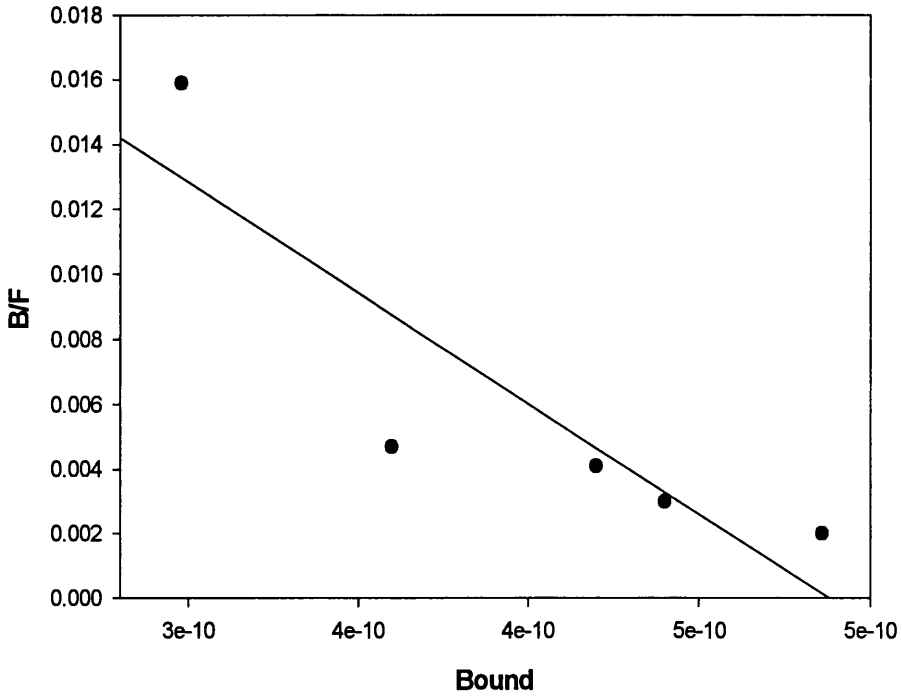


Fig.4.8. Plot of Scatchard transformed data of ^{125}I-LDL binding to HMCL assuming a one site model. Data was linearised using the LIGAND program. The calculated *Kd*=1.1x10^{-8} M and B_{max}=4.7x10^{-10}M.

4.3.2. Inflammatory cytokines induced LDL receptor transcription and expression in cell proliferation independent pathway

4.3.2.1. The effect of cytokines on LDL binding on HMCL

50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β increased specific binding of LDL to HMCL. Co-stimulation with above four cytokines also increased LDL binding to HMCL, however the effect was not additive (Fig.4.9.).

4.3.2.2. The effect of cytokines on expression of LDL receptor mRNA

Northern blot analysis of HMCL treated for 24 hours with four different cytokines showed increases in steady-state levels of LDL receptor mRNA consistent with the observed increases in specific binding (Fig.4.10.).

4.3.2.3. The effect of cytokines on HMCL proliferation

Usually, the activation of LDL receptor in response to growth factors reflects a requirement for cholesterol uptake or synthesis needed for the biosynthesis of new membranes in cells stimulated to proliferate. To determine whether the increased LDL receptor expression could be accounted for by changes in cell proliferation. We used relatively low concentrations of cytokines, which maximised LDL receptor gene expression, but minimised cell proliferation. Mitogenesis assays measuring ^{3}H-thymidine incorporation revealed that 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β did not significantly stimulate HMCL proliferation, but increased LDL receptor expression significantly during the 24 hours period (Fig.4.11.). ^{3}H-thymidine incorporation experiments were confirmed by cell-counting experiments (data not shown). These results

suggested that the up-regulation of LDL receptor transcription induced by TNF-α, TGF-β, PDGF, and IL-1β might be independent of cell proliferation.

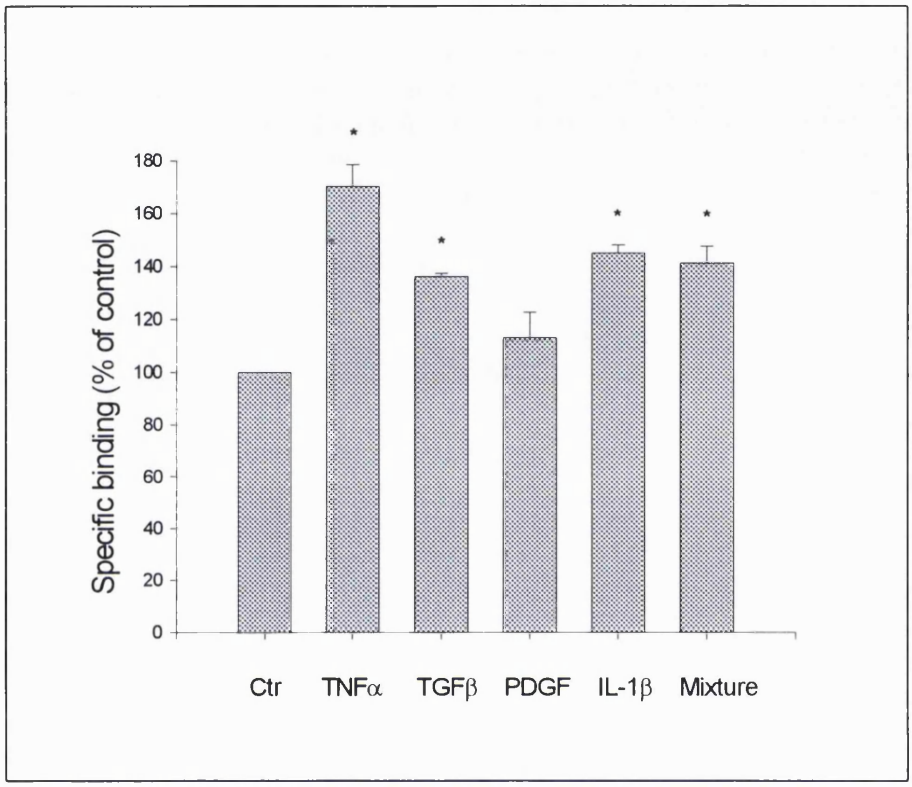


Fig.4.9. The effect of cytokines on LDL binding on HMCL. HMCL were grown to near confluence in 12-well plates and treated for 24 hours in serum free medium with or without with 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, IL-1β, and a mixture of above cytokines. LDL binding was assayed at 4°C for 2 hours with 5 μg/ml ^{125}I-LDL. Specific binding was calculated as the difference between ^{125}I-LDL binding with and without 50-fold excess unlabeled LDL and factorised to milligrams of protein. Four experiments were performed, then normalised to their respective control binding (100%). *$p<0.05$. vs control.

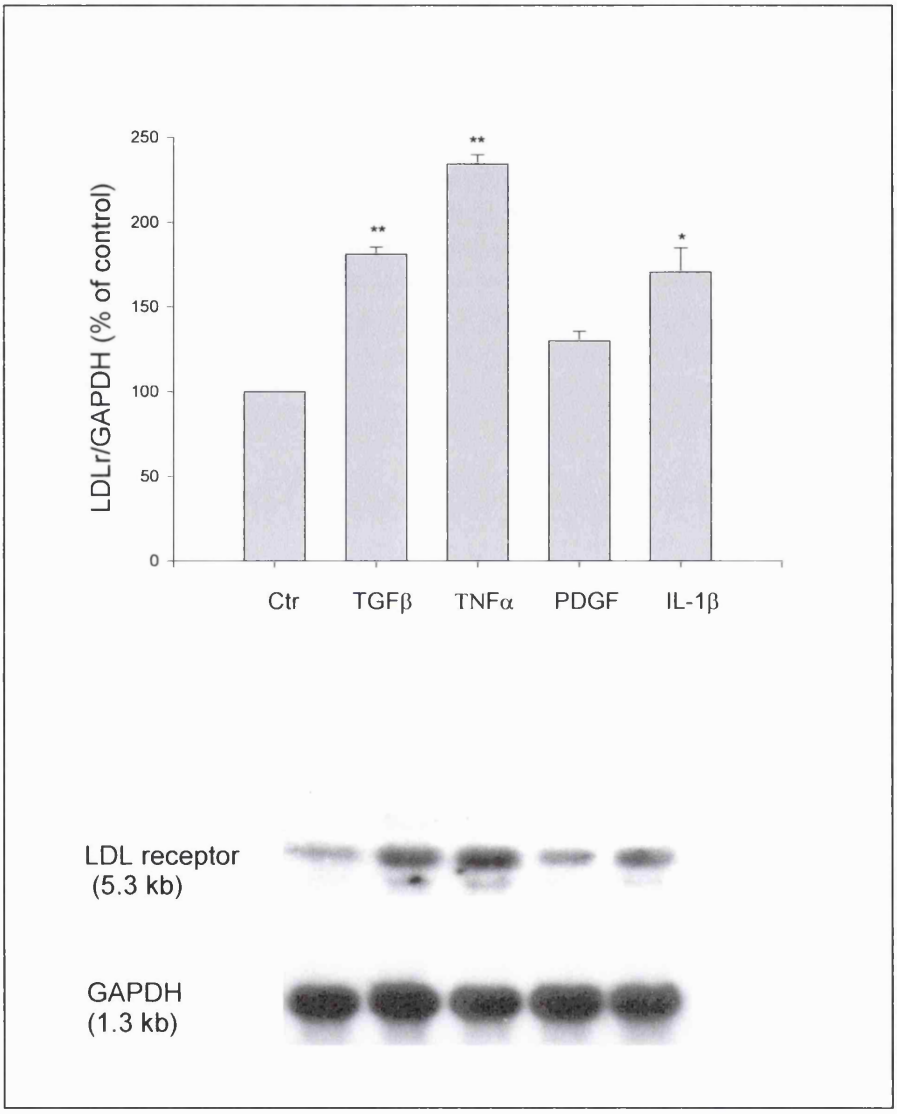


Fig.4.10. The effect of cytokines on expression of LDL receptor mRNA. HMCL were incubated in serum free medium (control) or serum free medium in the presence of 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β for 24 hours. Total cellular RNA was extracted and hybridised with ^{32}P-labelled LDL receptor and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probes. The histogram represent densitometric scanning of the LDL receptor mRNA band, normalised by comparison with GAPDH mRNA, and expressed as a percentage of control. *$p<0.05$, **$p<0.001$ vs control.

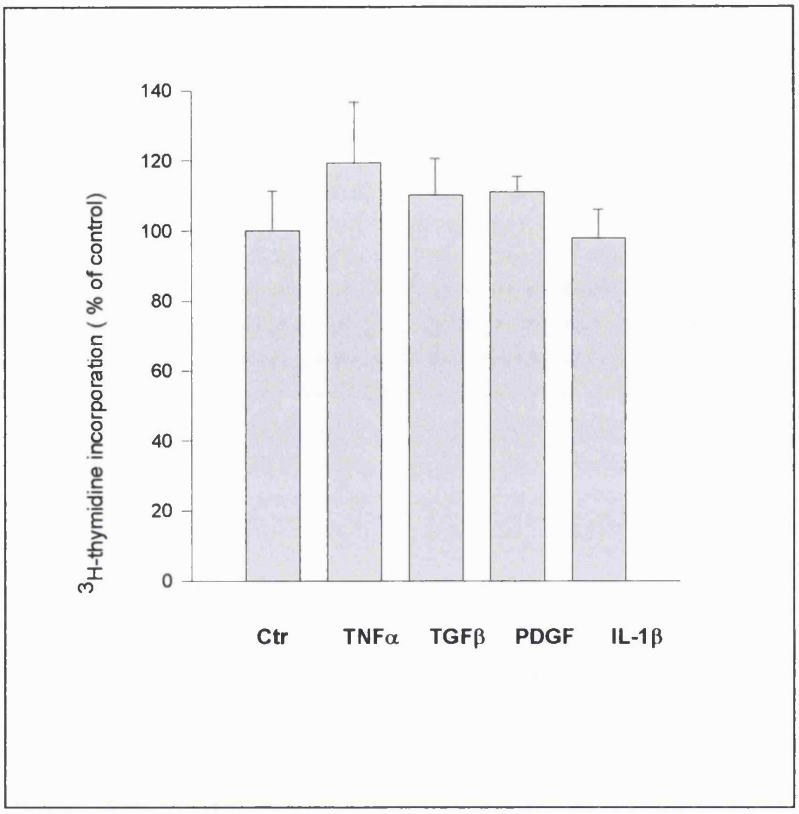


Fig.4.11. The effect of cytokines on HMCL proliferation. Quiescent HMCL were incubated for 24 hours in serum free medium (control), or serum free medium with 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β. The cells were harvested after labelling for 18 hours with ^{3}H-thymidine. Radioactivity associated with DNA of proliferating cells was measured in a scintillation counter. Results represent means±SEM of quadruplicate wells from four experiments. Results were normalised to their respective control (100%).

4.3.2.4. The effect of cytokines on activity of LDL receptor promoter

Using a luciferase assay system, treatment of HMCL with 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β increased LDL receptor promoter activity by 167.48±23.56%,

and 150.47±24.41%, 127.71±24.65%, 163.01±31.91 respectively, at 24 hours (control equal to 100%) (Fig.4.12.). These results were consistent with those found in terms of LDL receptor mRNA activity, and confirmed the LDL receptor protein binding experiments.

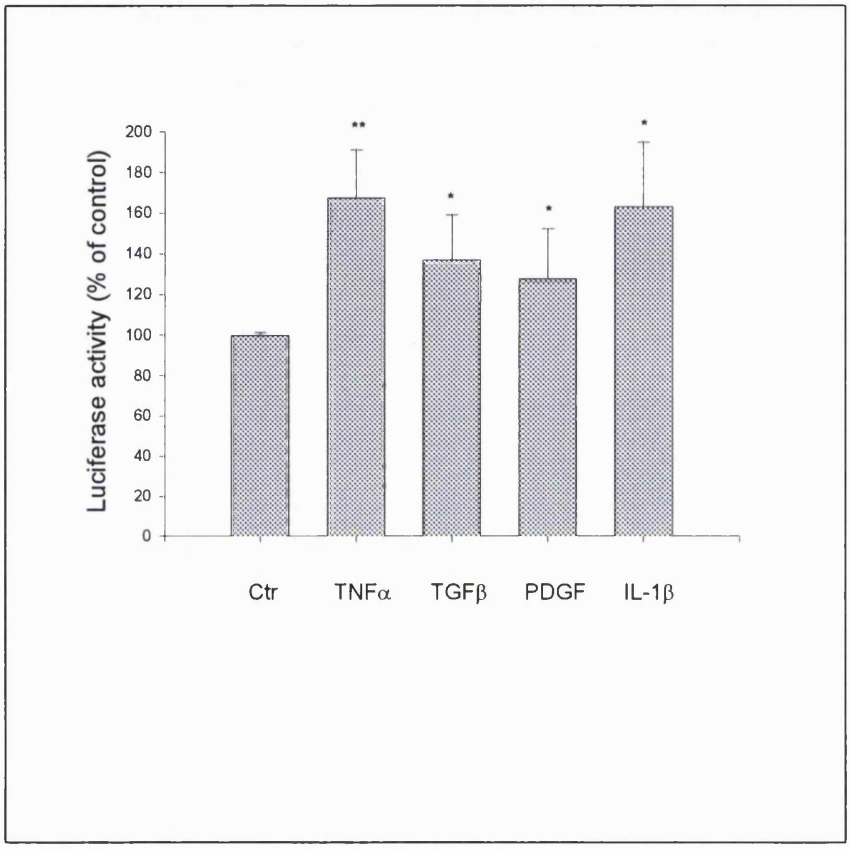


Fig.4.12. Cytokines stimulate LDL receptor promoter activity. HMCL were transfected with pGL3LDLR6500 construct, as a internal standard, psv-β-galactosidase plasmid was used. The transfected cells were cultured in a standard RPMI medium containing 5% FCS. After 24 hours, the cells were incubated in serum free medium without (control) or with 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β. Luciferase activity was measured and normalised by comparison with β-galactosidase activity. Results represent the mean±SEM of duplicate determinations from six experiments, which were normalised to their respective controls (100%). * $p<0.05$, ** $p<0.001$ vs control.

4.3.2.5. The effect of various signal transduction pathway inhibitors on scavenger receptor promoter activity

To determine the role of signal transduction pathway in the regulation of LDL receptor gene expression in cytokines-stimulated HMC, we evaluated the effects of various signal transduction inhibitors on LDL receptor transcription using the luciferase system. At non-cytotoxic concentrations of inhibitors, we observed that LDL receptor promoter activity in TNF-α, TGF-β, PDGF, and IL-1β treated HMCL in the presence of tyrosine kinase inhibitor (genistein) was significantly lower than in the absence of tyrosine kinase inhibitor, suggesting that tyrosine kinase transduction pathways were involved in LDL receptor up-regulation induced by all four cytokines used in this studies. Additionally, a serine/threonine kinase inhibitor (staurosporine) and G-protein inhibitor (pertussis toxin) significantly inhibited LDL receptor promoter activity induced by TGF-β. A calmodulin inhibitor (W-7), serine/threonine kinase inhibitor (staurosporine), and PKC inhibitor (calphostin C) significantly inhibited LDL receptor promoter activity induced by IL-1β (Table 4.1.). These results suggested that different cytokines regulated LDL receptor transcription via different signal transduction pathways and that tyrosine kinase may be a main pathway for LDL receptor up-regulation induced by those growth factors.

4.3.3. Inflammatory cytokines induce LDL receptor expression via a sterol-independent pathway

4.3.3.1. Visualisation of LDL uptake and lipid droplets

Foam cell formation will be considered to be evidence of functional transformation of LDL receptor. Experiments were performed to determine whether LDL receptor in the presence of cytokines is able to cause foam cell formation. Staining of HMCL with Oil Red O before (Fig.4.13A.) and after TNF-α (Fig. 4.13B.) & IL-1β (Fig.4.13C.)

stimulation in the presence of high concentration of LDL (250 μg/ml) showed that both TNF-α & IL-1β increased the number of intracellular Oil Red O stained lipid droplets and it could not be blocked by Poly I (Fig.4.13 D, E.), but was blocked by heparin (Fig.4.13 F,G.).

Table 4.1. The effect of various signal transduction inhibitors on cytokines-mediated luciferase activity driven by LDL receptor promoter

Treatment	Relative LDL receptor promoter -luciferase activity				
	Control	TNF-α	TGF-β	PDGF	IL-1β
No inhibitor	100 ±2.13	167.48 ±23.56	150.47 ±24.41	127.71 ±24.65	163.01± 31.91
Calmodulin inhibitor (W-7)	100 ±2.77	144.33±38.34	114.78 ±11.73	125.07 ± 10.59	114.78±14.74*
Tyrosine kinase inhibitor (Genistein)	100 ±2.15	105.07±20.67*	65.26±24.69*	82.47 ±24.50*	78.98±26.04*
PKC inhibitor (Calphostin C)	100 ±1.62	175.73 ±13.27	122.73 ±60	116.98 ±31.86	110.75 ±13.84*
Serine/threonine kinase inhibitor (Staurosporine)	100 ±6.96	136.40 ±19.59	87.88 ±18.26*	143.48 ±25.42	109.58±28.15*
G-protein inhibitor (Pertussis toxin)	100 ±5.78	142.56 ±27.91	99.15 ±7.21 *	123.31±41.70	119.10±45.95*

Transfected HMCL treated with serum free medium or serum free medium plus with 50 ng/ml of TNF-α, 5 ng/ml of TGF-β, PDGF, and IL-1β, in the presence or absence of different signal transduction pathway inhibitors: w-7, 25 μmol/L; genistein, 25 μmol/L; calphostin C, 100 nmol/L; staurosporine, 1 nmol/L; and pertussis toxin, 10 ng/ml. The relative luciferase activity of LDL receptor promoter is presented as percentages of experiments with serum free (control value). Results represent the mean±SEM of duplicate wells from four experiments, then normalised to their control values (100%). *$p<0.05$ vs no inhibitor

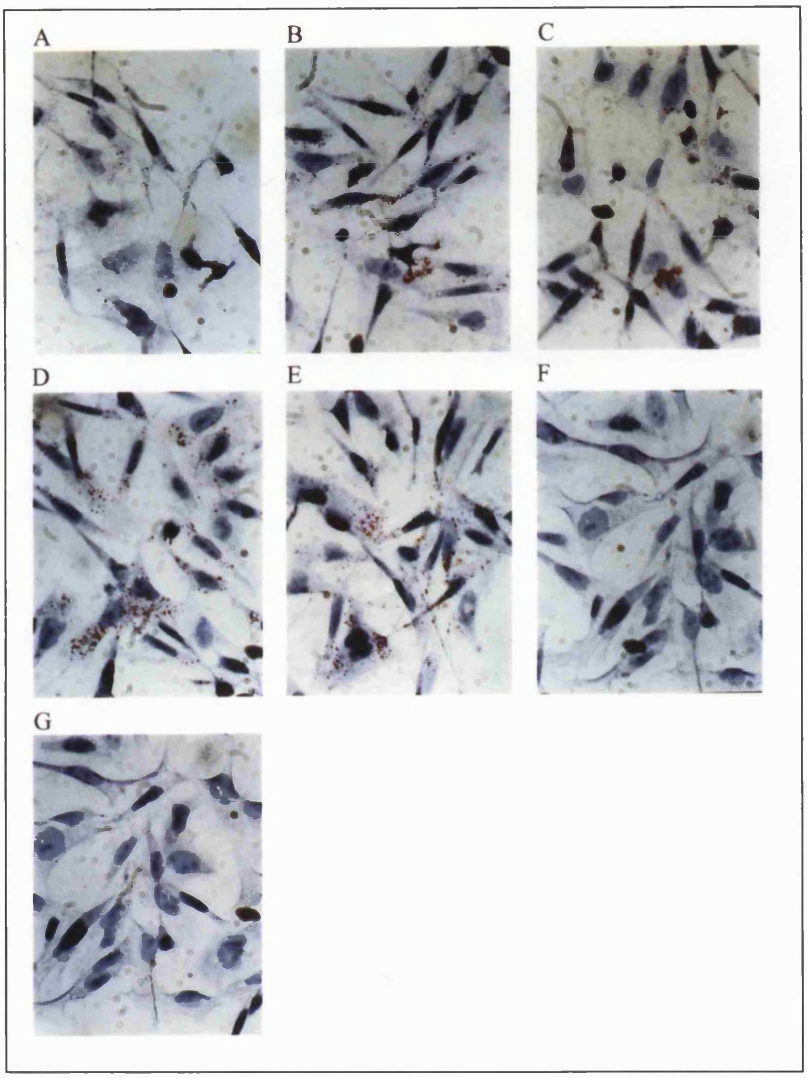


Fig.4.13. Visualisation of LDL uptake and lipid droplets in HMCL after TNF-α or IL-1β treatment. HMCL were incubated for 24 hours in serum free medium with 250 μg/ml of native LDL in the absence (A) or presence of 50 ng/ml of TNF-α (B) or 5 ng/ml of IL-1β (C), or TNF-α plus Poly I (D) or IL-1β plus Poly I (E) or TNF-α plus Heparin (F) or IL-1β plus Heparin (G). The cells were examined for lipid inclusions by Oil Red O staining. The cells were fixed and examined for fluorescence. The results are typical of those observed in four experiments.

The electrophoretic mobility of LDL from culture medium without antioxidants (EDTA & BHT) was increased in time dependent manner. However, it was the same as that of fresh LDL when the incubation performed in the medium with antioxidants as explained in the methods. All experiments described below were carried out in serum free medium containing 0.2% BSA, 100 μmol/l of EDTA and 20 μmol/l of BHT. It excluded the involvement of scavenger receptor and Ox-LDL (Fig.4.14.).

4.3.3.2. Expression of LDL receptor protein

We investigated LDL receptor protein expression by Western blot using LDL receptor monoclonal antibody. HMCL were cultured in serum free medium alone or with different concentrations of TNF-α or IL-1β in the presence of high concentration of LDL. Results showed that high concentration of LDL down-regulated LDL receptor protein in HMCL (Fig.4.13.). Both TNF-α and IL-1β significantly increased LDL receptor protein expression in a dose responsive manner, even in the presence of high concentration of LDL (250 μg/ml) (Fig.4.15). This suggested that inflammatory cytokines overrode LDL receptor protein suppression induced by a high concentration of LDL.

4.3.3.3. Analysis of flow cytometry

We used flow cytometry to demonstrate functional transformation of LDL receptor under the influence of cytokines. Results showed that TNF-α and IL-1β increased LDL uptake of HMCL, even in the presence of high concentration of LDL which confirmed that TNF-α and IL-lβ, specially IL-1β were able to override the suppression of LDL receptor induced by high concentration of LDL (Fig.4.16.). The influence of IL-1β was stronger than that of TNF-α in these experiments. These results suggest that native LDL

can be accumulated in HMCL via the LDL receptor pathway in the presence of inflammatory cytokines.

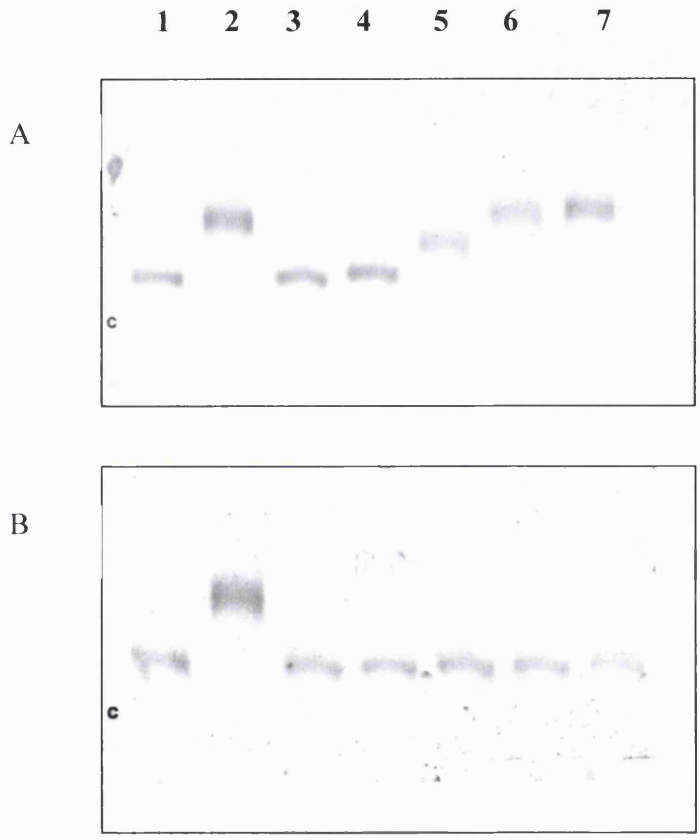


Fig.4.14. The electrophoretic mobility of LDL. HMCL were incubated for 24 hours in serum free RPMI1640 medium with 250 μg/ml of native LDL in the absence (A) or presence of 100 μM of EDTA and 20 μM of BHT (B) for periods indicated in the figures. The supernatants were collected and subjected to electrophoresis in BECKMAN Paragon LIPO Gel. Lane 1: fresh native LDL (negative control), lane 2: Ox-LDL (positive control), lane 3: 0 hours incubation with HMCL, lane 4: 24 hours, lane5: 48 hours, lane 6: 72 hours, lane 7: 96 hours.

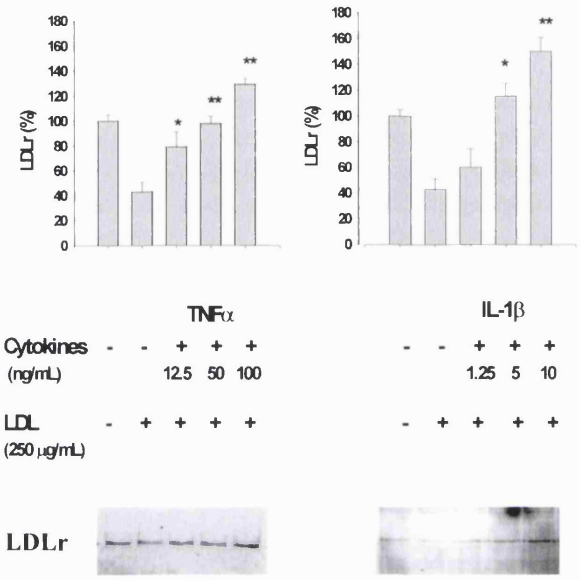


Fig.4.15. Inflammatory cytokines overrode the LDL receptor protein suppression induced by a high concentration of native LDL. HMCL were incubated for 24 hours in serum free medium alone (control) or with 250 µg/ml of native LDL in the absence or presence of various concentrations of TNF-α or IL-1β. (A): LDL receptor protein level was examined using Western blotting as described in methods. (B): The histogram represents means±SD of the densitometric scans of the LDL receptor protein bands from three experiments and expressed as a percentage of control. *$p<0.05$, **$p<0.001$ vs control.

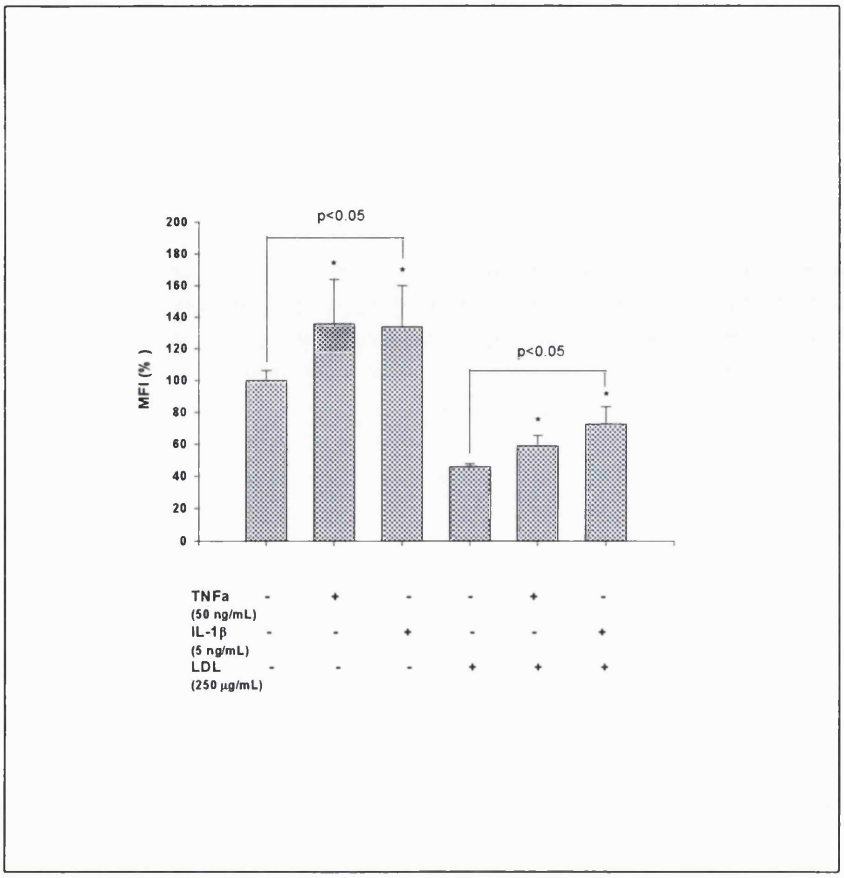


Fig.4.16. Analysis of the mean fluorescence intensity (MFI) in the TNF-α or IL-1β treated-HMCL. HMCL were incubated in serum free medium alone or with 50 ng/ml of TNF-α or 5 ng/ml of IL-1β in the absence or presence of a high concentration of native LDL (250 µg/ml) for 24 hours respectively, then the medium was replaced by fresh serum free medium containing 10 µg/ml DiI labelled LDL for 5 hours at 37°C. The cells were analysed by FACS. MFI was calculated by subtracting the autofluorescence intensity from the observed MFI of labelled cells. (B). Results represent means±SD of duplicate wells from four experiments * $p<0.05$ vs. control.

4.3.3.4. Expression of LDL receptor mRNA

To investigate the effect of inflammatory cytokines on LDL receptor mRNA expression in the presence of a high concentration of cholesterol, we isolated RNA from cultured HMCL stimulated by native LDL or LDL in the presence of TNF-α or IL-1β. Both TNF-α and IL-1β significantly increased LDL receptor mRNA expression, even in the presence of a high concentration of LDL (Fig.4.17.). This result was consistent with those found in terms of LDL protein and FACS analysis.

4.3.3.5. Activity of LDL receptor promoter

We also examined LDL receptor promoter activity using a luciferase assay system. HMCL were transfected with reporter gene pGL3LDLR6500 (Ruan et al. 1996; Ruan et al. 1998). This contains up to 6500 base pairs of 5' flanking DNA from the LDL receptor gene, which contains three imperfect direct, repeats of 16 bp and two TATA-like sequences of 7 bp each (Sudhof et al. 1987). The results showed that a high concentration of LDL (250 μg/ml) markedly decreased promoter activity to about 30% of control. TNF-α (50 ng/ml) and IL-1β (5 ng/ml) increased LDL receptor promoter activity by 167.48±23.56%, and 163.01±31.91%, respectively. TNF-α and IL-1β also increased promoter activity in the presence of high concentration of LDL (Fig.4.18.). This result confirmed that inflammatory cytokines increased LDL receptor expression at transcription levels, even in the presence of high concentration of cholesterol.

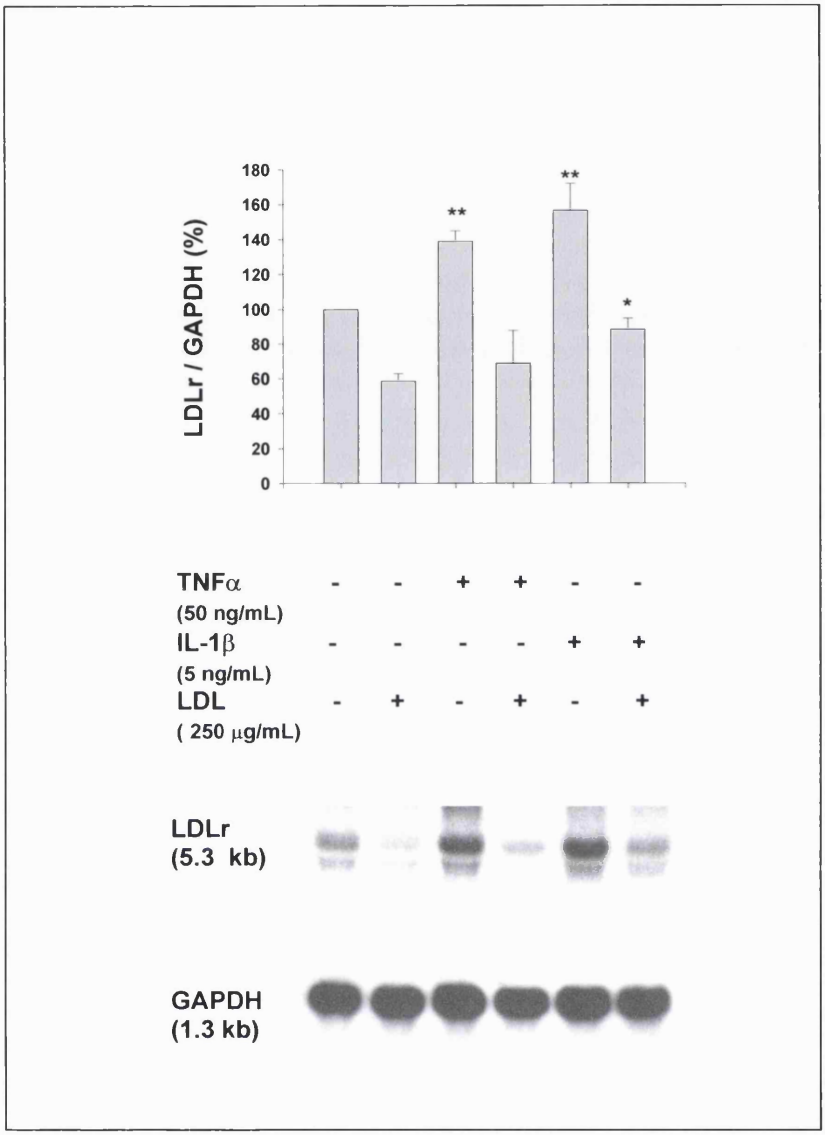


Fig.4.17. Inflammatory cytokines overrode the LDL receptor mRNA suppression induced by a high concentration of native LDL. HMCL were incubated in serum free medium alone (control) or with 250 μg/ml of native LDL in the absence or presence of 50 ng/ml of TNF-α or 5 ng/ml of IL-1β for 24 hours. A: LDL receptor mRNA expression was examined using northern blotting as described in methods. B: The histogram represent means±SD of the densitometric scans of the LDL receptor mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of control. *$p<0.05$ vs ctr+LDL, **$p<0.001$vs control.

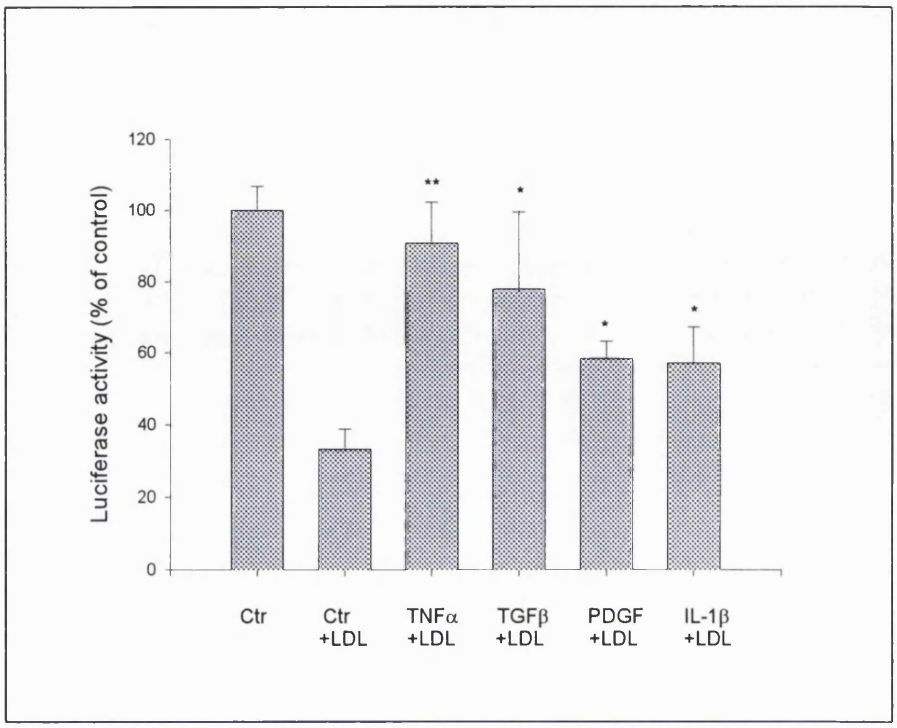


Fig.4.18. The response of LDL receptor promoter to TNF-α or IL-1β stimulation in the presence of a high concentration of native LDL. HMCL were transfected with pGL3LDL6500, as an internal standard, using a psv-β-galactosidase plasmid. The transfected cells were cultured in a standard RPMI medium containing 5% FCS. After 24 hours, the cells were incubated in serum free medium alone (control) or with 50 ng/ml of TNF-α or 5 ng/ml of IL-1β in the absence or presence of 250 μg/ml of native LDL. Luciferase activity was measured and normalised by comparison with β-galactosidase activity. Results represent the mean±SD of duplicate determinations from six experiments, which were normalised to the control (100%). * $p<0.05$, ** $p<0.001$ vs control+LDL.

4.3.3.6. Expression of SCAP mRNA

We therefore, investigated the molecular mechanisms by which cytokines are able to override the LDL receptor suppression induced by high concentrations of LDL. We examined the expression of SCAP in HMCL under influence of inflammatory cytokines and high concentrations of cholesterol. The results showed that cholesterol-depletion up-regulated SCAP expression up to 72 hours and those high concentrations of LDL down-regulated SCAP mRNA expression in a dose responsive manner. This suggested that intracellular concentrations of cholesterol affected SCAP expression at transcriptional level and that SCAP is the cholesterol sensor by which LDL receptor expression is regulated in HMCL (Fig.4.19). TNF-α and IL-1β increased SCAP mRNA expression in a dose responsive manner in the presence of high concentrations of cholesterol (Fig.4.20). These results focus on the role of SCAP in overriding the suppression of LDL receptor induced by high concentration of cholesterol.

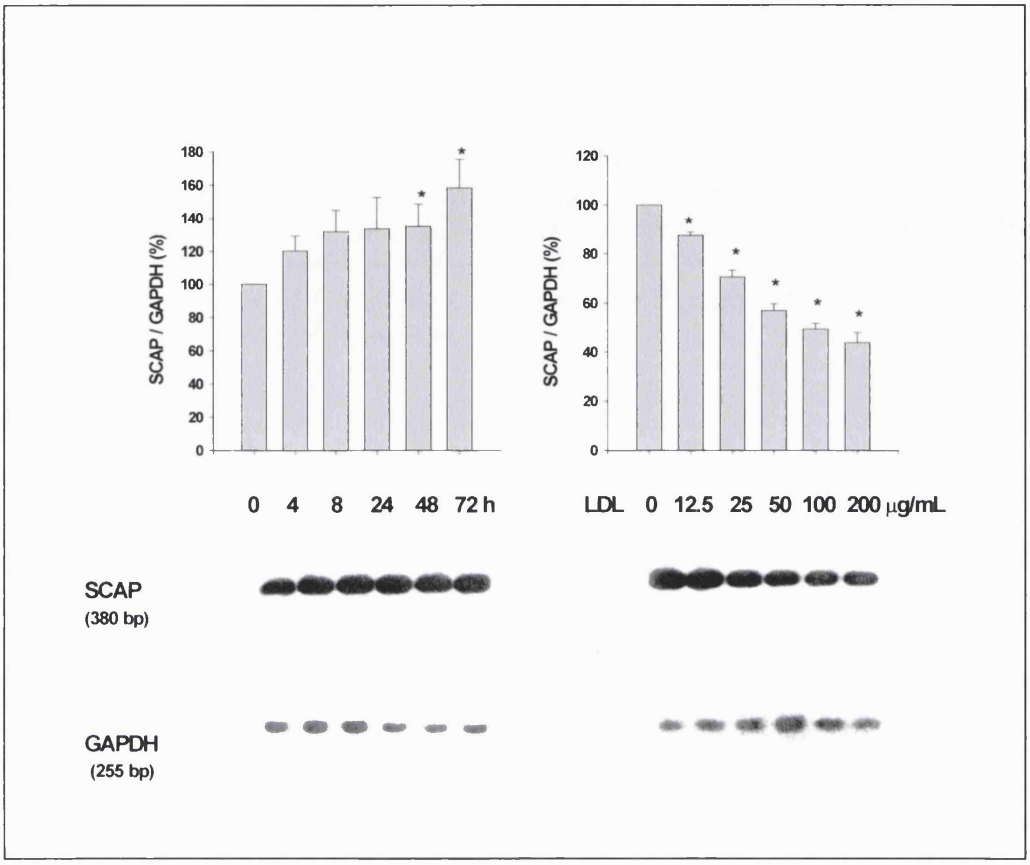


Fig.4.19. Intracellular concentrations of cholesterol regulated SCAP mRNA expression in HMCL. HMCL were incubated in serum free medium for 0 (control), 4, 8, 24, 48, 72 hours, or serum free medium without (control) or with various concentrations of native LDL (12.5, 25, 50, 100, 200 μg/ml) for 24 hours. (A): SCAP mRNA expression was examined using RT-PCR followed by Southern blotting as described in methods. (B): The histogram represent means±SD of the densitometric scans of the SCAP mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of control. *$p<0.001$ vs control.

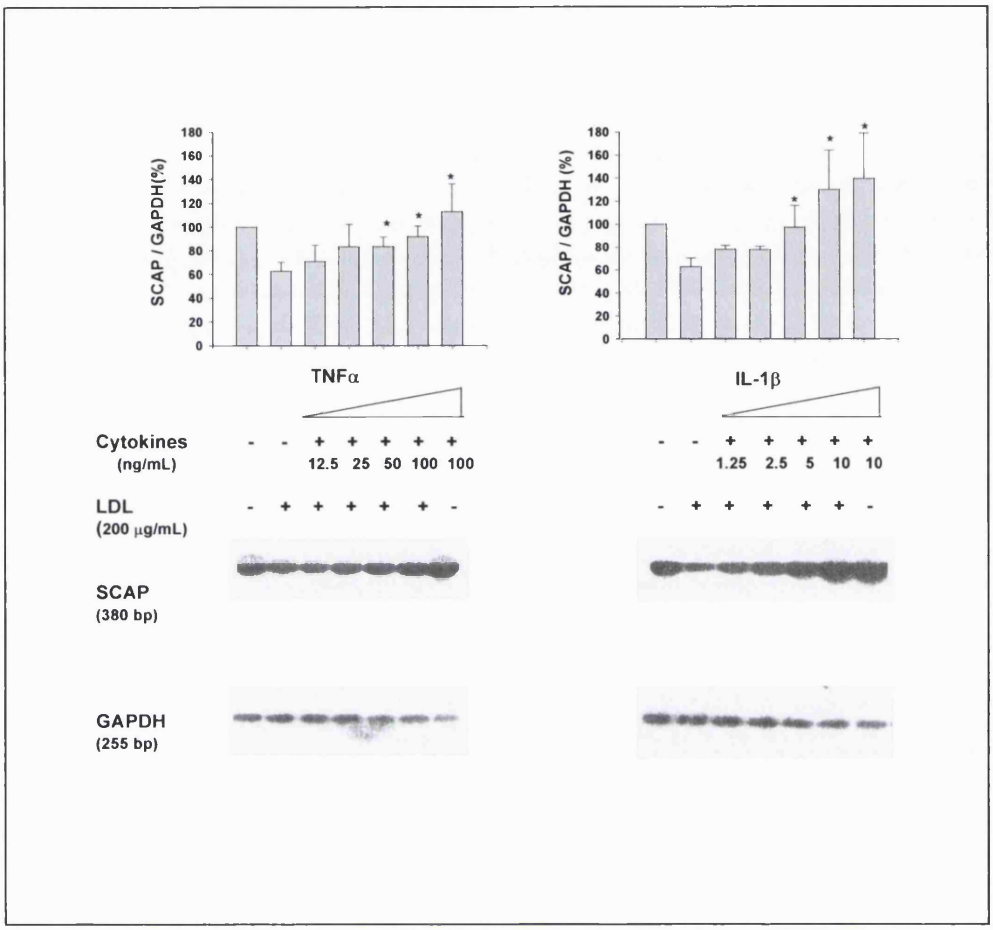


Fig.4.20. Inflammatory cytokines increased SCAP mRNA expression in HMCL. HMCL were incubated in serum free medium alone (control) or with 200 μg/ml of native LDL in the absence or presence of various concentrations of TNF-α or IL-1β. (A): SCAP mRNA expression was examined using RT-PCR followed by Southern blotting as described in methods. (B): The histogram represent means±SD of the densitometric scans of the SCAP mRNA band from three experiments, normalised by comparison with GAPDH mRNA, and expressed as a percentage of control. *$p<0.05$ vs control.

4.4. DISCUSSION

Mesangial cells have very important physiological functions, which include synthesis and secretion of matrix, eicosanoids, growth factors and cytokines. These cells are influenced from both autocrine and paracrine activity of above factors. However, there are only limited reports on the regulation of LDL receptor in HMC induced by cytokines. GrÖne found that PDGF promoted LDL binding and uptake of LDL by HMC. Our data extended these results and showed that sub-mitogenic concentration of cytokines TNF-α, TGF-β, PDGF and IL-1β significantly increased LDL receptor mRNA expression by increasing of LDL receptor gene promoter activity. These results were consistent with the observed increases in specific binding. This result suggests that inflammatory cytokines increase LDL receptor expression in a proliferation independent way.

The increase in steady-state levels of mRNA could be considered due to an increase in LDL receptor gene transcription or enhanced stability. Our previous study showed that cytokines TNF-α, TGF-β, PDGF, and IL-1β did not lengthen LDL receptor mRNA stability (Ruan, et al, 1998). Therefore, inflammatory cytokines increase LDL receptor expression by increasing of gene transcription.

The signal transduction pathways involved in upregulating LDL receptor in HMCL by TNF-α, TGF-β, PDGF, and IL-1β are unclear. Our results showed that tyrosine kinase second signal transduction pathway was essential for up-regulation of LDL receptor expression in HMCL induced by all four cytokines used in present experiments. The initial step of ligand-receptor interaction activates receptor tyrosine kinase activity and initiates a

phosphorylation cascade involving various protein kinases and subsequent signal transduction pathways. These results are similar to the reported effect of genistein in inhibiting PDGF-inducible genes in fibroblasts (Zwiller et al. 1991). In addition to tyrosine kinase, and serine/threonine kinase, G-protein seems to be involved in the up-regulation induced by TGF-β. Serine/threonine kinase, PKC, and calmodulin seem to be involved in IL-1β-mediated LDL receptor induction. Our limited studies with specific inhibitors of various signal transduction pathways demonstrate the complexities of the system involved in the regulation of LDL receptor.

In the classic pathway, cholesterol is the major regulatory element. The intracellular levels of cholesterol control up-take and synthesis of cholesterol through feedback regulation (Diamond and Karnovsky, 1988; Goldstein and Brown, 1985). The evidence from LDL receptor mRNA, protein, and promoter activity assay showed that normal feedback regulation is preserved in HMCL in culture, demonstrating the functional integrity of the LDL receptor. However, TNF-α and IL-1β override the suppression of LDL receptor induced by a high concentration of LDL. The process by which TNF-α & IL-1β increased the number of intracellular Oil Red O stained lipid droplets in HMCL could not be blocked by Poly I which blocks scavenger receptors (Krieger and Herz, 1994), but was blocked by heparin which blocks LDL receptor (Goldstein et al. 1976; Innerarity et al. 1986), implying LDL receptor pathway involvement and excluding the participation of scavenger receptor. It therefore appeared that the normally tight sterol-dependent feedback LDL receptor regulation in HMC was ineffective under the influence of TNF-α and IL-1β; pure native LDL was transported through LDL receptor to convert HMCL to foam cells. Additionally, all experimental incubation

mediums contained the anti-oxidants EDTA and BHT, both of which powerfully prevent oxidation of LDL by HMCL. The electrophoretic mobility of LDL from the culture medium was the same as that of fresh LDL, indicating that no oxidation of LDL took place during the culture. Therefore, there was no ligand for scavenger receptors in the culture medium and foam cell formation occurred through the dysregulation of LDL receptor.

We have shown that TNF-α, IL-1β increased LDL receptor expression in the presence of high concentration of LDL in this chapter, suggesting that sterol-resistant regulation of LDL receptor occur in HMC. Insulin also increases receptor mRNA concentrations in Hep G-2 cells in the presence of maximally suppressive concentrations of LDL (Wade et al. 1989). Similarly chorionic gonadotropin stimulates receptor production in granulosa cells in the presence of 25-hydroxycholesterol (Golos et al. 1986). The cyclic AMP-mediated up-regulation of LDL receptor is maintained at low concentrations of inhibitory sterols, but is eventually over-ridden at high concentrations of these sterols in human vascular smooth-muscle cells, skin fibroblasts and foetal-lung fibroblasts (Middleton, 1992). Stimulation with phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin increased native LDL receptor gene expression in the human leukemic T cell line Jurkat when cells were cultured in the absence of sterols and also increased nuclear accumulation of SREBP-1. PMA and ionomycin likewise increased LDL receptor mRNA levels when cells were cultured in the presence of suppressive concentrations of sterols, when neither SREBP-1 nor SREBP-2 was detectable in the nucleus. These findings indicated that mitogen-induced up-regulation of the LDL receptor gene could be independent of sterol-regulated transcription factors. The involvement of SRE-1 was analysed by transfection of LDL receptor promoter constructs. Promoter fragments of either the 5'

1472 or 142 base pairs induced reporter gene expression after mitogenic stimulation when cells were cultured in the absence or presence of sterols. Mutation of the SRE-1 sequence in either construct abolished sterol-mediated regulation of transcription. However, mutation of the SRE-1 sequence in the 1472 base pair promoter fragment did not alter mitogenic induction of transcription, whereas mutation of SRE-1 in the 142 base pair promoter fragment completely prevented up-regulation of transcription. Taken together, these results demonstrate that the LDL receptor promoter contains at least one 5' SRE-independent as well as an SRE-dependent response element. Furthermore, the data suggest that the SRE-independent response may not involve the action of either SREBP-1 or -2. Thus, mitogen-induced transcription of the LDL receptor promoter is regulated by diverse sterol-independent mechanisms (Makar et al. 1998).

Insulin-like growth factor–1 (IGF-1), follicle-stimulating hormone (FSH), or their combination increased LDL receptor mRNA expression. Hormonally stimulated accumulation was suppressed by 54-75% by the concurrent addition of LDL substrate (50 μg/ml). However, the combination of FSH and IGF-I significantly prolonged the message half-life, even in the presence of LDL suggesting that the combination of FSH and IGF-I can induce accumulation of LDL receptor mRNA in cultured granulosa cells even in the presence of sterol negative feedback and can do so mechanistically by a combination of promoter activation and increased mRNA stability. Huang reported that low density lipoprotein-containing immune complexes (LDL-IC) induced both transcriptional and post-transcriptional activation of the LDL receptor gene in PMA-treated THP-1 cells and this induction was independent of the free cholesterol content of these cells (Huang et al. 1997).

Leukemic cells from patients with acute myelogenous leukemia (AML) have higher LDL receptor activity than normal white blood and bone marrow cells. The underlying mechanism behind this is unclear. They studied the inhibitory effect of sterols on induction of LDL receptor activity in leukemic cells from 27 patients with AML and in white blood cells from 13 healthy individuals. The high affinity degradation rate of ^{125}I-labeled LDL was determined in mononuclear blood cells directly after isolation from blood and after incubation for 2 days in medium with 10% lipoprotein-deficient serum with or without various concentrations of 25-hydroxycholesterol + cholesterol. The median sterol concentration for 50% inhibition (IC50) of induction was more than five times higher for leukemic cells than for normal mononuclear cells. At the highest sterol concentration (400 μg/ml 25-hydroxycholesterol + 8 mg/ml cholesterol), the LDL receptor activity was abolished in cells from all healthy individuals while the induction of LDL receptor activity in cells from three acute myeloid leukemia (AML) patients was unaffected. These results demonstrate a decreased feedback regulation of LDL receptor activity by sterols in AML cells and support the conclusion that elevated LDL receptor activity is associated with sterol resistance and cell proliferation. These findings are of potential interest for diagnosis and specific treatment of leukemia (Tatidis et al. 1997). Thus there are other evidences supporting our finding for sterol-independent modulation of LDL receptor synthesis.

The mechanism of sterol-independent regulation of LDL receptor is unclear. SRE-1, a decamer (5'-ATC-ACCCCAC-3') flanking the LDL receptor gene, activates transcription in sterol-depleted cells and is silenced by sterols. Overexpression of

SREBP-1 abolished sterol regulation (Yokoyama et al. 1993). It suggests that SREBP-1 is regulated by an unknown factor that is overwhelmed when SREBP-1 is overexpressed. We investigated the molecular mechanisms by which inflammatory cytokines overrode the normal cholesterol suppression of LDL receptor by examining the expression of human SCAP. The high expression of SCAP mRNA in sterol-depleted HMCL was suppressed by high concentrations of LDL, suggesting that SCAP contributed to LDL receptor regulation as a cholesterol sensor in HMCL. Cytokines increased SCAP mRNA expression in the presence of high concentrations of LDL. Lawler recently reported that TNF-α was capable of inducing SREBP-1 proteolysis independently of the presence of sterols, and that the product of sterol-independent SREBP-1 proteolysis is capable of nuclear translocation and binds to the sterol regulatory element in human hepatocytes (Lawler, Jr. et al. 1998). This data supports our finding that sterol-independent SREBP activation could take place in the presence of inflammatory cytokines. We provide evidence that this mechanism can at least partially explain why inflammatory cytokines were able to override the suppression of LDL receptor induced by high concentrations of cholesterol.

The second regulatory element which probably involved in sterol-independent regulation of LDL receptor SP-1. TNF simultaneously induced both LDL receptor and SP-1 genes in human endothelial cells, indicating a possible link between SP-1 induction and LDL receptor gene transcription (Hamanaka et al. 1992). It is conceivable that TNF may activate a tyrosine kinase signal transduction pathway, then induce synthesis of SP-1 which then activates LDL receptor gene transcription. Recently, Basheeruddin reported that PDGF stimulation of quiescent cells lead to enhanced SP-1 binding to the LDL receptor gene, this enhanced binding could participate in PDGF induction of LDL receptor gene

transcription (Basheeruddin et al. 1995). Additional transfection studies utilising constructs with mutations of the SP-1 binding sites of the LDL receptor promoter would be necessary to further test this hypothesis. In vitro and vivo, Liu described a sterol-independent regulation of LDL receptor transcription by the cytokine oncostatin M (OM) in HepG2 cells. Using a luciferase reporter system comprising either the native LDL receptor promoter including repeats 1, 2, and 3, or a synthetic promoter vector containing repeats 2+3 only, to directly examine OM effects on individual elements. Specific mutants in repeats 1, 2, and 3 were made to facilitate the mapping of the OM effect on the promoter. Wildtype and mutant constructs were assayed for cholesterol and OM regulation. The results show that mutation within the core SRE-1 element of repeat 2 totally abolished cholesterol regulation but had no effect on OM inducibility. Interestingly, a mutation within repeat 1 reduced basal transcription activity to 10% of the native promoter, but OM induction was unaltered. However, the identical mutation engineered in repeat 3 significantly decreased OM induction of LDL receptor promoter activity. These results suggest a novel regulatory role for the repeat 3 element in LDL receptor transcription (Liu et al. 1997).

Another possible mechanism by which inflammatory cytokines override LDL induced LDL receptor suppression is that inflammatory cytokines directly affect LDL receptor promoter by specific transcription factor in addition of SCAP-SREBP or SP-1 pathway. Further work will be needed to identify the specific sterol-independent regulatory element in LDL receptor promoter.

In summary, we have now provided experimental evidence that inflammatory cytokines may independently modulate LDL receptor function. The one of the

mechanism may be that inflammatory cytokines influence the cholesterol sensor SCAP, enabling unregulated synthesis of LDL receptor in the presence of a high concentration of intracellular cholesterol. Such dysregulation would give the LDL receptor a 'scavenger-like function' and permit the unregulated accumulation of LDL with foam cell and potentially fatty streak formation. In the kidney, this process may specifically contribute to progressive renal disease and chronic renal transplant dysfunction. In the arteries, the same process may result in atherosclerosis. The implications of this finding are that inflammatory cytokines are risk factors for atherogenesis, and that no cholesterol concentration is 'safe' in the presence of acute or chronic inflammation because of the dysregulation of LDL receptor. This finding may partly explain the ineffectiveness of cholesterol lowering strategies for arresting the progression of renal disease. Therefore, anti-inflammatory drugs may be useful therapeutic agents in addition to anti-oxidants and cholesterol-lowering drugs.

CHAPTER 5. THE EFFECTS OF CALCIUM CHANNEL BLOCKERS ON THE REGULATION OF LDL RECEPTOR IN HUMAN MESANGIAL CELLS

5.1. INTRODUCTION

As we discussed in chapter 4, inflammatory cytokines can break LDL receptor feedback regulation and the LDL receptor itself then acquires scavenger functions to accumulate unmodified native LDL particles in an unregulated way to form foam cells and possibly fatty streaks. Since 1970, calcium channel blockers (CCBs) have been used effectively to lower high blood pressure, and showed an inhibitory effect on atherosclerotic lesion formation in a variety of animal models (Weinstein and Heider, 1989; Nayler, 1999). The ability of CCBs to slow atherosclerotic lesion formation is a class specific effect that is independent of their blood pressure-lowering effect and occurs without any significant change in the plasma lipid profile (Holzgreve and Burkle, 1993). It is accompanied by a reduction in vessel wall cholesterol and calcium and is maintained over prolonged periods of treatment. The mechanisms that may be involved include inhibition of smooth muscle cell proliferation and migration, slowed platelet aggregation, restructuring of cholesterol-enriched cell membranes, enhanced gene expression for low-density lipoprotein receptor protein, inhibition of growth factor release, slowed calcium uptake, and restoration of endothelium-dependent relaxation (Schachter, 1997a; Schachter, 1997b).

Several clinical trials have attempted to determine the efficacy of these drugs in human atheromatous disease. The emerging evidence suggests that an antiatherosclerotic action can be demonstrated in patients but the extent and clinical importance of this effect have yet to be fully evaluated. In the Montreal Heart Institute trial, nicardipine did not influence the overall rate of progression and regression; however, patients treated with nicardipine experienced significantly less progression of minimal lesions, defined as stenosis of less than or equal to 20%

severity. In the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT), nifedipine had no effect on overall progression and regression but, by one method of analysis, reduced the rate of appearance of new coronary lesions. In a preliminary report, diltiazem prevented the development of coronary atherosclerosis in heart transplant recipients. These studies indicate that calcium channel blockers retard the development of early atherosclerosis not only in animal models but also in human coronary arteries. Other studies recently completed or now under way will help to clarify the clinical role of calcium channel blockers in antiatherosclerotic therapy (Waters and Lesperance, 1994).

Glomerulosclerosis is the final common glomerular lesions in many renal diseases. A beneficial effect of calcium antagonists on the progression of glomerulosclerosis has also been observed in various animal studies (Haller, 1993). In mesangial cells it has been demonstrated that calcium antagonists decrease the expression and secretion of matrix proteins (Haller, 1993). However, it is unclear whether CCBs can affect LDL receptor in human mesangial cells (HMC). CCBs and antagonists of calmodulin action increase the LDL receptor protein and mRNA content of cultured fibroblasts (Filipovic and Buddecke, 1986a; Eckardt et al. 1988). The present study was undertaken to answer the specific question, how do CCBs modify native apoB/E LDL receptors on HMC? An understanding of this mechanism may help to decide whether CCBs have a major effect in influencing lipid mediated glomerular injury and modifying the progression of glomerulosclerosis.

5.2. METHODS

5.2.1. Cell culture

An established stable human mesangial cell line (HMCL) was used (kindly donated by Dr.J.D.Sraer, Hopital Tenon, Paris). HMC were immortalised by transfection with T-SV40 and H-ras oncogene. It retains many morphological and physiological features of normal HMC (Sraer et al. 1996). Experiments were carried out in serum free RPMI medium containing 0.2% bovine serum albumin (BSA, Sigma, Poole, Dorset, UK). All reagents for cell culture were obtained from Gibco BRL (Paisley, UK).

5.2.2. Preparation of Lipoprotein

Plasma was collected from a healthy human volunteer and LDL was isolated by sequential ultracentrifugation as described in chapter 2.

5.2.3. Iodination of LDL

LDL was iodinated using the enzymobead method as described in chapter 2.

5.2.4. Binding of ^{125}I-LDL to HMCL at 4 ^{0}C

Lipoprotein binding experiments were carried out using the methods as described in chapter 2.

5.2.5. Northern Blot Analysis

Total RNA was isolated and electrophoresis performed as described in chapter 2. For hybridisation, a fragment of the LDL receptor cDNA, and as a control, glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA were used. The LDL receptor cDNA probe (2.8 kb) was prepared by digesting the plasmid pLDLr3 (American Type Culture

Collection, Rockville, MD, USA) with the restriction endonucleases Hind III and Sma I. The GAPDH cDNA probe (0.8 kb) was prepared by digesting plasmid pHcGAP (American Type Culture Collection, Rockville, MD, USA) with the restriction endonucleases Pst I and Xba I. The cDNA probes were labelled with ^{32}P-dCTP (3000 Ci/mmol, Amersham, Buckinghamshire, UK) by nick translation as previously described in chapter 2. Hybridisation was performed using a standard method as described in chapter 2 and chapter 4.

5.2.6. Cell proliferation assay

HMCL were plated in 96-well plates at a density of 6000 cells/well in RPMI containing 5% FCS and cultured until nearly confluent. The medium was then changed to serum free medium for 48 hours and each well was washed with PBS. Cells were incubated for 0, 24, 48, and 72 hours in serum free RPMI 1640 medium or RPMI 1640 medium plus 5% FCS with or without different concentrations of CCBs (1 to 100 μmol/l). The cells were labelled for 18 hours using 1 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK), then washed with PBS, trypsinised for 30 minutes and harvested. Radioactivity associated with DNA of proliferating cells was measured in a scintillation counter. Determinations were performed in quadruplicate for each experiment. For cell counts, HMCL were subcultured at 20,000 cell/well into 24-well culture plates (Falcon, Oxford, UK) in standard growth medium. At confluency, the cells were synchronised to the quiescent state as described above. After 48 hours, viable cells/well (assessed by trypan blue stain) were counted using a haemocytometer (time 0). Then cells were incubated for 24, 48, and 72 hours in serum free RPMI 1640 medium or RPMI 1640 plus 5% FCS, and different concentrations of CCBs (1 to 100 μmol/l). Cell counts were performed. In addition

to cell counts and ^{3}H-thymidine incorporation, potential cytotoxic effects of CCBs were also assessed by using the trypan blue exclusion method and by measuring the release of lactate dehydrogenase (LDH) into the supernatant (Moldeus et al. 1978).

5.2.7. LDL receptor promoter-report gene constructs

The LDL receptor promoter-luciferase fusion gene pGL3LDLR6500 was constructed using standard molecular clone techniques as described in chapter 4. The LDL receptor promoter (a gift from Dr. D. Russell, University of Texas) was subcloned into the unique HindIII site of pGL3Luciferase vector, a recombinant plasmid that contains a SV40 enhancer (Promega, Southampton, UK). pGL3LDLR6500 contains up to 6500 base pairs of 5' flanking DNA from the LDL receptor gene which contains three imperfect direct repeats of 16 bp and two TATA-like sequences of 7 bp each (Sudhof et al. 1987).

5.2.8. Transient Expression Assay

HMCL in 6×10^{7}/ml were transfected with 100 μg of supercoiled pGL3LDLR6500 and 100 μg control plasmid of psv-β-galactosidase by electroporation. Standard electroporation conditions were 340 volts and 125 uF with a Gene Pulser (Bio-Rad, Herts, UK). After electroporation, the cells were placed into 6 well plates with growth medium. Approximately 24 hours after replating, cells were washed with PBS and the medium was replaced with RPMI 1640 or RPMI 1640 plus treatment with different concentrations of CCBs for 24 hours. The cells then were washed twice with PBS and lysed by cell lysis buffer (Promega, Southampton, UK). The luciferase activity in the supernatants was measured and normalised by comparison with β-galactosidase enzyme activity using Promega luciferase and β-galactosidase assay systems.

5.2.9. The effect of various signal transduction inhibitors on CCBs-mediated activity of LDL promoter

Using the transient expression assay system described above, the electroporated HMCL were incubated for 24 hours in serum free RPMI 1640 medium or RPMI 1640 medium with 100 μmol/l of diltiazem, and verapamil, in the presence or absence of different signal transduction pathway inhibitors: 25 μmol/l W-7 (calmodulin antagonist), 25 μmol/l genistein (tyrosine kinase inhibitor), 100 nmol/l calphostin C (PKC inhibitor), 1 nmol/l staurosporine (serine/threonine kinase inhibitor), 10 ng/ml pertussis toxin (G-protein inhibitor). The activities of luciferase driven by LDL receptor promoter were measured.

5. 3. RESULTS

5.3.1. The effect of CCBs on LDL binding on HMCL

At 4 ^{0}C, LDL only binds to cell surface receptors and is not significantly internalised. Binding of LDL to its receptor on HMC at 4 ^{0}C has been shown to reach equilibrium at 4 h and the experiments described were carried out using this time period. Incubation of ^{125}I-LDL with HMCL at 4 ^{0}C resulted in a dose dependent increase in cell associated radioactivity which was saturable.

Diltiazem and verapamil increased specific binding of LDL to HMCL in a dose-dependent manner. Specific binding of LDL to nifedipine treated cells was not significantly different from controls (Fig.5.1.).

5.3.2. The effect of CCBs on expression of LDL receptor mRNA

Northern blot analysis showed that diltiazem and verapamil increased steady-state levels of LDL receptor mRNA in a dose dependent manner (Fig.5.2a & 5.2b.). These results were

consistent with the observed increases in specific binding of ^{125}I-LDL. Nifedipine at concentrations of 1 to 100 μmol/l did not increase LDL receptor mRNA expression (Fig.5.2.).

5.3.3. The effect of CCBs on HMCL proliferation

Mitogenesis assays measuring ^{3}H-thymidine incorporation revealed that CCBs significantly inhibited the proliferation of HMCL induced by 5% FCS. Nifedipine at concentrations of 1 to 100 μmol/L, diltiazem and verapamil at concentration of 100 μmol powerfully inhibited HMCL proliferation in culture at 24 hours (Fig.5.3a.), Diltiazem and verapamil at low concentrations (1-10 μmol/L) inhibited HMCL proliferation at 48 and 72 hours (data not shown). These results were confirmed by cell counting (Fig.5.3b.). 24 hour cytotoxicity experiments showed that CCBs in the concentration range from 1 to 100 μmol/L had no cytotoxic effects on HMCL as measured by LDH release (data not shown). These results suggest that the upregulation of LDL receptor transcription induced by CCBs was independent of cell proliferation.

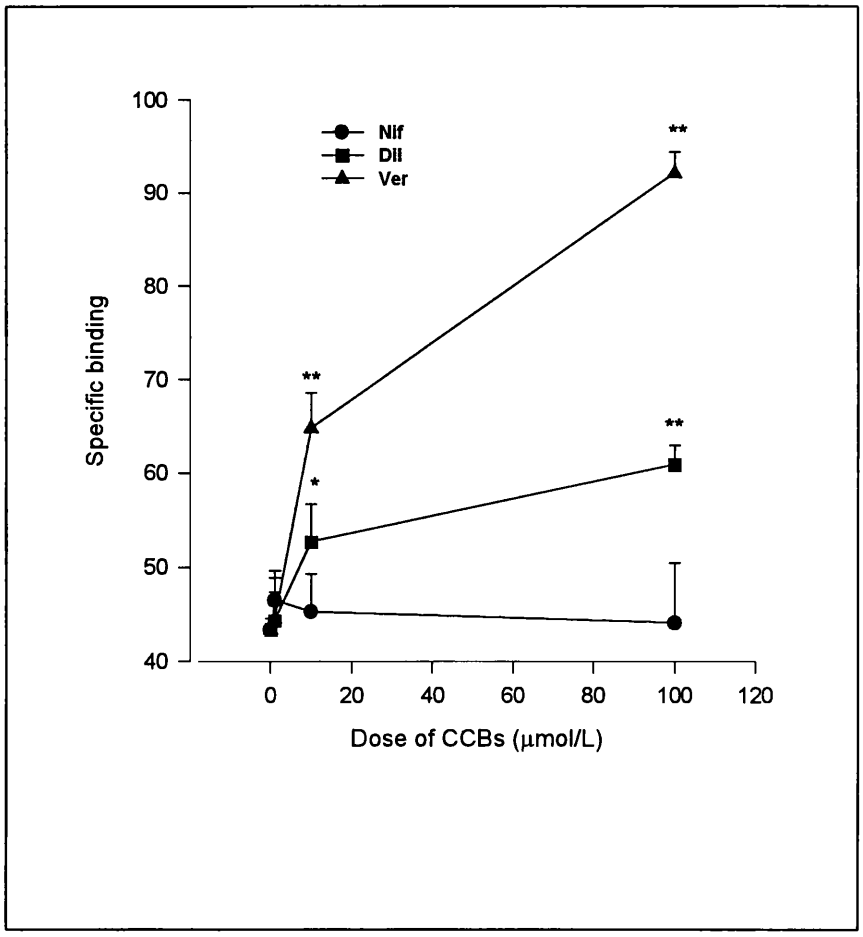


Fig.5.1. The effect of CCBs on LDL binding on HMCL HMCL were grown to near confluence in 12-well plates and treated for 24 hours in serum free medium with or without different concentrations of nifedipine (Nif), diltiazem (Dil), or verapamil (Ver). LDL binding was assayed at 4°C for 2 hours with 5 μg/ml ^{125}I-LDL. Specific binding was calculated as the difference between ^{125}I-LDL binding with and without 50-fold excess unlabelled LDL and factorised to milligrams of protein. The results represent means±SD of duplicate wells from four experiments, then normalised to their respective control binding (100%). Asterisk indicates significant difference *versus* control (serum free). *$p<0.001$, tested for statistical significance using one way analysis of variance (*ANOVA*).

5.3.4. The effect of CCBs on activity of LDL receptor promoter

Using a luciferase assay system, treatment of HMCL with diltiazem, and verapamil increased LDL receptor promoter activity in a dose dependent manner. A concentration of 100 μmol/L led to increases of promoter activity of 126.72 ±10.68%, and 166.41 ±11.41%, respectively, at 24 hours (control equal to 100%) (Fig.5.4.). However, stimulation with nifedipine at low concentration (1 &10 μmol/L) had no significant effect on LDL receptor promoter activity, while at high concentration (100 μmol/L), nifedipine had an inhibitory effect (Fig.5.4.). These results confirmed those found for LDL receptor mRNA activity and LDL receptor protein binding experiments with ^{125}I-LDL in that there was no increase in either LDL receptor mRNA expression or LDL binding to HMCL at all concentrations of nifedipine.

5.3.5. CCBs were not able to override the suppression of LDL receptor induced by a high concentration of LDL

Incubation of transfected cells with a high concentration of LDL (250 μg/ml) markedly decreased promoter activity to about 33% of control. Results of experiments, performed with HMCL cocultured with LDL (250 μg/ml) and each CCB, showed that none of the CCBs were able to override the sterol-suppressive effects of LDL on the LDL receptor promoter (Fig.5.5.).

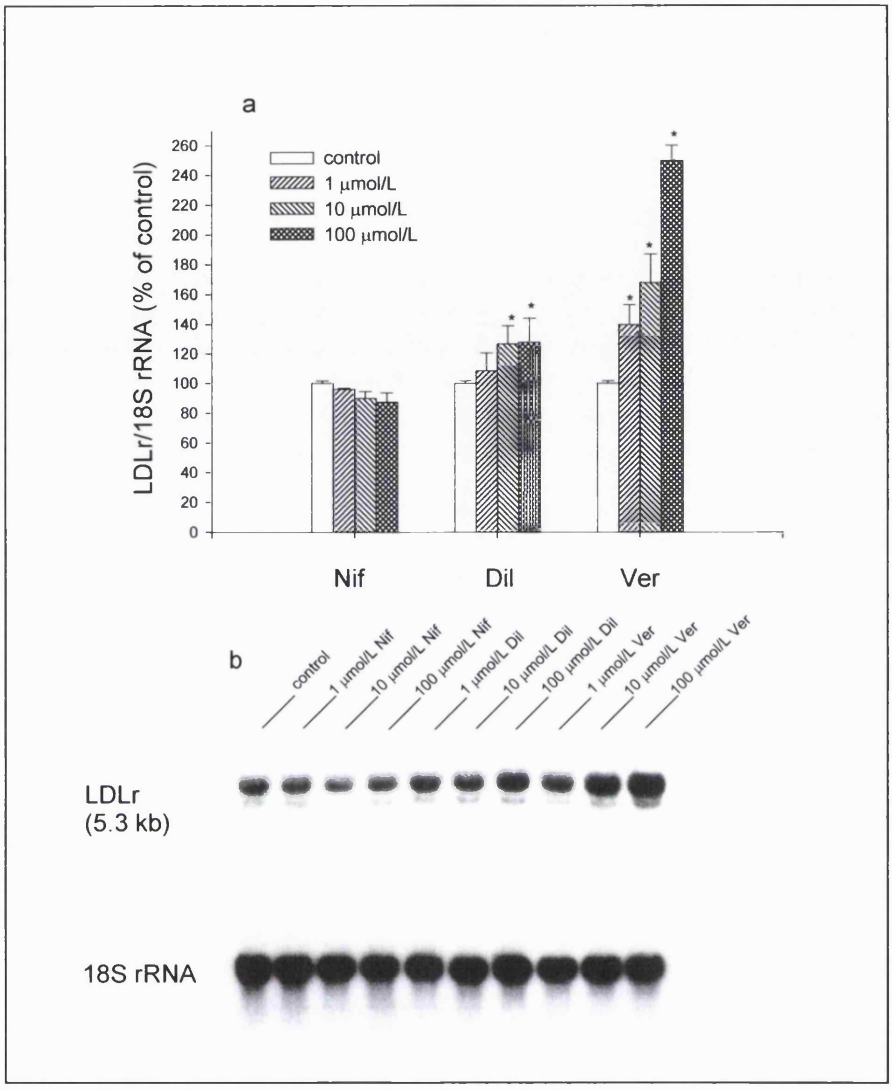


Fig.5.2a & 5.2b. The effects of CCBs on expression of LDL receptor mRNA.

HMCL were incubated with different concentrations (from 1 to 100 μmol/L) of nifedipine (Nif), diltiazem (Dil), or verapamil (Ver) for 24 hours. Total cellular RNA was extracted and hybridised with ^{32}P-labeled LDL receptor and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probes. The histogram represents densitometric scanning of the LDL receptor mRNA band, normalised GAPDH, and expressed as a percentage of control. The value represent means±SD from four experiments. $p<0.001$ vs control.

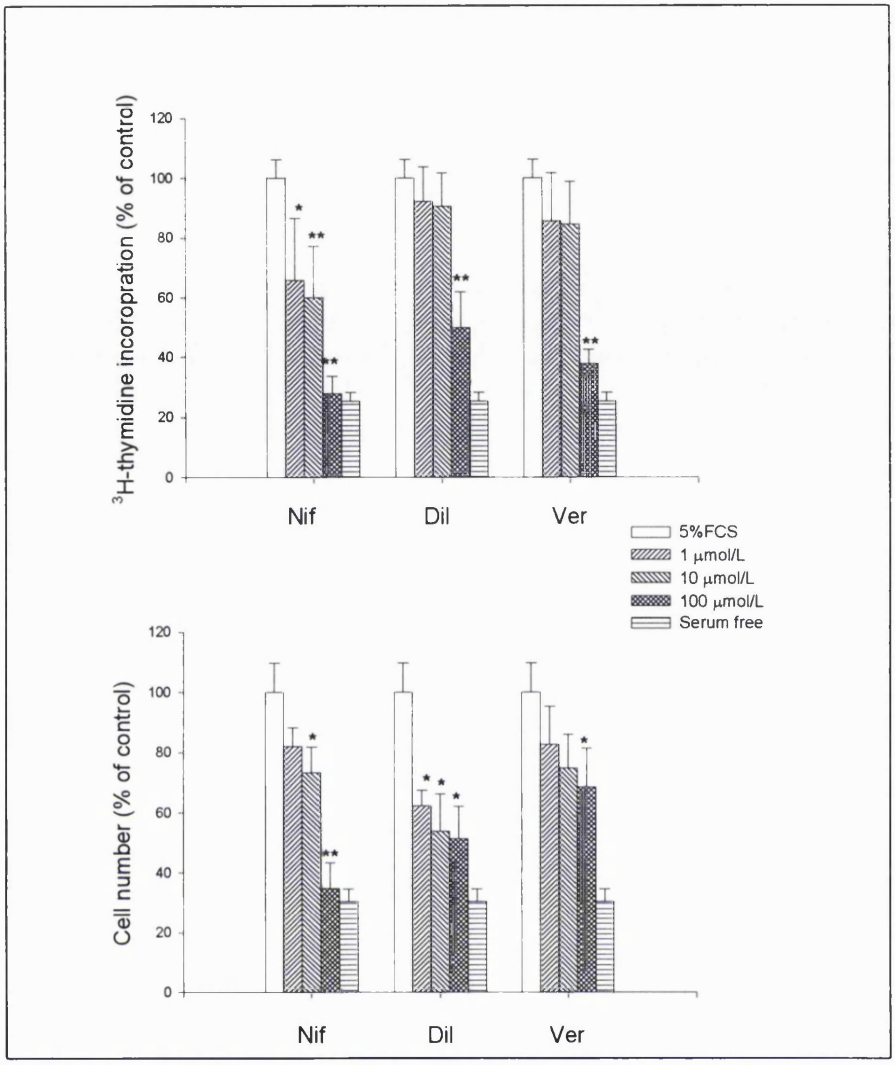


Fig.5.3a & 5. 3b. The effect of CCBs on HMCL proliferation.

(a) Quiescent HMCL were incubated for 24 hours in serum free medium (negative control), or serum free medium containing 5% FCS and different concentrations (1 to 100 μmol/L) of nifedipine (Nif), diltiazem (Dil), or verapamil (Ver). The cells were harvested after labelling for 18 hours with ^{3}H-thymidine. Radioactivity associated with DNA of proliferating cells was measured in a scintillation counter. Results represent means±SD of quadruplicate wells from four experiments, which were normalised to their respective control (100%). (b) Quiescent HMCL were treated for 24 hours with the same medium as described in the ^{3}H-thymidine incorporation. Viable cells/well (assessed by trypan blue staining) were counted using a haemocytometer. The value represent means±SD from four replicates. Asterisk indicates significant difference *versus* 5% FCS experiments. * $p<0.05$, ** $p<0.001$, tested for statistical significance using one way analysis of variance (*ANOVA*).

5.3.6. The effect of various signal transduction pathway inhibitors on CCBs-mediated activity of LDL promoter

To determine the role of signal transduction pathways in the regulation of LDL receptor gene expression in CCB-stimulated HMCL, we evaluated the effects of various signal transduction pathway inhibitors on LDL receptor transcription using the luciferase system. At non-cytotoxic concentrations of inhibitors, we observed that calmodulin seem to be involved in upregulation of the LDL receptor gene induced by diltiazem and verapamil. Tyrosine kinase and PKC may also be involved in upregulation of LDL receptor gene induced by verapamil. However, the promoter activity of LDL receptor in nifedipine-treated HMCL showed no significant difference in the presence or absence of inhibitor, suggesting that these signal transduction pathways were not involved in LDL receptor regulation induced by nifedipine. (Table 5.1).

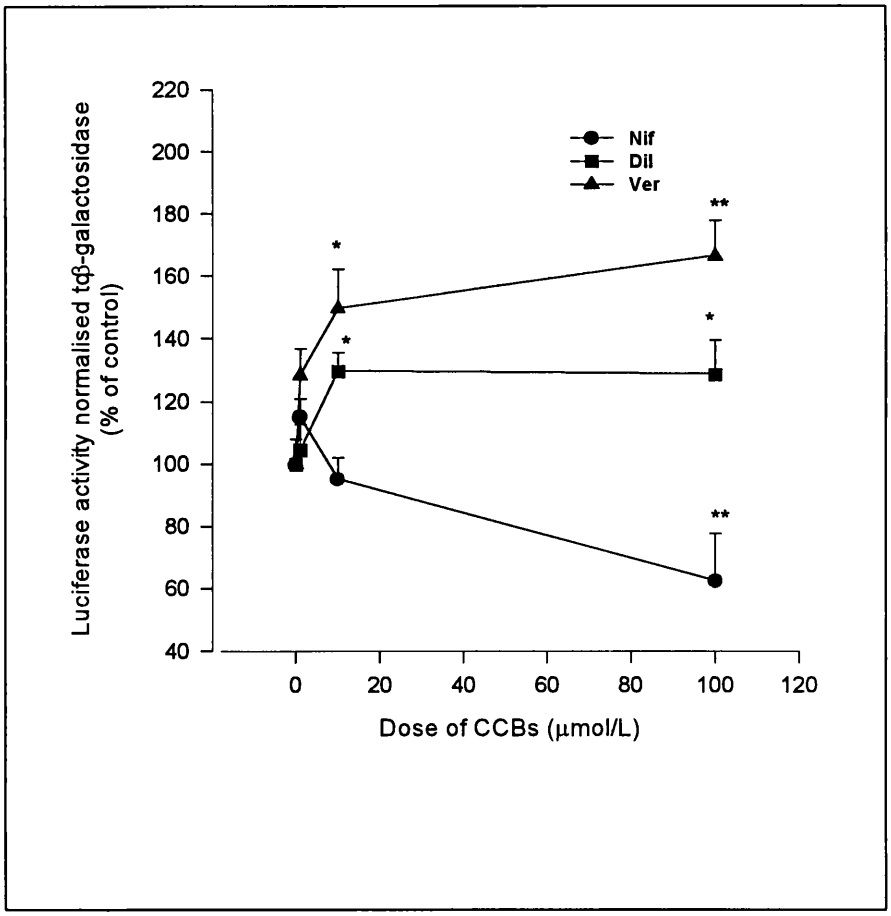


Fig.5.4. CCBs stimulate LDL receptor promoter activity HMCL transfected with pGL3LDLR6500 construct, as a internal standard, psv-β-galactosidase plasmid was used. The transfected cells were cultured in a standard RPMI 1640 medium containing 5% FCS. After 24 hours, the cells were incubated in serum free medium without (control) or with different concentrations of nifedipine (Nif), diltiazem (Dil), or verapamil (Ver). Luciferase activity was measured and normalised by comparison with β-galactosidase activity. Results represent the mean±SD of duplicate determinations from six experiments, which were normalised to their respective control (100%). Asterisk indicates significant difference *versus* their control (serum free). * $p<0.05$, ** $p<0.001$, tested for statistical significance using one way analysis of variance (*ANOVA*).

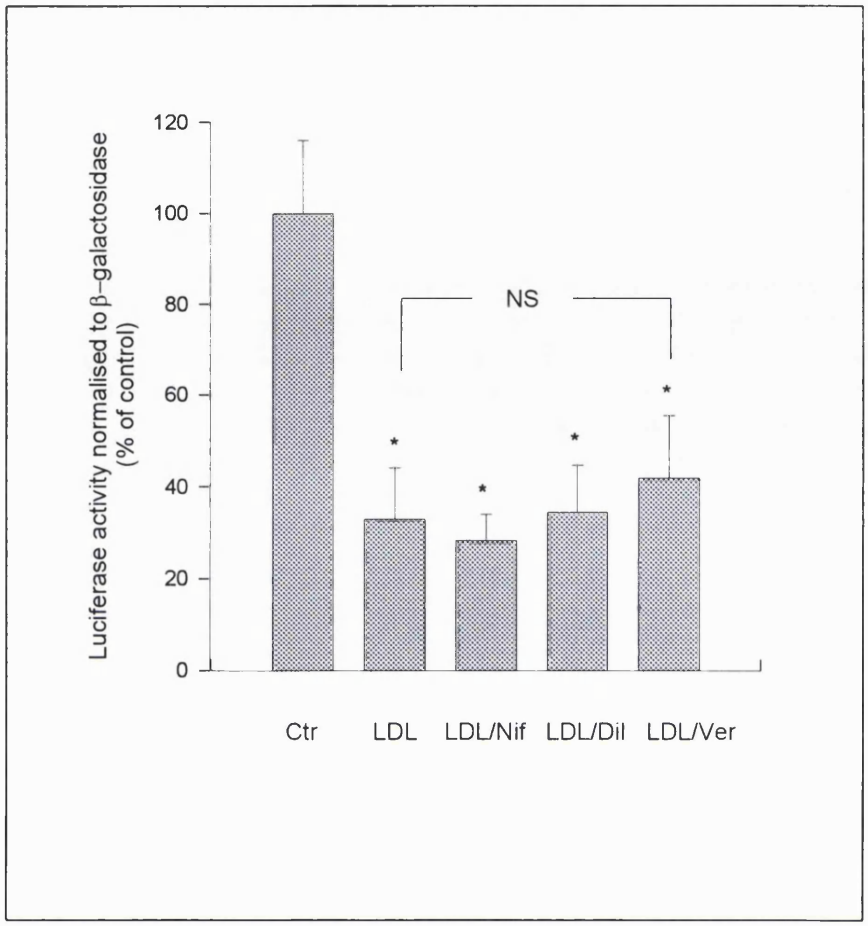


Fig.5. 5. The effect of CCBs on LDL receptor promoter activity in the presence of LDL.

Transfected HMCL were incubated for 24 hours in serum free medium (control) or serum free medium containing LDL (250 μg/ml), LDL plus 1μmol/L nifedipine (Nif), 100 μmol/L diltiazem (Dil) or verapamil (Ver), and luciferase activity was measured and normalised by comparison with β-galactosidase activity. LDL was added 30 min before the addition of CCBs. The results represent the mean±SD of duplicate determinations from four experiments, then normalised to their control value. (100%). Asterisk indicates significant difference *versus* their control (serum free). * $p<0.001$, tested for statistical significance using one way analysis of variance (*ANOVA).* There were no significant differences between LDL alone and LDL plus treatment CCBs.

Table 5.1. The effect of various signal transduction inhibitors on CCBs-mediated luciferase activity driven by LDL receptor promoter

Treatment	Relative LDL receptor promoter-luciferase activity			
	Serum free	Nif	Dil	Ver
No inhibitor	100	68.31±7.63	126.72 ±10.48	166.41± 11.41
Calmodulin (W-7)	100±6.22	55.92±4.70	78.60±23.20*	100.71±26.84**
Tyrosine kinase (Genistein)	100±3.79	66.90±10.67	99.95±16.59	124.02±13.06*
PKC (Calphostin)	100±5.46	71.14±10.00	108.64±40.61	126.99±18.48*
Serine/threonine kinase (Staurospine)	100±8.07	62.28±7.26	102.54±12.91	138.30±32.01
G-protein (Pertussis toxin)	100±10.84	79.77±14.56	111.29±27.35	136.33±17.61

Transfected HMCL treated with serum free medium or serum free medium plus 100 μmol/L of nifedipine (Nif), diltiazem (Dil), verapamil (Ver), in the presence or absence of different signal transduction pathway inhibitors: w-7, 25 μmol/L; genistein, 25 μmol/L; calphostin C, 100 nmol/L; staurosporine, 1 nmol/L; Pertussis toxin, 10 ng/ml. The relative luciferase activity of LDL receptor promoter is listed as percentage of serum free control values. The results represent the mean±SD of duplicate wells from four experiments, then normalised to their control value (100%). Groups of data were evaluated for significance by *ANOVA*. Asterisk indicates significant difference *versus* CCBs treatment without inhibitor. * $p<0.05$, ** $p< 0.005$.

5.4. DISCUSSION

Control of LDL receptor function occurs at both the transcriptional and post-transcriptional levels. The expression of LDL receptor gene could be regulated by factors such as intracellular levels of cholesterol, oxysterols, and of several growth factors (Hoogerbrugge et al. 1996; Streicher et al. 1996; Srivastava et al. 1995; Srivastava et al. 1993). The LDL receptor is present on HMC (Grone et al. 1992; Lee and Koh, 1994). Several reports have shown that CCBs and calmodulin antagonists increased both the apparent rate of synthesis and the endocytic capacity of the LDL receptor, and increased binding of LDL to cultured human fibroblasts, smooth muscle cells and endothelial cells. This effect was apparently specific for the CCBs and was deduced from an increase in LDL receptor mRNA (Eckardt et al. 1988; Filipovic and Buddecke, 1986a; Block et al. 1991; Filipovic and Buddecke, 1986b). This is the first time studies of LDL receptor regulation with CCBs carried out in HMCL. The current experiments showed that diltiazem and verapamil upregulated LDL receptor mRNA expression in HMCL and increased specific binding of LDL to HMCL. LDL receptor promoter analysis showed that the molecular mechanism of induction of LDL receptor by diltiazem and verapamil results from an increasing of LDL receptor gene promoter activity. However nifedipine did not increase the mRNA level of LDL receptor, consistent with the analysis of LDL receptor promoter activity and binding of LDL to receptors on HMCL. The mechanism by which nifedipine gives different results to verapamil or diltiazem is unclear and the clinical significance of this finding remains to be determined.

Recently, Hajjar reported that some cytokines, e.g. bFGF, TGFβ, TNF-α and IL-1 stimulated the LDL receptor gene in HepG2 and smooth muscle cells (Hsu et al. 1994;

Stopeck et al. 1993; Nicholson and Hajjar, 1992), possibly to satisfy the demand for cholesterol during cell proliferation. However, our results showed that CCBs could significantly stimulate LDL receptor gene transcription and expression in HMCL even in the absence of cell proliferation since HMCL DNA synthesis as measured by ^{3}H-thymidine incorporation and cell counting was significantly inhibited by all CCBs. Hence, up-regulation of the LDL receptor gene may be divided into three types: proliferation-dependent, proliferation-independent and modulated by a falling intracellular cholesterol concentration. The biological significance of proliferation-independent LDL receptor upregulation remains to be determined. However, in patients with high plasma LDL, the anti-proliferative effects of CCBs evident in our study may have a beneficial effects in preventing the development of glomerulosclerosis.

The signal transduction pathways involved in CCBs mediated up-regulation of LDL receptor gene expression are unclear. Most of the cellular effects of CCBs in vitro experiments seem to be mediated by their effect on trans-membrane calcium flux through voltage-operated calcium channels (Negre-salvayre and Salvayre, 1992). However, the existence of voltage-operated calcium channels has not been convincingly demonstrated in several cell types, such as platelets, macrophages and mesangial cells, suggesting that CCBs may exert additional effects on intracellular messenger systems. A recent report suggested that in non-excitable tissues, including mesangial cells, this Ca^{2+} entry pathway is voltage independent and directly receptor operated or ligand gated (Ma et al. 1996). Using patch-clamp studies, Ma reported that cultured rat mesangial cell possess abundant low conductance 1-pS Ca^{2+} channels (3,000-4,000 channels per cell). This channel has the following properties: (a) baseline channel activity is very low, but dramatic activation occurs when PDGF-BB is applied inside the path by pipette; (b) channel kinetics are only

slightly voltage dependent; and (c) the channel is cation non-selective, but with a higher divalent than monovalent permeability(Ma et al. 1996). On the other hand, the participation of calcium in LDL receptor gene expression was excluded by the facts that modulation of extracellular concentration of Ca^{2+} was without effect, and addition of the Ca^{2+} ionophore A23187 or the calcium antagonist TMB, thought to block intracellular Ca2+ mobilisation, failed to alter the rate of LDL receptor synthesis (Block et al. 1991). We measured LDL receptor promoter activity in the absence and presence of various signal transduction inhibitors. The results showed that W-7 (calmodulin inhibitor) inhibited the activity of LDL receptor promoter induced by diltiazem and verapamil, suggesting that calmodulin was a common pathway for upregulation of LDL receptor by diltiazem and verapamil. In addition, the tyrosine kinase and PKC signal transduction pathways were involved in LDL receptor induction by verapamil in HMCL. However, signal transduction pathway inhibitors used in this study did not affect the regulation of LDL receptor induced by nifedipine. These findings suggest that various second messengers are involved in signal transduction pathway induced by CCBs. There are reports that support the idea that CCBs are "promiscuous" receptor blockers (Insel, 1988). Interactions of CCBs have been reported with benzodiazepine binding sites, muscarinic and alpha- and beta-adrenergic receptors, serotonin receptors and calmodulin (Weinstein and Heider, 1989). This suggests that the established effect of CCBs, i.e. inhibition of Ca^{2+} influx via potential operated channels may not be relevant to their stimulatory influence on the LDL receptor gene. Essentially all calcium antagonists are complex hydrophobic chemical entities that bind to serum proteins (including lipoproteins), and have the potential to diffuse through and accumulate in the lipid bilayer of cell membranes and may perturb the normal membrane bilayer structure (Shi and Tien, 1986). These properties may be responsible for many of the non-calcium channel effects observed at the cell surface membranes and at the

intracellular membrane sites that regulate cell metabolism. CCBs may have a potential anti-glomerulosclerotic activity apart from their established ability to block Ca^{2+} influx via voltage-operated channels.

CCBs suppress the influx of Ca^{++} into cells and calmodulin acts as a down-regulator of LDL receptor synthesis (Eckardt et al. 1988; Filipovic and Buddecke, 1986b). CCBs could upregulate LDL receptor through inhibition of calmodulin and modulates phosphorylation of several nuclear proteins that are possibly involved in the regulation of transcription or stimulation of some protein kinases. Furthermore, it has been reported that regulation of LDL receptor genes involves action of the negative regulatory protein. Thus, macrophages treated with cycloheximide have an increased expression of LDL receptor genes. In that CCBs have an inhibitory effect on DNA synthesis in HMC, it's possible that they act primarily by increasing expression of the LDL receptor gene through a negation of the effect of the negative regulatory protein, such as p53 and Rb (Takuwa et al. 1993; Takuwa et al. 1992). So, we reason that up-regulation of LDL receptor gene divided into two types: one is proliferation-dependent upregulation which is required for cell growth, another is inhibition-related which may relate to cell differentiation or something else, such apopotosis. More interestingly, CCBs could amplify PDGF-BB-induced expression of LDL receptor mRNA and inhibit the proliferative effect produced by PDGF-BB (Block et al. 1991). This would suggest that two different upregulation steps acted upon by different signal transduction pathways have a cumulative effect. In summary, CCBs have a potential antiglomerulosclerotic activity apart from their established ability to block Ca2+ influx via potential-operated channels. CCBs may increase the transcription of LDL receptor gene by Phosphatidyl inositol (PI) pathway and a negative regulatory protein.

Additionally, we focused on the mechanism of LDL receptor up-regulation through LDL receptor gene promoter activity. Our studies showed that treatment with diltiazem or verapamil increased LDL receptor promoter activity significantly, suggesting that diltiazem or verapamil increased mRNA and protein levels by increasing LDL receptor gene transcription.

In summary, our experiments show that diltiazem and verapamil can upregulate LDL receptor gene expression by stimulating LDL receptor gene promoter activity and also increase LDL receptor protein binding. We also show a differential response between nifedipine and other two CCBs (diltiazem and verapamil) in terms of gene expression of LDL receptor and protein binding. It is difficult to envisage how up-regulation of LDL receptor activity by the action of CCBs in cells of non-hepatic tissue such as kidney can be anti-atherosclerotic or anti-glomerulosclerotic. To answer this question, probably prospective clinical studies would be necessary. However, this study has some relevance in understanding the lipoprotein-mediated renal damage. It is reasonable to suggest that the anti-atherosclerotic effects of diltiazem and verapamil in the peripheral tissues may be partly mediated through its anti-proliferative effects. The anti-proliferative effect of CCBs and their induction of LDL receptor may influence physiologically regulated LDL transport through mesangial cell. This is particularly significant because we have shown that CCBs cannot overcome the inhibitory effect of a high cholesterol concentration in down-regulating the expression of LDL receptor on HMCL. This could imply that CCBs preferentially favour the transport of LDL through LDL receptor instead of using the relatively unregulated foam cell forming scavenger receptor pathway. Thus, the anti-atherogenic effect of CCBs may be more pronounced in the presence of cholestero-lowering drugs. Furthermore, our studies show that diltiazem and verapamil are useful

agents in studying the proliferation independent expression of LDL receptor. On the other hand, Sugiura reported that CCBs inhibit rat mesangial cell proliferation and matrix production through the suppression of AP-1 and CREB transcription factors (Sugiura et al. 2000). It will be of interest to study whether CCBs have any inhibitory effect on scavenger receptor since we have shown that AP-1 is a specific response element in reduction of scavenger receptor in HMCL. Yoshisa et al showed that lipophilic Ca^{+}-antagonist nilvadipine significantly inhibited the oxidative modification of LDL in human (Sugiura et al. 2000). Sanchez showed that nifedipine & verapamil had a greater anti-inflammatory effect in rat (Sanchez et al. 1998; Lee et al. 1997). It would be of great interest to study whether CCBs could inhibit inflammatory cytokines-induced upregulation of LDL receptor. Therefore, it is possible that the major anti-glomerulosclerotic effect of CCBs may be through the inhibition of cellular proliferation, inhibition of scavenger receptor, anti-oxidant activity and anti-inflammatory effect.

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSION

The lipid nephrotoxicity hypothesis suggests that hyperlipidaemia is a potentiating factor for the progression of initial glomerular injury and eventual progression to glomerulosclerosis (Moorhead et al. 1982). Results presented in this thesis help us to extend the hypothesis further and suggest how the influence of inflammation may modify intracellular cholesterol homeostasis. I have shown in chapter 3 the ability of inflammatory cytokines to induce scavenger receptors in renal mesangial cells in culture. The evidence presented in chapter 4 shows the influence of inflammatory cytokines in disrupting the sterol-dependent regulation of LDL receptor so that native LDL can enter these cells in an unregulated fashion to convert them to foam cells. In chapter 5, I have demonstrated both cell proliferation independent and sterol dependent regulation of LDL receptor. This new finding is important in understanding why lipid lowering strategies alone are not adequate in arresting the progression of renal diseases and provides adequate grounds for concluding that hyperlipidaemia, oxidative modification of lipid, and inflammation are three key factors in the lipid-mediated progression of renal diseases.

6.1. INFLAMMATION IS A KEY FACTOR IN GLOMERULAR ATHEROSCLEROSIS.

Based on clinical and experimental evidence, it has been hypothesised that infection and inflammation play a fundamental role in atherogenesis and acute thrombosis, probably also in glomerulosclerosis. Although first suggested at the turn of the 20th century, there is renewed interest in the infectious theory of atherosclerosis. Studies done in many laboratories around the world over last few years have shown an association between markers of inflammation and atherosclerosis with an exacerbation

of the inflammatory process during acute myocardial ischemia, particularly in the early stages of reperfusion (Mehta et al. 1998).

The previous understanding of association between inflammation and atherosclerosis was primarily based on 'the response to injury hypothesis', whereby inflammation causing endothelial injury and platelet-mediated haemodynamic damage (Ross and Glomset, 1973). One of the earliest events in both human and experimental atherosclerosis is adhesion of monocytes and T lymphocytes to the endothelial surface followed by their migration into the intima. This intimal recruitment of blood derived cells, coupled with enhanced endothelial permeability to plasma proteins, indicates a potential role for inflammatory mechanisms in early atherogenesis (Watanabe et al. 1996). Chemokines or chemotactic cytokines represent an expanding family of structurally related small molecular weight proteins, recognised as being responsible for leukocyte trafficking and activation. About a decade ago, soon after the discovery of this class of cytokines, monocyte chemoattractant protein-1 (MCP-1) was found to be highly expressed in human atherosclerotic lesions and postulated to be central in monocyte recruitment into the arterial wall and developing lesions. Other chemokines such as RANTES, MIP-1alpha and MIP-1beta have also been implicated in atherosclerotic lesion formation as are a number of more recently discovered chemokines like MCP-4, ELC and PARC (Reape and Groot, 1999). Co-localisation of T lymphocytes and macrophages in all stages of human atherosclerosis, from grossly normal pre-lesional intima to fully advanced atheromatous plaques, and expression of cytokines and MHC class II antigens by many types of cells involved in the lesion provide further evidence that atherosclerosis has both inflammatory and immune components. The presence of T lymphocytes and macrophages in close

contact with each other suggests that cognate cell to cell interaction also plays a pivotal role in the pathogenesis of atherosclerosis. It seems conceivable that the T lymphocyte-macrophage interaction takes place particularly in areas where atherosclerotic lesions are in progress or active. The pathogenic potentials of immunological factors are fruitful subjects for further investigation (Watanabe et al. 1996).

In kidney, several recent studies have focused on the role of cytokines and growth factors produced by infiltrating macrophages and intrinsic glomerular cells in promoting glomerulosclerosis. Ding et al. demonstrated that glomerular and peritoneal macrophages obtained from PAN nephrotic or hypercholesterolaemic animals displayed higher expression of transforming growth factor β (TGF-β) mRNA in contrast to macrophages obtained from control animals (Ding et al. 1994). Another study demonstrated that tumour necrosis factor (TNF) and interleukin-1 (IL-1) were present in glomerular cells using immunohistochemical methods 14 days after PAN administration (Diamond and Pesek, 1991). They also demonstrated that proliferating mesangial cells and infiltrating glomerular macrophages were present in the glomerulus during this acute nephrotic phase. Essential fatty acid free diet significantly reduced TNF and IL-1 positive glomerular cells. Work carried out using the spontaneous murine lupus model provided further evidence that macrophage derived peptide growth factors participate in the process of FSGS. This model is characterised by mesangial cell proliferation and macrophage infiltration into the glomerulus. It has been demonstrated that infiltrating macrophages in the mouse lupus nephritis model release pro-inflammatory mediators such as TNF-α, IL-1β (Boswell et al. 1988). Brennan has also shown that infusion of cytokines into the peritoneum of the above

nephritic mice caused accelerated renal disease and a higher mortality (Brennan et al. 1989).

Most of the above studies suggest a role for accessory cells. But another theory suggests that mesangial cell proliferation precedes infiltration of macrophages into the glomerulus and glomerulosclerosis (Wilens et al. 1951). Experiments carried out using the 5/6 nephrectomised rat model suggest a phenotypic switch of mesangial cell from a contractile type to proliferating and secreting type. Mesangial cells proliferation precedes influx of monocyte/macrophages into the glomerulus (Floege et al. 1992). Mesangial cell proliferation also precedes glomerular and interstitial fibrosis following an immunological insult (French et al. 1967). Mesangial cell proliferation is thought to involve complement and platelet factors and may involve the basic fibroblast factor (bFGF). Mesangial cell proliferation is maintained by autocrine factors that involve the up-regulation of mesangial cell platelet derived growth factor (PDGF) and PDGF receptors. These early changes precede up-regulation of genes that code for extracellular matrix components, mesangial matrix expansion and sclerosis (French et al. 1967). Strategies that retard mesangial cells proliferation such as complement and platelet depletion, administration of heparin and anti PDGF antibodies reduce matrix expansion (Floege et al. 1993). These evidences suggest that inflammation also contribute to glomerular atherosclerosis by contributing to the lipid deposition in the extracellular matrix.

The present study shows that the inflammatory cytokines can modify intracellular lipid homeostasis by dysregulating lipoprotein receptors in a human mesangial cell line (HMCL). This report is also the first to address the question of the very low

expression of scavenger receptor in normal mesangial cells. However, I found that inflammatory cytokines induced macrophage scavenger receptor expression in these cells, resolving previous controversy regarding the expression of scavenger receptor in normal mesangial cells. The present data also show that inflammatory cytokines dysregulated the LDL receptor in human mesangial cells (HMC), it acquires scavenger-like functions. The cells then accumulate native LDL in an unregulated way and become foam cells. This study therefore, demonstrates a fundamental difference in LDL receptor expression induction between central and peripheral tissues. It may be that the over expression of LDL receptors in hepatocytes is not damaging because the native LDL taken up through these receptors does not accumulate, but is rapidly excreted in the form of bile salts. There is no such exit route for the accumulated cholesterol molecules in the peripheral cells and therefore they become foam cells. Inflammation is a key event in activating this pathway.

6.2. INFECTION AND ATHEROSCLEROSIS

In certain genetically susceptible people, infection with very common organisms, such as Chlamydia pneumoniae or cytomegalovirus, may lead to a localised infection and a chronic inflammatory state. Persistence of infection may relate to the degree of inflammation and severity of atherosclerosis. Early trials with appropriate antibiotic agents in some patients with a recent history of acute myocardial infarction have yielded promising results. If patients with an infectious basis of atherosclerosis can be identified, therapy directed at eradication of the offending organism may be appropriate (Mehta et al. 1998). Herpes viruses cause atherosclerosis in experimental animals and can also be detected in atherosclerotic lesions in humans. Cytomegalovirus may play a role in arteriosclerosis in transplanted hearts, and this

virus, together with tumour suppressor protein p53, can be found in re-stenosis lesions following angioplasty. Chlamydia pneumoniae and dental infections are associated with coronary heart disease in cross-sectional and longitudinal studies, and ischaemic stroke is associated with preceding respiratory infections. Infections may favour formation of atherosclerosis and thrombosis by elevation of blood levels of fibrinogen, leukocytes, clotting factors, and cytokines and by alteration of the metabolism and functions of endothelial cells and monocyte macrophages. Low-grade infections may also be one of the causes of the inflammatory reaction observed in atherosclerotic lesions and acute ischaemic symptoms, reflected in elevated levels of C-reactive protein (Mattila et al. 1998). There is an increased incidence of death from myocardial infraction in winter months in comparison to summer months due to increased incidence of respiratory infections, this is particularly so in older population.

6.3. MARKERS OF INFLAMMATION

From an epidemiological perspective, corroboration of inflammatory theory has been provided by a series of prospective cohort studies which demonstrate that inflammatory parameters, such as C reactive protein, cytokines (interleukin-6), fibrinogen, and serum amyloid A, and cellular adhesion molecules ICAM-1, are all elevated over baseline among patients at risk for future coronary occlusion. The acute-phase reactants, C-reactive protein and interleukin-6, and markers of the fibrinolytic state (plasminogen activator inhibitor-1 and tissue plasminogen activator) are also elevated in the acute coronary syndromes and in healthy individuals who are at increased risk for developing coronary artery disease (Kinlay et al. 1998). The acute-phase (AP) serum amyloid A proteins (A-SAA) are multifunctional apolipoproteins which are involved in cholesterol transport and metabolism, and in

modulating numerous immunological responses during inflammation and the acute-phase response to infection, trauma or stress. During the acute-phase response the hepatic biosynthesis of A-SAA is up-regulated by pro-inflammatory cytokines, and circulating concentrations can increase by up to 1000-fold. In the later stages of the acute-phase response, A-SAA expression is effectively down-regulated via the increased production of cytokine antagonists such as the interleukin-1 receptor antagonist (IL-1Ra) and of soluble cytokine receptors, resulting in less signal transduction driven by pro-inflammatory cytokines (Jensen and Whitehead, 1998). These markers may reflect vascular inflammation. Their measurement may pinpoint the mechanisms of benefit of cholesterol-lowering therapy and other interventions designed to reduce coronary risk, and potentially could offer a new method for monitoring coronary risk factor reduction in patients (Kinlay et al. 1998).

6.4. FUTURE WORKS

This research has created a link between inflammation and atherosclerosis, and established a new concept that inflammatory cytokines are risk factors for atherogenesis and that no cholesterol concentration is 'safe' in the presence of acute or chronic inflammation because: 1) inflammatory cytokines dysregulate LDL receptor regulation and change the LDL receptor function into a 'scavenger-like function'; 2) inflammatory cytokines induce scavenger receptor expression. However, further work is needed in this area to collect more evidence clinically and experimentally. Current work in the department is directed to the following areas:

6.4.1. Explore the role of SCAP as cholesterol sensor in different cell types

SCAP maintains a constant level of cholesterol in hepatocytes and other cells through feed back regulation (Goldstein and Brown, 1985). However inflammatory cytokines were able to disrupt the feedback regulation in HMC by over expression of SCAP. Currently we are exploring the relationship of cytokines, SCAP, SREBP and LDL receptor in SMC, HepG2 cells, HMC, and fibroblasts. We will be establishing stable cell lines which over express human SCAP by transfection of one or three copies of human SCAP cDNA into these cells to provide a direct evidence that over-expression of SCAP will disrupt normal LDL receptor feed-back regulation.

6.4.2. Identification of specific response elements in LDL receptor promoter region.

Using LDL receptor reporter system, a cis-active element responsible for transcriptional activation of the LDL gene in response to cytokines will be identified. A screening assay will be established to search cytokine-dependent minimum responsive element and enhancer in genomic sequences located further upstream of the LDL receptor promoter from +35 to –6500. Finally, specific sterol-independent response elements to TNF-α, and IL-1β in the LDL receptor promoter region will be identified.

6.4.3. Explore the signal transduction pathways involved in scavenger receptor regulation

We have demonstrated that Ap-1/ets pathway was involved in scavenger receptor inducible expression in HMC induced by PMA, but not by the inflammatory cytokines TNF-α and IL-1β. We will be exploring the relationship between other transcription

factors and scavenger receptor expression and will also focus on cell cycle proteins, mitogen-activated protein, and proliferator-activated receptors.

6.4.4. Identification of specific response elements to TNF-α and IL-1β in scavenger receptor promoter region

Using reporter gene techniques, we will be identifying the cis-active elements responsible for transcriptional activation of the scavenger receptor gene in response to cytokines. A screening assay will be established to search for a cytokine-dependent minimum responsive element and enhancer in genomic sequences located further upstream of the scavenger receptor promoter from +46 to –700. Finally, specific response elements to TNF-α, IL-1β in scavenger receptor promoter region will be identified.

6.4.5. Animal model

A suitable animal model is required to explore the possibility of using anti-receptor antibody against specific inflammatory cytokines.

6.5. CONCLUSION

In summary, these studies provide a link between inflammation and intracellular cholesterol homeostasis and show how inflammatory cytokines induce scavenger receptors on renal mesangial cells and how these cytokines dysregulate the LDL receptor pathway. These studies help to resolve the paradox of why lipid-lowering strategies have not been shown to be of major benefit in arresting the progression of renal disease or controlling chronic graft dysfunction. The importance of this observation is that there are a number of mechanisms involved in the accumulation of

lipid in intracellular and extra cellular matrices. It is clear from these studies that no cholesterol concentration is safe in the context of acute or chronic inflammation. Chronic renal disease itself is an inflammatory state, hence pre-existing atherosclerotic processes and the inflammatory state of renal disease may create a vicious circle leading to progression of both renal disease and aggravation of atherosclerosis. Therefore combined use of anti-inflammatory agents, antioxidants and lipid lowering drugs may be necessary to prevent or ameliorate lipid- mediated progression of renal disease.

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