Interaction Of Monocytes With Glomerular Mesangial Cell Matrix In The Pathogenesis Of Glomerular Injury

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ABSTRACT

Acute inflammatory kidney diseases may resolve, leaving limited residual damage or progress to cause chronic renal scarring characterized by glomerulosclerosis and interstitial fibrosis. Understanding the mechanisms that control inflammation within the kidney may facilitate the development of treatment strategies to prevent irreversible kidney damage and slow progression of chronic kidney disease. Infiltration of mononuclear cells is recognized as an early event in many different conditions that may ultimately lead to kidney injury. Having extravasated from blood vessels at sites of injury, these multifunctional cells differentiate into tissue macrophages, which depending on their phenotype, have the potential to both promote resolution of inflammation or to cause scarring, making them an attractive target for therapy. Having left the glomerular capillary lumen, mononuclear cells are very likely to encounter the mesangial matrix. It was therefore hypothesized that interactions between monocytes and matrix components might modify the behavior of the infiltrating cells and thereby modify the outcome of the inflammatory process.

The work presented in this thesis demonstrates that mesangial matrix activates monocytes leading to expression of peroxisome proliferators activated receptor γ and the CD36 scavenger receptor, both markers of macrophage differentiation. Since LDL accumulation in the mesangium may contribute to glomerular injury, the interaction between this lipoprotein and the matrix was also examined. These studies demonstrated that LDL becomes oxidized when exposed to matrix components, possibly due to loss of protective antioxidants. The presence of oxidized LDL has the potential to induce

mesangial cell chemokine production, which is likely to promote further monocyte influx into the glomerulus. Furthermore, matrix-activated monocytes internalized oxidized LDL via CD36 scavenger receptor, leading to foam cell formation, a recognized characteristic feature of glomerular injury. Foam cell formation may in turn amplify and perpetuate the disease process by driving further production of cytokines and growth factors.

Finally, to establish that these observations were relevant to human glomerular disease, the presence of macrophages expressing PPAR-γ and the CD36 scavenger receptor in human kidney biopsy samples taken from patients with inflammatory glomerular disease was demonstrated, using sections from non-inflamed kidneys as controls. These observations imply that monocyte-matrix interactions are important in the context of glomerular disease and may represent a potential target for therapies designed to limit injury resulting from glomerular inflammation.

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Chana RS, Martin J, **Rahman EU**, Wheeler DC. Monocyte adhesion to mesangial matrix modulates cytokine and metalloproteinase production. *Kidney Int. 2003 Mar;63(3):889-98.*

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LIST OF ABBREVIATIONS

Ac-LDL: Acetylated LDL

AgII: Angiotensin II

BHT: Butylated hydroxytoluene

BSA: Bovine serum albumin

CD: Cluster of differentiation

CM: Conditioned medium

CR3: Complement receptor type 3

Dil-Ac-LDL: Ac-LDL, labeled with 1,1'-dioctadecy-3,3,3',3',-tetramethylindocarbocyanine

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EDTA: Ethylene diamine tetra acetic acid

ESRD: End-stage renal disease

FACS: Fluorescence-activated cell sorter analysis

FCS: Foetal calf serum

FITC: Fluorescein isothiocyanate

FN: Fibronectin

GBM: Glomerular basement membrane

GFR: Glomerular filtration rate

Gp:	Glycoprotein
GS:	Glomerulosclerosis
GM-CSF:	Granulocyte monocyte colony stimulating factor
H_2O_2	Hydrogen peroxide
Hep	Heparin
HLA-DR:	Histocompatibility leukocyte antigen-differentiation region
HMCL:	Human mesangial cell line
ICAM:	Intracellular adhesion molecule
IL:	Interleukin
Ig:	Immunoglobulin
IL-1β:	Interleukin-1β
LDL:	Low density lipoprotein
LDH:	Lactate dehydrogenase
LFA:	Lymphocyte functional-associated antigen
LPS:	Lipopolysaccharide
MadCAM:	Mucosal addressin cell adhesion molecule
MAC-1:	Adhesion molecule CD11b/CD18
MFI:	Mean fluorescence intensity
MC:	Mesangial cell
M-CSF:	Monocyte colony stimulating factor
MCP-1:	Monocyte chemotactic protein

MDA:	Malondialdehyde
MMP:	Matrix metalloproteinase
NaBr:	Sodium bromide
NaCl:	Sodium chloride
Ox-LDL:	Oxidised low-density lipoprotein
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDGF:	Platelet derived growth factor
PGE:	Prostaglandin E
PKC:	Phosphokinase C
PMA:	Phorbol 12-myristate 13-acetate
Poly I:	Polyinosinic acid
PPAR:	Peroxisome proliferator activated receptor
RGD:	Arginine-Glycine-Aspartic Acid
RNA:	Ribonucleic acid
RPMI 1640 Medium:	Roswell Park Memorial Institute 1640 medium
RT-PCR:	Reverse transcriptase-polymerase chain reaction
SCr:	Scavenger receptor
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SD:	Standard deviation
SMC:	Smooth muscle cell

TBARS: Thiobarbituric acid reactive substances

TBA: Thiobarbituric acid

TBS: Tris buffered saline

TGF-β: Tumour growth factor-β

Th: T-helper

TIMP: Tissue inhibitors of metalloproteinase

TNF-α: Tumour necrosis factor-α

UKTS: United Kingdom transplant sharing scheme

VCAM: Vascular Cell Adhesion Molecule

VLA: Very Late Antigen

CHAPTER 1

GENERAL INTRODUCTION

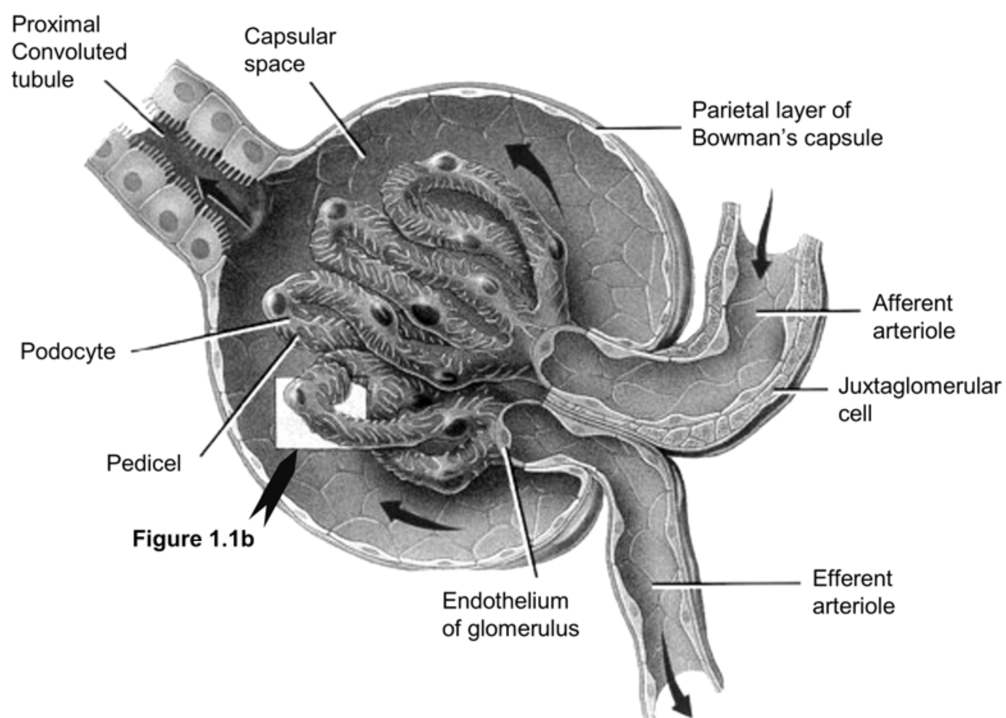
Glomeruli are susceptible to a variety of inflammatory, metabolic, haemodynamic, toxic and infectious insults which induce similar clinicopathologic presentations. Despite advances in understanding the factors that initiate glomerular injury, efforts to stop or slow the progression of established chronic renal disease have proved largely unsuccessful. The fact that multiple pathogenic mechanisms result in a similar histological endpoint suggests that the glomerulus has only a limited repertoire of responses to injury and that renal scarring can be considered to represent a secondary phenomenon rather than a specific disease process. Glomerulosclerosis is the final result of a number of interrelated events leading to permanent glomerular injury. Histological features of many chronic progressive renal diseases are evidenced by the accumulation of matrix, macrophages and cholesterol in sclerotic glomeruli. Several factors act independently or together to play a pivotal role in determining whether the final outcome of an acute inflammatory glomerular lesion leads to complete resolution or permanent scarring. The study of these factors may suggest new targets for therapeutic intervention.

1.1. THE NORMAL GLOMERULUS

Blood enters the glomerulus through the afferent arteriole, which branches into tiny clusters of looping blood vessels, which comprise the glomerular ("capillary") tuft (which is actually a highly specialized section of an arteriole). As blood passes through the tuft, the plasma is filtered through fenestrations in the endothelial cells and the glomerular basement membrane (GBM), then through spaces between the podocyte foot processes (in the slit membrane) into Bowman's space. The capillary endothelium, GBM and the slit membrane constitute the filtration barrier that collectively filters plasma

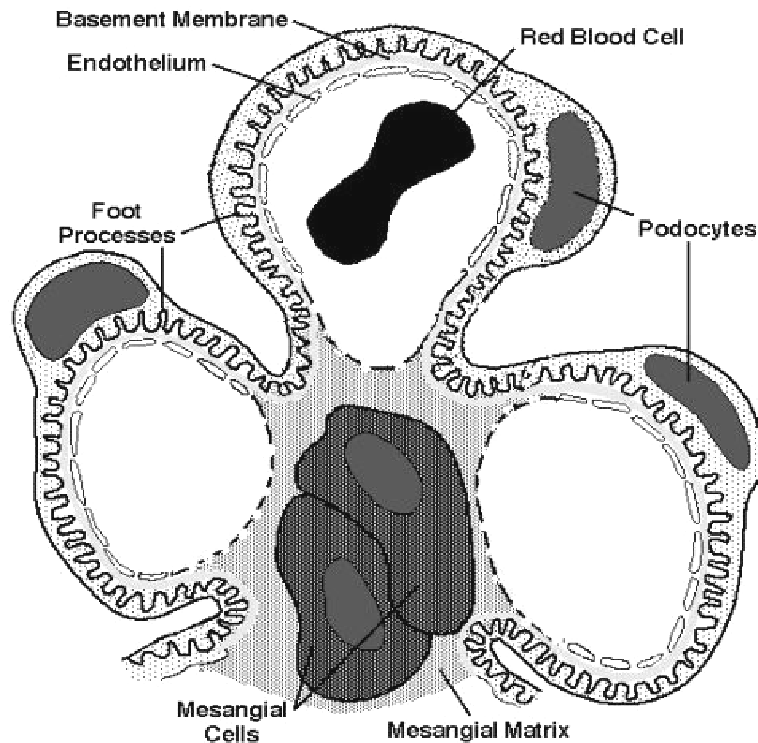
allowing passage of water, small solutes such as sodium, urea, and glucose, small proteins and small organic molecules. The filtered blood is then drained from the glomerular tuft through the efferent arteriole. The GBM is continuous throughout the glomerulus, surrounding each capillary loop and the ‘stalk’ region of the glomerular tuft. At the vascular pole, the GBM is continuous with Bowman’s capsule. Between the glomerular capillaries lie the mesangial cells and the mesangial matrix, which together provide structural support by surrounding the glomerular capillaries (Figure 1.1a and 1.1b). Some mesangial cells are located outside the glomerulus, between the afferent and efferent arterioles and are known as Lacis or Goormaghtigh cells.

Figure 1.1a. The Normal Glomerulus



(Image courtesy of PB Works nephrology images)

Figure 1.1b. Schematic Diagram of a Single Capillary Tuft within Bowman's Capsule



(Image courtesy of PB Works nephrology images)

Much of the original work describing the mesangium was concerned with the ultra-structural appearance of cross-sections of the glomerulus using electron and light microscopy. According to current understanding, the mesangium consists of mesangial cells and mesangial cell matrix, which are capable of various tasks Table 1.1. The interstitial areas of each lobule join at the glomerular stalk and are thereby in direct continuity with the renin-secreting juxtaglomerular apparatus.

Table 1.1. Functions of the Glomerular Mesangium

The table below gives a summary of the various functions of the Mesangial cell and Mesangial cell matrix within the mesangium, as reviewed by Professor Detlef Schlondorff (Schlondorff 1996; Schlondorff and Banas 2009).

Component of Mesangium	Function
Mesangial Cell	• Production of Matrix. • Production of tissue inhibitor of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) which control turnover of mesangial matrix. • Influence GFR by regulating blood flow through the glomerular capillaries or by altering capillary surface area. • Biological handling and clearance of macromolecules; advanced glycated end-products (AGE's), immune complexes and lipids. • Exhibit phagocytic activity. • Production of vasoactive agents; angiotensin II (Ag II), nitric oxide (NO), prostaglandin E2 (PGE2) and inflammatory mediators.
Mesangial Cell Matrix	• Structural support. • May potentially trap LDL. • Has the capacity to oxidise LDL. • Interacts with mesangial cells and infiltrating cells via ligands. • Sequesters cytokines and growth factors.

1.2. GLOMERULAR MESANGIAL CELLS

Mesangial cells are similar to vascular smooth muscle cells and are believed to have several functions in vivo. They contain large amounts of actin, myosin and tropomyosin indicative of a contractile function. The presence of receptors for the vasoactive peptide angiotensin II (AgII) support this role (Kreisberg 1983), which is important in the regulation of glomerular haemodynamics. When glomeruli are damaged, mesangial cells produce chemotactic factors such as monocyte chemotactic protein (MCP-1) (Cushing, Berliner et al. 1990), monocyte colony stimulating factor (m-CSF) (Rajavashisth, Andalibi et al. 1990) and interleukin-1β (IL-1β) (Ku, Thomas et al. 1992). Mesangial cells also secrete matrix, which creates the structural framework for the glomerular tuft as well as enzymes (and enzyme inhibitors) that maintain the balance between synthesis and degradation (Michael, Keane et al. 1980; Davies, Coles et al. 1990; Sugiyama, Kashihara et al. 1998; Fogo 1999). Additionally the mesangial cell possesses phagocytic properties that contribute to the clearance and uptake of macromolecules from the glomerulus (Schreiner, Kiely et al. 1981; Davies 1994). Thus, inappropriate activation of mesangial cells may lead to excess matrix production, as well as release of chemotactic factors resulting in monocyte influx into the mesangium.

Expansion of the mesangium, both due to the deposition of matrix proteins and an increase of mesangial cell numbers, is seen in kidney disease. Estimates of proliferation of mesangial cells in vivo suggest a low rate of about 1% per day (Pabst and Sterzel 1983; Davies 1994). Mesangial cell proliferation appears to play a role in the progression of glomerular pathology, particularly in the early stages. Matrix expansion is

generally preceded by mesangial cell proliferation in experimental models of glomerulonephritis. Persistent mesangial cell hyperplasia, caused by repeated injury, is believed to lead to irreversible scarring and eventual loss of glomerular function (Floege, Eng et al. 1993; Shimizu, Kawachi et al. 1999). The proliferation of mesangial cells is presumed to be a necessary physiological response required for the reconstitution of renal tissue.

1.3. The Mesangial Cell Matrix

The mesangial extracellular matrix fills the spaces between the mesangial cell and the perimesangial basement membrane, in addition a small amount of matrix may be found beneath the endothelium. In ultra-structural studies, this matrix has been characterized as a dense network of elastic microfibrils similar to the connective tissue of many other organs, and contains a network of intercellular channels that traffic macromolcules. In immunohistochemical studies, fibronectin is detected within the mesangium, along with laminin and type IV collagen, whilst type III collagen is found in the tubulointerstitium and type V collagen in the mesangial interstitium and the GBM (Oomura, Nakamura et al. 1989; Sugiyama, Kashihara et al. 1998). Fibronectin and Collagen type IV comprise the major protein components within the mesangial matrix, and were therefore the components studied in the experimental work described in this thesis. Minor components present in mesangial matrix include proteins such as laminin, vitronectin, entactin and proteoglycans. Fibronectin is the only component to be present exclusively within mesangial matrix and is specifically localized to areas immediately

surrounding mesangial cell processes. The other protein components mentioned are found distributed throughout the mesangial matrix, tubular basement membranes and Bowman's capsule (Madri, Roll et al. 1980).

Fibronectin is closely associated with microfibrils, thus providing a link between mesangial cells and other matrix structures (Reale, Luciano et al. 1981; Brown, Andres et al. 1982; Cohen and Ku 1984). This microfibrillar network also appears to provide a solid base of contact between mesangial cells and the perimesangial GBM. Microfibrils are attached to sites in the mesangial cell membrane that serve to anchor intracellular actin filaments and penetrate the lamina densa to connect with the GBM. Not only does fibronectin serve to interconnect cells and matrix components, but also interconnects the microfibrils at their crossing points, so further stabilizing the entire matrix (Schwartz, Goldfischer et al. 1985). As a result of these interconnections, the microfibrillar network has sufficient three-dimensional tensile strength to balance distending forces acting in all directions. The importance of the mesangial matrix as a connecting structure between mesangial cells and the GBM is demonstrated by studies in which the failure of such connections produces miroaneurysms of the glomerular capillary tuft (Mosher 1984; Cohen, Saini et al. 1987; Proctor 1987; Yasuda, Kondo et al. 1996).

Due to its nature, mesangial matrix has the potential to trap large molecules including lipoproteins such as LDL (Gupta, Rifici et al. 1992; Wheeler and Chana 1993). The involvement of additional factors such as intra-renal hypertension, and inflammation are necessary for the induction and progression of lipid-induced renal dysfunction. Foam cells and lipid deposits are found in focal segmental sclerosis in human renal biopsies (Lee, Lee et al. 1991). Many of the features of progressive

glomerular and tubulo-interstitial diseases share biological mechanisms with those of atherosclerosis.

This may be relevant in the pathogenesis of glomerular disease, particularly following an insult that has altered the glomerular filtration barrier permeability, allowing excess amounts of LDL to penetrate the mesangium. This process may potentially contribute to the formation of foam cells within the mesangium as explored later in the thesis.

1.4. The Glomerulus in Disease

Glomerular injury results from an initial pathogenic insult and may heal without consequences or lead to altered function of intrinsic glomerular cells and invasion of monocytes/macrophages from the circulation. Such changes result initially in mesangial cell proliferation, but if not resolved, continued cell proliferation leads to hyperplasia and the concomitant increase in matrix production to glomerulosclerosis. The term glomerulosclerosis is a non-specific finding on light microscopic examination that can be seen in any primary glomerular, tubulointerstitial, or vascular kidney disease.

The process may initially involve only a small proportion of glomeruli (focal) and within these, only certain segments of the tuft (segmental). These localized lesions may progress to involve the whole glomerulus. The sclerotic areas consist of collapsed capillary loops obscured by an excess of mesangial matrix.

A variety of early changes are recognized to precede glomerular obliteration. The basement membrane become thickened, is often detached from the overlying epithelial

cells and may be adherent to Bowman's capsule. Epithelial cells are hypertrophied or absent at the sites of adhesions and electron microscopy reveals fusion of foot processes. Subendothelial eosinophilic deposits of hyaline material are found in both sclerotic and non-sclerotic areas of the glomerular tuft. Mesangial hypercellularity may result both from an increase in the number of contractile mesangial cells and from invasion of inflammatory macrophages. The mesangial area is also expanded by an excess deposition of mesangial cell matrix (Couchman, Beavan et al. 1994).

Lipid deposition is seen within mesangial and epithelial cells both of sclerotic and non-sclerotic capillary loops but may also occur in interstitial regions (Chana, Wheeler et al. 2000). These histological changes correlate with the clinical manifestations of progressive glomerulosclerosis (Klahr, Schreiner et al. 1988; Magil and Frohlich 1991; Moorhead 1991). Proteinuria usually precedes a reduction in glomerular filtration rate and renal blood flow. Deterioration of renal function progresses and is frequently associated with the development of hypertension. Tubular atrophy leads to a reduction of the renal concentrating ability and impaired acid secretion.

1.5. MESANGIAL CELL MATRIX IN GLOMERULAR DISEASE

The mesangial matrix is no longer seen as a static scaffold in which cells reside; but has been shown to be involved in cell proliferation, migration and cell-cell interactions. Turnover of the different extracellular matrix components is recognised as an active process with multiple levels of regulation (Sterzel, Schulze-Lohoff et al. 1992;

Yasuda, Kondo et al. 1996). Net deposition of matrix proteins, as seen in glomerulosclerosis, results from both quantitative and qualitative changes to mesangial matrix (Bruijn, Hogendoorn et al. 1988; Klahr, Schreiner et al. 1988; Olgemoller and Schleicher 1993; Harendza, Schneider et al. 1999). Changes in mesangial matrix may modify glomerular function by changing cell-cell interactions and by promoting infiltration and entrapment of macrophages.

Matrix construction and remodelling involves three factors, matrix metalloproteinases (MMPs), plasmins that activate latent MMPs and tissue inhibitors of MMPs (TIMPs) (Raines 2000; Keeling and Herrera 2008). In inflammatory conditions, levels of growth factors such as TGF-β increase and can act to suppress the expression of matrix degrading plasminogen-activator inhibitor (PAI), and increase the activity of tissue inhibitors of metalloproteinases (TIMPs), thus favoring matrix accumulation.

One consequence of mesangial matrix remodelling is that changes within this complex three-dimensional structure can reveal hidden sites previously unrecognizable to various adhesion receptors on the surface of cells coming into contact with the matrix. For example the RGD sequence of the fibronectin molecule is able to bind very late antigen (VLA)-5 present on the cell surface of monocytes (Pierschbacher and Ruoslahti 1984; Hemler 1990). Also the CS-1 domain of fibronectin binds VLA-4 on monocytes by an RGD-independent mechanism (Wayner, Garcia-Pardo et al. 1989). Matrix remodelling, by exposing these sites, may promote monocyte adhesion to this matrix component.

1.6. THE MONOCYTE – MACROPHAGE LINEAGE

Monocytes and macrophages comprise a family of phagocytic cells that are widely distributed throughout the body and are generally referred to as the mononuclear phagocyte system because of their common origin, similar morphology and common functions. Monocytes originate in the bone marrow, but become widely distributed in tissues where they mature into macrophages and take on specialist roles. Macrophages are well recognized for their ability to phagocytose, a property that enables them to eliminate pathogens and other foreign materials. However, these cells play a pivotal role in a variety of processes including inflammation, the induction and regulation of specific immune responses and tissue remodelling and repair. There are several basic properties of these cells that are relevant to their role in glomerular injury.

Firstly, mononuclear phagocytes are highly mobile and have the capacity to adhere to various biological substrates, a function that facilitates their migration to sites of inflammation. Secondly, these cells secrete a range of soluble mediators that modulate functions of many other different types of cells. Thirdly, mononuclear phagocytes ingest and degrade various materials including senescent cells and tissue debris. Finally, mononuclear phagocytes can be activated by the external environment.

Whilst mononuclear phagocytes play a critical role in host defense, these cells may also injure the host while exercising their defensive role. For example, monocytes have been shown to contribute to tissue damage by releasing proteolytic enzymes (Campbell, Silverman et al. 1989; Senior, Connolly et al. 1989), toxic oxygen metabolites (Carp and Janoff 1980; Campbell, Senior et al. 1982), pro-fibrotic cytokines

(Martinet, Rom et al. 1987; Shaw 1991) and other mediators (Tracey, Lowry et al. 1986).

Extravasation of monocytes from the vasculature into the mesangium involves directional migration of cells in response to chemoattractant factors. Since the mesangium forms the core of each tuft and is in direct contact with plasma constituents without an intervening membrane, monocytes directly encounter matrix during the migration stage. It is clear that regulated and reversible adherence of monocytes to extracellular matrix components is a prerequisite for the accumulation of these cells at sites of tissue inflammation (Snyderman and Goetzl 1981); however, little is known about the biological and pathological factors that regulate monocyte adherence to extracellular matrix and the resulting changes that occur to the monocyte.

1.7. MONOCYTES

Monocytes represent 3 to 8% of peripheral blood leukocytes. These mature cells measure 12 to 15 μM in diameter and posses a characteristic kidney-shaped nucleus. Their cytoplasm contains a well-developed Golgi apparatus, numerous lysosomal granules, microtubules and actin-containing filaments (which are cross-linked by actin-binding protein and myosin). Monocytes are slowly motile, exhibit phagocytic activity and have a strong tendency to adhere and spread on glass surfaces (Lasser 1983). Monocytes give a positive reaction for non-specific esterases and contain peroxidase, acid phosphatase, lysozyme, aryl sulphatase and glucuronic acid (Yam, Li et al. 1971). Monocytes express HLA-DR antigens on their surface (McKinney, Boto et al. 1980;

Smith and Ault 1981) along with receptors for Fc component of IgG, complement C3 (Huber, Polley et al. 1968; Schwartz, Bianco et al. 1975) and insulin (Schwartz, Bianco et al. 1975).

1.8. MACROPHAGES

Macrophages measure 20 to 80μM in diameter and contain a large vacuolated nucleus often with prominent nucleoli. Their cytoplasm contains a large well-developed Golgi apparatus, abundant rough endoplasmic reticulum and ribosomes, large mitochondria, microtubules, microfilaments and numerous lysosomes rich in hydrolytic enzymes.

The transition from monocyte to macrophage is associated with increases in: 1) the number of lysosomes and mitochondria; 2) the activity of mitochondrial enzymes and the rate of cellular respiration; 3) phagocytic activity; 4) protein synthesis; 5) the capacity to interact with lymphocytes (Lasser 1983); 6) the expression of Scavenger receptor-A (Xu, Yu et al. 2006) and 7) the expression of Peroxisome proliferator-activated receptor-gamma (PPAR-γ) (von Knethen and Brune 2003).

Macrophages are also facultative anaerobes, with the exception of the pulmonary alveolar macrophage. They are highly motile and have marked phagocytic activity. In contrast to monocytes, macrophages have been shown to proliferate in response to certain stimuli in vitro (Diesselhoff-den Dulk, Crofton et al. 1979; Lasser 1983). As with monocytes, macrophages also express receptors for the Fc component of IgG, C3 (Griffin, Spertini et al. 1990) and insulin (Bar, Kahn et al. 1977) however macrophages

also express receptors for IgE which is important in mediating host immunity to various parasites (Dessaint, Torpier et al. 1979; Melewicz and Spiegelberg 1980). Some macrophages express HLA-DR antigens and can function as antigen presenting cells for lymphocytes. The expression of HLA-DR antigen varies with the type of macrophage; only 15% of peritoneal macrophages express HLA-DR antigen compared with 50% of spleen and thymus macrophages (Cowing, Schwartz et al. 1978; Beller and Unanue 1980).

Macrophages are widely distributed throughout the body, but are particularly prominent in the spleen, lymph nodes, liver (Kupffer Cells), peritoneum, skin (Langerhans cells) and pulmonary alveoli. Macrophages resident in different tissues have widely differing morphological and functional properties. It has been postulated that the profile of local stimuli, to which macrophages are exposed in a particular tissue, influences their maturation and thereby accounts for their diversity of form and function (Cline, Lehrer et al. 1978).

1.8.1. Macrophage Heterogeneity

Local factors are important in determining the phenotype adopted by the recruited monocyte. The resultant tissue macrophages can be broadly divided into two groups; 'resident macrophages' and 'inflammatory macrophages'(Gordon 2003).

Tissue macrophages are heterogeneous and those isolated from different anatomical sites differ in function, presumably because of adaptive responses to the local microenvironment. Inflammatory macrophages are derived largely from circulating

monocytes which infiltrate damaged tissue; some also arise by local cell division. Different macrophage activation states have been recognized and result from exposure to specific stimuli that initiate differentiation into A) classically or B) alternatively activated macrophages (see table 1.2).

Classically activated macrophages exhibit a Th1-like phenotype, promoting inflammation, extracellular matrix destruction, and apoptosis, while alternatively activated macrophages display a Th2-like phenotype, promoting extracellular matrix construction, cell proliferation, and angiogenesis (Erwig and Rees 1999; Duffield 2003). Although both phenotypes are important for clearance of pathogens and apoptotic cells, the classically activated macrophage tends to elicit chronic inflammation and tissue injury whereas the alternatively activated macrophage tends to resolve inflammation and facilitate wound healing.

Table 1.2. Stimuli for classically and alternatively activated macrophages.

The table below summarises the various stimuli giving rise to a particular macrophage activation status as reviewed by Dr. Jeremy Duffield, a prominent author within the field inflammation research (Duffield 2003).

Macrophage Activation Status	Stimulus
Classical Activation	Pro-inflammatory cytokines Abnormal matrix Hypoxia Bacterial DNA
Alternative Activation	IL-4 IL-10 IL-13 TGF-β

As a result of their opposing phenotypic states, macrophages play a central role in innate protection both through the clearance of infective pathogens and through the repair of tissue injury that occurs, in part, as a consequence of this response. For example, the initial response of infiltrating monocytes to bacterial infection results in macrophage differentiation favoring a classically activated phenotype and so is cytotoxic and proinflammatory; then, once the infection is under control, macrophages phagocytose cellular debris and apoptotic bodies and begin tissue repair (Duffield 2003; Erwig, Kluth et al. 2003).

1.9. MONOCYTES/MACROPHAGES IN GLOMERULAR INJURY

In the normal kidney there are small numbers of interstitial leukocytes thought to perform an immune surveillance function. These are predominantly monocytes. Only a small fraction of leukocytes in the normal kidney comprise B cells, T cells, natural killer cells, and neutrophils.

In an acutely inflamed glomerulus, the predominant leukocyte is the macrophage. In human glomerular disease, macrophage numbers correlate with the extent of histological damage at the time of biopsy and predict renal outcome in certain disease settings (Ootaka, Saito et al. 1997). Macrophages expressing activation and proliferation markers have been identified in more aggressive forms of human and experimental glomerulonephritis and correlate with disease severity (Kerr, Nikolic-Paterson et al. 1994; Lan, Nikolic-Paterson et al. 1995; Yang, Isbel et al. 1998). Strategies that limit disease progression in this setting include (A) the systemic depletion of macrophages; (B) Inhibition of pro-inflammatory cytokines that both activate and are produced by activated macrophages; and (C) the blocking of factors that promote the recruitment of macrophages to tissue sites (e.g; blockade of cytokines and adhesion molecules).

1.9.1. The Potential For Monocyte-Matrix Interactions To Influence Glomerular Injury

The mesangial matrix has the potential to play a key role in monocyte differentiation (Sugiyama, Kashihara et al. 1998; Jacob, Shastry et al. 2002). There are

two main mechanisms by which matrix can influence cell behavior. Firstly matrix harbours growth factors and growth factor binding proteins (Ignotz, Heino et al. 1989; Lee and Streuli 1999). These factors are passively sequestering but may be actively released by remodelling enzymes such as the MMPs and may thereby influence the behaviour of cells that are exposed to matrix components. Secondly, matrix can directly regulate cells via receptor-mediated signalling (Schoecklmann, Rupprecht et al. 1996; Gauer, Yao et al. 1997; Hamerski and Santoro 1999). Since monocytes enter the mesangium through the fenestrated endothelium, they are unlikely to have undergone endothelial activation and therefore may encounter mesangial matrix in an inactivated state. The first activation signals that these cells encounter may therefore take the form of receptor-mediated signalling by matrix components. This monocyte-matrix interaction may play a key role in influencing monocyte behaviour within the glomerulus and the resulting macrophage phenotype is likely to be highly dependant on the local microenvironment within the glomerulus. For example, in a disease situation, matrix remodelling may be disrupted, leading to qualitative and quantitative changes in matrix composition which in turn may influence the sequestration of growth factors. A change in the composition of matrix components may potentially trigger different signal transduction cascades which, in turn, may influence monocyte behavior. (Wesley, Meng et al. 1998; Ingram, Ly et al. 1999; Urushihara, Takamatsu et al. 2010). Furthermore, subtle changes in the matrix composition resulting from enzymatic digestion may release bioactive matrix fragments or expose sequestered growth factors.

As an example, fibronectin, one of the major matrix proteins, binds to very late antigen (VLA)-5 integrin subunits on the surface of monocytes. Binding of fibronectin

to this receptor depends on its conformation. Since fibronectin production is increased in diseased mesangial matrix, it is conceivable that an excess of this matrix component or conformational changes might influence these signalling pathways and thereby modulate monocyte activation and behaviour. The same may be true of collagen type IV, another matrix protein that is increased in diseased mesangial cell matrix. The RGD sequence of the fibronectin molecule binds VLA-5 present on the cell surface of monocytes thus signalling to the cell (Pierschbacher and Ruoslahti 1984; Hemler 1990). Likewise the CS-1 domain of fibronectin binds VLA-4 on monocytes by an RGD-independent mechanism (Wayner, Garcia-Pardo et al. 1989).

1.10. Lipids in Glomerular Injury

The pathological effects of lipoprotein in progressive kidney disease may be similar to those in atherosclerosis. In recent years, an improved understanding of atherosclerosis has illuminated the pathology of glomerulosclerosis and supported the concept of lipoproteins as mediators of renal disease (Moorhead, Brunton et al. 1997). The possible role of lipoproteins in progressive renal disease may be understood in the more familiar context of atherosclerosis.

Atherosclerosis results from a complex sequence of events in which normal cycling of LDL through the vascular endothelium is altered, leading to trapping of LDL. In addition, monocytes are recruited from the blood, smooth muscle cells proliferate and fibrous tissue is deposited. Trapped LDL may become oxidized, partly as a result of pro-oxidant factors produced by monocytes (Parthasarathy, Printz et al. 1986; Quinn,

Parthasarathy et al. 1987; Boullier, Bird et al. 2001). The similarity between atherosclerosis and glomerulosclerosis is based on the assumption that the glomerulus posses cell types which are known to respond to lipoprotein injury, namely monocytes, or which resemble smooth muscle, namely the mesangial cell. Thus the mechanisms involved in atherosclerosis may also apply to glomerulosclerosis.

Oxidised LDL (Ox-LDL) has been demonstrated to be more cytotoxic when compared with unmodified native LDL (Fernando, Varghese et al. 1993). Ox-LDL has also been proven to be important in lesion progression in atherosclerosis since uptake by macrophages via scavenger receptors causes the generation of foam cells. A similar mechanism may play an important role in lipid-mediated glomerulosclerosis. Ox-LDL has also been shown to stimulate monocyte influx (Pai, Kirschenbaum et al. 1995) by inducing mesangial cells to release chemotactic cytokines.

Another prominent feature of lipid-induced glomerular injury is the accumulation of mesangial cell matrix. Studies in vitro indicate that lipid-activated mesangial cells produce excess matrix (Schlondorff 1993; Wheeler and Chana 1993; Lee 1999) as has been described in the atheromatous artery (Ross 1984). Lee et al has demonstrated that LDL stimulates mesangial cells through the induction of the phosphokinase C (PKC) pathway to synthesize TGF-β, which favours matrix production (Lee 1999). Chana et al reported that LDL also selectively enhances the synthesis of specific proteoglycans and hyaluronan in mesangial cells (Chana, Wheeler et al. 2000). The incubation of mesangial cells with native LDL (25-100 μg/ml) increased the synthesis and secretion of both fibronectin and laminin in a dose-dependant manner. Similarly, oxidized forms of LDL

(25-100 μg/ml) increased fibronectin and laminin and had a greater effect than native LDL (Roh, Kamanna et al. 1998).

Therefore, lipoproteins and modified lipoproteins may get trapped in the mesangial matrix, become oxidized and get taken up by infiltrating monocyte/macrophages which form foam cells.

1.11. MACROPHAGE DEACTIVATION AS A TARGET FOR THERAPY

The exact mechanism of macrophage activation and accumulation within the glomerulus is largely unknown, although there is a considerable amount of experimental evidence implicating adhesion molecules as being relevant in the setting of glomerulosclerosis. Many of the interactions between adhesion molecules and infiltrating macrophages have been successfully blocked and could serve as targets for therapeutic interventions (Adler and Brady 1999; Allen, McHale et al. 1999; Chana and Wheeler 1999; Cook, Khan et al. 2002). For example, the beta-1 integrin, $\alpha 4\beta 1$, also known as very late antigen 4 (VLA-4), is present on macrophages and binds to VCAM-1, which has been shown to be up-regulated in the glomerular endothelium in experimental glomerulonephritis. Blocking using anti-α_4 antibodies can prevent experimental crescentic glomerulonephritis as demonstrated by Allen et al (Allen, McHale et al. 1999) and has been shown to halt progression of established disease (Khan, Allen et al. 2003), making it a very attractive candidate for therapy. The humanized version of this anti-$\alpha 4$ monoclonal antibody known as Natalizumab has been used successfully in multicenter double-blind controlled studies in Crohn's disease and multiple sclerosis (Ghosh, Goldin et al. 2003; Miller,

Khan et al. 2003; Miller, Soon et al. 2007; Targan, Feagan et al. 2007). Despite its initial approval Natalizumab was withdrawn from the market by its manufacturer after it was linked with three cases of the rare neurological condition progressive multifocal leukoencephalopathy (PML) when administered in combination with interferon β-1a, another immunosuppressive drug often used in the treatment of multiple sclerosis (Tyler and Khalili 2005; Ransohoff 2007). After a review of safety information and no further deaths, the drug was returned to the US market in 2006 under a special prescription program. As of June 2009, ten cases of PML were known. However, twenty-four cases of PML had been reported since its reintroduction by October 2009, showing a sharp rise in the number of fatalities and prompting a review of the chemical for human use. By January 2010, 31 cases of PML were attributed to natalizumab, however it was not withdrawn from the market because its clinical benefits outweighed the risks involved (Ransohoff 2010; Steiner 2010).

The beta-2 integrins include CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1/Complement receptor 3). A humanized monoclonal antibody to CD18 known as Efalizumab, which blocks CD11a/CD18 and CD11b/CD18 has been reported to reduce infiltrating leukocytes and improve vasculitic ulcers in four of five patients with systemic vasculitis (Lockwood, Elliott et al. 1999). Despite these beneficial effects Efalizumab was withdrawn from the market ten years later as it was associated in some cases with fatal brain infections (Major 2010).

1.12. AIMS OF THIS WORK

Monocyte/macrophage accumulation within the glomerular mesangium is a recognized feature of glomerular injury in man. Although the mechanisms of macrophage trafficking and activation within the glomerulus are not properly understood, it is generally assumed that these cells are derived from circulating monocytes that migrate from the glomerular capillary lumen. During this process the monocyte encounters many extracellular signals that promote differentiation to a macrophage and other cellular responses. Recent studies suggest that such interactions may program macrophages, thereby potentially modifying their behavior in the setting of acute or chronic glomerular disease (Erwig, Kluth et al. 1998; Min, Lyons et al. 2009).

To begin to address the pathobiological importance of alterations in monocyte phenotype following interaction with matrix components, there is the need to firstly identify the nature of the interaction taking place and the resulting changes in monocyte phenotype. A representative matrix component (fibronectin) was used to conduct blocking studies to examine the extent to which binding to matrix modifies the secretory behavior of monocytes.

Secondly to address the extent of monocyte differentiation into macrophages upon exposure to matrix, three macrophage specific markers were studied: a) the peroxisomal proliferator-activated receptor-γ (PPAR–γ), a nuclear receptor that acts as a transcriptional mediator for genes involved in lipid metabolism and adipogenesis

((Moore, Rosen et al. 2001), b) CD36, a class B scavenger receptor and c) Scavenger receptor class-A.

Finally since LDL accumulation in the mesangium may contribute to glomerular injury, the interaction between this lipoprotein and the mesangial matrix was examined.

CHAPTER 2

GENERAL METHODS

2.1. Human Mesangial Cell Culture

Techniques used for the isolation and maintenance of mesangial cells in vitro have been refined since these cells were first cultured in vitro in the late 1970's. The two methods for mesangial cell culture used are enzymatic isolation (Striker and Striker 1985) and explantation (Kreisberg and Karnovsky 1983; Striker, Lange et al. 1987). The starting material used for both methods is glomeruli isolated by differential sieving techniques.

Enzymatic isolation uses collagenases to partially digest away the glomeruli, thus exposing the glomerular 'cores' comprising capillary loops and mesangium depleted of endothelial cells and the majority of epithelial cells. These cores are then explanted in to plastic cell culture flasks, and give rise to a heterogeneous outgrowth of cells within 2-4 days.

The choice of a culture medium with a high serum concentration (10-20%) promotes growth of mesangial cells rather than endothelial or glomerular epithelial cells, which require lower serum concentrations. The fact that mesangial cells attach more readily to plastic than endothelial and epithelial cells also aids in their purification. Thus within 2-3 passages, homogenous mesangial cell cultures are obtained.

The alternative method of mesangial cell isolation (the explantation method), involves plating of undigested glomeruli into plastic flasks and use of high concentrations of foetal calf serum (FCS). Due to the different growth rates of intrinsic glomerular cell, the timing of subculture is used to select the cell type of interest. Both the enzymatic isolation and explantation methods provide reproducible and reliable

mesangial cell cultures, although cells have to be subcultured several times before sufficient numbers can be obtained for use in experiments. These cells retain many of the morphological and functional characteristics of mesangial cells *in vivo* (Lee 1995).

2.1.1. Materials

1) RPMI 1640 (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) ITS (insulin-human transferrin-sodium selenite) (Sigma, Dorset, UK)

7) Trypsin, ethylene diamine tetra acetic acid (EDTA) (0.025% and 0.01% respectively)

8) 75 cm^2 and 25 cm^2 conical flasks (Falcon, UK)

9) Sterile stainless steel sieves with the following mesh sizes: 425µm, 180µm and 125µm.

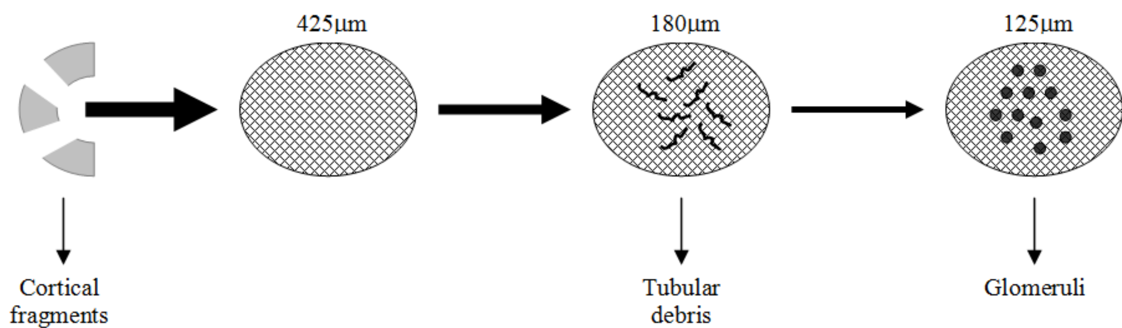
10) Glass syringe plunger (20ml)

11) Collagenase (Type 1A) (Sigma, Dorset, UK)

2.1.2. Method for Human Mesangial Cell (HMC) culture

Human cadaver kidneys that could not be used for transplantation for technical reasons were used as a source of cultured cells with permission from the United Kingdom Transplant Sharing Scheme (UKTS). Under sterile conditions, the capsule was removed and the cortex excised from the underlying medulla. Cortical fragments were minced to a pulp and pushed through a 425μm mesh stainless steel sieve using a plastic syringe (20ml) plunger. The material on the underside of the sieve was washed through a 180μm sieve and the glomeruli retained on the top surface of a third sieve with a mesh diameter of 125μm (Figure 2.1).

Figure 2.1. Separation of Human Glomeruli by Differential Sieving



These glomeruli were collected by aspiration, pelleted by centrifugation at 1000g, placed in collagenase (type 1A) solution (400-600 units/ml) and digested for 20 minutes at 37°C. The digestion was arrested by adding medium containing 20% FCS, the

glomeruli were harvested by centrifugation and plated on $25cm^2$ or $75cm^2$ Falcon tissue culture flasks depending upon the number of glomeruli obtained.

2.1.3. Media and growth condition

Growth medium consisted of 80% RPMI-1640 (Gibco BRL, Paisley, UK) and 20% FCS (Life Technologies, Paisley, UK), supplemented with insulin (5μg/ml) human transferrin (5μg/ml), and sodium selenite (5ng/ml) (Insulin-Transferrin-Sodium selenite media supplement, Sigma). Benzyl penicillin (100 units/ml), and Streptomycin sulphate (50 μg/ml) were added to minimise the risks of infection.

2.1.4. Passaging cells

Primary cultures were left undisturbed for 9 days after which time the growth medium was changed. Glomeruli attached to the plastic within 3-4 days and after an initial outgrowth of epithelial cells (day 7-14), human mesangial cells began to predominate and outgrew the epithelial cells within 3 weeks of plating (Fernando, Varghese et al. 1993). Cells were subcultured when they reached confluence. Growth medium was removed and cells washed with PBS. Trypsin and ethylene diamine tetra acetic acid (EDTA), (0.025% and 0.01% respectively dissolved in sterile PBS, Life Technologies) was then added and the cells incubated for 3-5 minutes at 37^oC. Cell detachment was assessed by phase-contrast microscopy and enhanced by vigorous

agitation. The enzymatic action of trypsin was arrested by adding growth medium; next the detached cells were pelleted by centrifugation at 1000g for 10 minutes. The cell pellet was then re-suspended in fresh growth medium. The cells detached from one $25cm^2$ flask were placed in two new flasks of the same size at a plating density of 1-5 x 10^6 cells/ml. Medium was changed every 3-4 days and subsequent passages carried out at 7-14 day intervals when confluence was reached. Mesangial cells from passages 2-10 were used in characterisation studies and for the experiments described in the following chapters.

2.2. MESANGIAL CELL MATRIX ISOLATION

Mesangial cells were grown to approximately 90% confluence, washed 3 times with RPMI medium then growth arrested in serum-free RPMI medium for 48 hours. The cell layer was removed by addition of 2.5 mM NH_4OH and 0.1% Triton X-100 for 3 minutes, leaving behind cell matrix (Weiss and Regiani 1984). This matrix layer was then washed 3 times with PBS before commencing adhesion experiments.

For experiments requiring solubilised matrix, the isolated matrix layer described above was collected by mechanical scraping and sonicated for 30 seconds using an ultrasonic probe to apply ultrasound energy.

Protein concentration of matrix was carried out using a modified Lowry method (Lowry OH 1951), the matrix was then re-suspended in RPMI medium at concentrations of 10, 50, 100 and 500 μg/ml. Matrix was isolated from mesangial cells derived from 4 different glomerular preparations and used on the day of isolation. The matrix contained

very low concentrations of TGF-β (<0.05 pg/μg), and virtually undetectable amounts of TNF-α (<0.02 pg/μg), IL-1β (<0.003 pg/μg) and IL-6 (<0.003 pg/μg) as measured by enzyme-linked immunosorbent assay (Felisaz, Boumediene et al.) (R&D Systems, Abingdon, Oxon, UK) according to the manufacturer's instructions. Individual matrix proteins; collagen type IV, fibronectin and laminin were sourced from Sigma (Sigma Chemical Co, Poole, Dorset, UK). All reagents and materials including matrix and buffers were tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Sigma) and proved negative

2.3. Immunohistochemical Labelling

These studies were designed to confirm that matrix preparations were free of mesangial cells and contained fibronectin. Human mesangial cells were grown on glass cover slips, then removed from the underlying matrix as described in section 2.2. The remaining matrix was then fixed with 100% ethanol and exposed to a mouse monoclonal anti-human fibronectin antibody for 30 minutes at room temperature. Cells and matrix were then exposed to a bridging rabbit anti-mouse antibody for one hour, followed by an alkaline phosphatase-conjugated mouse anti-alkaline phosphatase complex. After rinsing, the coverslips were developed using fast red for 5 mins and counter-stained for nuclei with Mayer's acid alum haematoxylin.

2.4. FIBRONECTIN ASSAY

The synthesis of fibronectin by mesangial cells was measured by ELISA (Burton, Combe et al. 1996). After washing, the cell layer was solubilized overnight in wash buffer (0.3M NaCl, 0.1% Triton X-100 in PBS) containing 1% P40.

The standards and appropriately diluted cell layer extracts were then incubated overnight in wells that had been pre-coated with a rabbit polyclonal anti-human fibronectin antibody (1:1000, Sigma). This was followed by the addition of a mouse monoclonal anti-human fibronectin (1:500, Sigma) and a horseradish peroxidase-conjugated anti-mouse antibody (1:1000, Dako) for 2 hours each.

Plates were then developed using a phenylenediamine substrate. After the color had appeared, the reaction was stopped by adding 1M sulphuric acid and the absorbance recorded at 492 nm. All fibronectin concentrations were measured as ng/ml and then expressed as % of control (growth arrested cells).

2.5. CULTURE OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

2.5.1. Materials

1) RPMI 1640 with L-glutamine containing 25mm HEPES

2) 10% FCS (fibronectin free)

3) 1% L-glutamine (200mM)

4) 1% Penicillin-streptomycin solution (10,000 units and 10,000 μg/ml respectively)

5) Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden

6) 0.15 mmol/L sterile NaCl solution

7) 5 mmol/L sterile EDTA

2.5.2. Methods of peripheral human blood mononuclear cell culture

Peripheral blood was collected into lithium-heparin tubes and diluted with an equal volume of sterile 0.15 mol/L NaCl solution. The diluted blood (30ml) was layered over ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) (15ml) and centrifuged at 900 g for 30 min at room temperature. The mononuclear cells were harvested and washed twice with 20 ml of sterile 0.15 mol/L NaCl solution. The cells were re-suspended at 2 x 10^6 cells/ml in supplemented RPMI 1640 medium, containing 10% fibronectin free foetal calf serum, L-glutamine and penicillin-streptomycin. An adherent step was then performed to separate the monocytes, which are the adherent cells, from the lymphocytes which are the non-adherent cells (Ackerman and Douglas 1978). This involved plating aliquots (1 ml) of the mononuclear suspension into 35 x 10 mm tissue culture dishes. The dishes were incubated at 37°C for 2 hours in a humidified incubator at 37°C, 5% CO_2. Non-adherent cells were removed by washing four times with 2 ml of sterile 0.15 mol/L NaCl solution. The adherent cells were then detached by incubating the dishes with sterile 0.15 mol/L NaCl solution containing 5 mmol/L EDTA for 45 min at 4°C whilst gently agitating. The dishes were again washed three times with 2 ml sterile 0.15 mol/L NaCl solution. The suspension of previously adherent cells was centrifuged (600 g for 5 min at 4°C) and the supernatant was discarded.

The cells were re-suspended in 2 ml of RPMI medium, counted three times using a haemocytometer and the mean value was determined. Cell viability was shown to be between 90 and 95% when assessed by cellular exclusion of trypan blue under phase-contrast microscopy.

For experiments the cells were re-suspended at 1.5 x 10^6 cells/ml in supplemented RPMI medium with 5% fibronectin-free homologous serum. All reagents and materials used in the experiment had been tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Sigma) and proved negative.

2.6. CULTURE OF THE HUMAN MONOCYTE CELL LINE THP-1

The THP-1 cell line was originally derived from the peripheral blood of a one year old male with acute monocytic leukaemia (Tsuchiya, Yamabe et al. 1980). These cells were sourced from ATCC (Middlesex, UK). The cells are grown in continuous suspension in the medium described below and can be differentiated into macrophages by exposure to phorbol esters (Tsuchiya, Yamabe et al. 1980; Tsuchiya, Kobayashi et al. 1982).

2.6.1. Materials

Medium A

1) RPMI 1640 with L-glutamine containing 25mm HEPES

2) 10% FCS

3) 1% L-glutamine (200mM)

4) 1% Penicillin-streptomycin solution (10,000 units and 10,000 μg/ml respectively)

5) 40 nmol/ml mercaptoethanol

Medium B

1) RPMI 1640 with L-glutamine containing 25mm HEPES

2) 10% FCS

3) 1% L-glutamine (200mM)

4) 1% Penicillin-streptomycin solution (10,000 units and 10,000 μg/ml respectively)

5) 40 nmol/ml mercaptoethanol

6) 125 nmol/ml phorbol ester myristate acetate (Chinetti, Griglio et al.)

2.6.2. Method of human monocyte THP-1 cell culture

Cells were grown to a concentration of 2-4 x 10^6 cells/ml during a 7 day growth period and were then harvested by centrifugation for 10 min at 400 g and re-suspended in medium A to give a concentration of 1 x 10^6 cell/ml.

To differentiate THP-1 monocytes into macrophages, cells were re-suspending in medium B at a concentration of 5.25 x 10^5 cells/ml and 1 ml of this suspension placed into each well of a 12-well plate. The medium was then replaced with 1ml/well of medium B every 2 days and the plates incubated in a humidified incubator at 37°C, 5% CO_2 for 5 days. For experiments the cells were re-suspended at 1.5 x 10^6 cells/ml in

supplemented RPMI medium with 5% fibronectin-free homologous serum. All reagents and materials used in the experiments including matrix and buffers had been tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Sigma) and proved negative.

2.7. Culture Of The Human Monocyte Cell Line U937

Human myelomonocytic leukaemia cells of the U-937 cell line (European Collection of Cell Culture, Salisbury, UK) were grown in supplemented RPMI 1640 medium as above. Cultures were expanded by seeding approximately 2 x 10^6 cells into 15 ml 20% foetal calf serum supplemented RPMI medium in T75 culture flasks (Falcon Scientific supplies, London, UK) and medium changed every 4 days. For experiments, cells were centrifuged at 180g for five minutes. The monocyte pellet was rinsed twice in RPMI medium. For experiments the cells were re-suspended at 1.5 x 10^6 cells/ml in supplemented RPMI medium with 5% fibronectin-free homologous serum. All reagents and materials used in the experiments including matrix and buffers had been tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Sigma) and proved negative.

2.8. Flow Cytometry Analysis of Monocytes

Monocyte associated cell surface markers, CD 69 and late HLA-DR expression were assessed on peripheral blood and U-937 monocytes. A change in the expression of

these antigens would allow us to identify if the monocytes have become activated. Briefly, 200μl of monocyte cell suspension (1.0 x 10^6 cells/ml) was aliquoted and centrifuged at 400g for 5 minutes. The pellet was resuspended in 80μl of PBS/10% FCS/0.1% sodium azide. Then 10μl of anti-CD 14 antibody coupled to fluorescein isothiocyanate (FITC) (Becton Dickinson, Cowley, UK) together with 10μl of either anti-CD 69 antibody coupled to phycoerythrin (PE) (Becton Dickinson, Cowley, UK) or anti-HLA-DR PE antibody (Becton Dickinson, Cowley, UK) was added and samples incubated in the dark at room temperature for 30 minutes. Cells were then washed with PBS containing 1% BSA and fixed for flow cytometric analysis in equal amounts of PBS/10% FCS/0.1% NaN_3 and 2% paraformaldehyde solution. Data were acquired on a FACS 440-flow cytometer using an argon ion laser at 488nm. FITC fluorescence was assessed using a 530 + 15 nm filter and PE using a 575 + 15nm filter. Collected data were edited and analysed using in house software.

2.9. SCANNING ELECTRON MICROSCOPY

I thank Professor Alan Phillips at the Department of Paediatric Gastroenterology, Royal Free Hospital for the initial training and subsequent use of his departmental scanning electron microscope. Coverslips measuring 1cm in diameter were placed at the bottom of 24 well plates. Mesangial cells were grown to confluence within the wells and relevant wells were subsequently treated with Trypsin and ethylene diamine tetra acetic acid (EDTA), (0.025% and 0.01% respectively dissolved in sterile PBS, Life

Technologies) to expose the underlying matrix and incubated with human PBMCs for 48 hours in a humidified incubator at 37°C, 5% CO_2.

After the culture period, tissue specimens were washed three times with fresh culture medium to remove any non adherent cells, fixed in 3% phosphate-buffered glutaraldehyde, and post fixed in 1% aqueous osmium tetroxide. For scanning electron microscopy (SEM), specimens were taken through a graduated series of ethanol and critical point dried in liquid CO_2, using a Polaron E3000 critical point drying apparatus. Samples were then sputter coated with gold-palladium in a Polaron E5100 series II coating system and examined in a JOEL JSM-5300 scanning electron microscope. Scans of 5 random fields of view for each specimen were taken.

2.10. Monocyte Adhesion Assay

The binding of U-937 and peripheral blood-derived monocytes to mesangial matrix was determined using a colorimetric method as described by Mené et al (Mene, Fais et al. 1995). Mesangial cells plated at a density of 25,000 cells/well in 24 well plates were grown for 96 hours then growth arrested for 48 hours in serum free medium. To determine the impact of matrix accumulation, mesangial cells were prestimulated with TGF-β (10 ng/ml), TNFα (10 ng/ml) or a combination of both cytokines for 48 hours. After thorough washing, the cell layer was removed by adding 2.5 mM NH_4OH and 0.1% Triton X-100 for 10 minutes as described by Weiss and Regiani leaving behind the cell matrix which was also washed extensively (Weiss and Regiani 1984).

Monocytes (2 x 10^6 cells/well) were then incubated with the residual matrix produced by the pre-stimulated or non-stimulated mesangial cells for one hour at 37°C in serum free medium. Non-adhered monocytes were removed by washing. Adhered cells were fixed with 1.0% glutaraldehyde and stained with a crystal violet solution (0.1% (w/v). Cells were washed, solubilized overnight in Triton X-100 (1.0%) and absorbance readings recorded at 595nm. The results obtained were corrected by subtracting background staining of the underlying matrix. The corrected values were then taken to be proportional to the number of monocytes bound to matrix.

To confirm the role of fibronectin and specific monocyte integrins in the binding process, anti-human fibronectin (25 μg/ml, Sigma), anti-VLA-4 (10 μg/ml, AMS Biotechnology) and anti-VLA-5 (5 μg/ml, AMS Biotechnology) antibodies were used in blocking experiments. In each case, the optimal antibody concentrations were chosen on the basis of preliminary experiments using a range of dilutions. The anti-human fibronectin antibody was pre-incubated with matrix whilst either U937 or peripheral blood-derived monocytes were pre-exposed to the anti-integrin antibodies for one hour at room temperature prior to the adhesion assays.

2.11. CYTOKINE PRODUCTION

IL-1β, IL-6 and TNFα secreted by peripheral blood-derived monocytes following exposure to matrix and individual protein components were measured by ELISA (R&D Systems). Briefly monocytes (0.5 x 10^6 cells/ml) were added to 24 multiwell plates containing mesangial matrix or to empty plastic wells for 24 hours.

Alternatively, solubilized matrix, fibronectin, laminin and collagen IV were added to monocytes pre-plated in 24 well plates. For positive and negative controls, monocytes were incubated in plastic wells for 24 hours with or without lipopolysaccharide (LPS, 10 μg/ml) respectively. In each case, the resulting conditioned medium was collected and spun at 6500 rpm for 5 mins on a microfuge to remove non-adherent monocytes. Samples were stored at -80°C prior to the analysis. All reagents and materials used in the experiments including matrix, matrix components and buffers were tested for endotoxin contamination using a Limulus amebocyte lysate test kit (Sigma) and proved negative.

2.12. THYMIDINE INCORPORATION

The ability of mesangial cell matrix to induce proliferation of blood-derived and U-937 monocytes was assayed by measuring the incorporation of ^{3}H-thymidine. Briefly, ^{3}H-thymidine (3.6 Ci/ml) was added to peripheral-blood derived (0.25 x 10^{6} cells/ml) or U-937 monocytes (1.25 x 10^{6} cells/ml) plated on matrix or in empty plastic culture plates for up to 96 hours. At the end of the incubation period, the adherent and non-adherent cells were collected, washed with PBS and treated with 50% methanol containing 10% glacial acetic followed by 10% Trichloroacetic acid. After further washes, the cells were solubilized in 0.1 M NaOH containing 1% SDS and the radioactivity incorporated determined by liquid scintillation counting.

2.13. GELATINOLYTIC ACTIVITY

The secretion of gelatinolytic enzymes by peripheral blood-derived monocytes incubated with mesangial cell matrix and matrix proteins was determined by zymography as previously described (Martin, Davies et al. 1989). Briefly, mesangial cells grown in 24 well plates were removed from the underlying matrix and incubated with human monocytes (0.5 x 10^6 cells/ml) for 24 hours in the absence of foetal calf serum. The resulting conditioned medium was isolated, centrifuged and stored at –80°C for batch analysis.

The enzyme activity of MMP-9 was assessed by SDS-PAGE using a 7.5% gel, incorporating gelatin (1 mg/ml), under non-reducing conditions. Following electrophoresis the gel was washed with 2.5% Triton X-100 for 1 hour and incubated in 50 mM Tris HCl pH 7.6 containing $CaCl_2$ (10 mM) and Brij (0.05%) at 37°C overnight. The gel was then fixed and stained with Coomassie blue and enzymatic activity demonstrated by zones of lysis, analysed by comparison to controls and quantified by densitometry.

2.14. ANALYSIS OF TIMP I AND TIMP II

Concentrations of TIMP I and TIMP II secreted by blood-derived monocytes into conditioned medium were measured by ELISA (Baker, Tickle et al. 1994; Martin, Steadman et al. 1998). Standards of TIMP 1 (0.15 to 150 ng/ml) and TIMP II (0.15 to 300 ng/ml) were prepared using purified TIMP kindly supplied by Prof. T. E. Cawston

(Department of Medicine, University of Newcastle, UK). Antibodies to TIMP were kindly provided by Dr. A Docherty, Cell Tech, Slough, and Dr G Murphy, University of East Anglia, Norwich, U.K.

2.15. RNA ISOLATION

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

2.15.1. Materials

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

2) 0.75M sodium citrate (pH 7.0): 11.029g of citrate 3 Na (Sigma, Dorset, UK) was dissolved in dH_2O and reconstituted to 50ml. Next 0.1ml of DEPC was added and the mixture stirred for 2 hours, then autoclaved.

3) 10% Sarcosyl: 10g of n-lauroylsarosine sodium salt (Sigma, Dorset, UK) was dissolved in dH_2O and reconstituted to 100ml. Then 0.2ml of DEPC was added and the mixture was stirred for about 2 hours, then autoclaved.

4) Denaturing solution: 250g of guanidinium thiocyanate (GTC) (Sigma, Dorset, UK) (final concentration 4M) was mixed with 17.6ml of 0.75M sodium citrate (pH 7.0) (final concentration 25mmol/l), 26.4ml of 10% sarcosyl (final concentration 0.5%) and 293ml of RNase-free water. The mixture was stirred at 65°C until GTC was dissolved. Before use, 2-mercaptoethanol was added (Sigma, Dorset, UK) 0.36ml per 5ml solution.

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

6) 0.5M EDTA: 186.1g of disodium ethylene diamine tetraacetate (Sigma, Dorset, UK) was added to 800ml of dH_2O and stirred vigorously. The pH was adjusted to 8.0 with NaOH. The solution was dispensed into aliquots and sterilised by autoclaving.

7) 3M Sodium acetate (pH 5.2): 408.1g of sodium acetate was dissolved in dH_2O and pH adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 litre. The solution was dispensed into aliquots and sterilised by autoclaving.

8) Phenol: Chloroform:Isoamyl Alcohol (125:24.1, pH 4.7) (Sigma, Dorset, UK)

9) Isopropanol (Sigma, Dorset, UK)

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

2.15.2. Steps to avoid Ribonuclease contamination

Sterile disposable plastic ware was used for handling RNA. Non-disposable glassware and plastic ware were treated before use to ensure that they were RNase-free. Glassware was baked at $200^{o}C$ overnight. Plastic ware 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 treated by the addition of diethyl pyrocarbonate (DEPC) 0.1% overnight at room temperature, and then autoclaved for 30 minutes to remove any traces of DEPC. Tris buffers were prepared using containers which were designated for Tris only and which had been treated with DEPC and autoclaved.

2.15.3. Total RNA purification

Human mesangial cells were cultured in 75 cm^2 flasks. Before RNA extraction, cells were pelleted and washed once in ice-cold phosphate-buffered saline and lysed in ice-cold denature solution using 600µl per 1 flask (about $5x10^6$). Cell lysates were sheared eight times through a 21-gauge needle. Next, 60µl of 2M sodium acetate (pH 4.0) was added to the lysate in each flask and mixed thoroughly by inverting 4-5 times. One volume of phenol:chloroform:isoamyl alcohol was then added. The mixture was 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 $4^{o}C$. The aqueous phase was carefully transferred to a fresh RNase free tube, taking

care not to touch the interface. To this an equal volume of isopropanol was added and mixed, then precipitated by incubating at –20°C for 2 hours. The crude RNA pellet was recovered by centrifugation at 12,000 rpm for 20 minutes at 4°C and washed by resuspension in 1ml of 75% ice-cold ethanol.

The RNA was recovered by centrifugation at 12,000 rpm for 10 minutes at 4°C. The pellet was then dried in air and resuspended in 150µl RNase free water. 10µl of 3M sodium acetate (pH 5.2) was added and mixed thoroughly by inverting the tube 4-5 times. One volume (150µl) of phenol:chloroform:isoamylalcohol was added. The mixture was then vortexed for 10 seconds, then centrifuged for 10 minutes at 4°C. The aqueous phase was carefully transferred to a fresh RNase free tube, then an equal volume of ethanol (100%) was added, this was then mixed and precipitated by incubating at –20°C for 2 hours. The concentration of RNA was determined by measuring the absorbance at 260nm using a spectrophotometer.

2.16. RT-PCR

The RNA sample was reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. (Becker-Andre and Hahlbrock 1989).

2.16.1. Materials

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

1) 10x PCR buffer II: 500mmol/l KCl, 100mmol/l Tris/HCl

2) 25 mmol/l $MgCl_2$ solution

3) dNTPs: 10mmol/l deaoxyribonucleoside triphosphates

4) Random hexamers 50μmol/l

5) RNase inhibitor (20 Unit/ l)

6) M-MLV reverse transcriptase (50 Unit/ l)

7) *Taq* DNA polymerase (5 Unit/ l)

2.16.2. Method of RT-PCR

Total RNA (500ng) was used as a template for RT-PCR. The RT reaction was set up in a 20μl mixture containing 50mmol/l KCl, 10mmol/l Tris/HCl, 5mmol/l $MgCl_2$, 1 mmol/l of each of the dNTPs, 2.5μmol/l random hexamers, 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 10μl aliquots for separate amplification of

cDNA using specific primers. For PCR, the final concentrations of the PCR reaction mixture were 50mmol/l KCL, 10mmol/l Tris/HCl, 2mmol/l $MgCl_2$, 200μmol/l dNTPs, 0.125-0.25μmol/l of primers, 1.25U *Taq* DNA polymerase. After incubation at 145 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.

2.17. ANALYSIS OF PPAR-γ, CD36 AND SCAVENGER RECEPTOR-A GENE EXPRESSION USING RT-PCR

Based on previous experiments, monocytes were incubated in the presence of soluble matrix for various times up to 120 hours (5 days) at 37°C in a humidified atmosphere of 5% CO_2. PMA (125 nM) and cell culture grade bovine serum albumin (BSA, 500 μg/mL) (Sigma) served as positive and negative controls respectively. Cells were trypsinised and recovered by centrifugation. Total RNA (approximately 500 ng) was extracted from the cell pellet by a SDS/double phenol extraction method (Ruan, Varghese et al. 1999). The RNA was used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) and the resultant cDNA was amplified for PPAR-γ, CD36 and scavenger receptor-A by PCR, using β-actin as a control. The following primers were used: PPAR-γ upper primer 5'-GGC AAT TGA ATG TCG TGT CTG TGG AGA TAA 3' and PPAR-γ lower primer 5'-AGC TCC AGG GCT TGT AGC AGG TTG TCT TGA-3', CD36 upper primer 5' CAG CCT CAT TTC CAC CTT TTG TT and CD36 lower primer 5' GTT GAC CTG CAG CCG TTT TG, scavenger receptor-A upper primer 5' TCG CTC AAT GAC AGC TTT GC 3' and scavenger receptor-A lower

primer 5’ CCA TGT TGC TCA TGT GTT CC 3’, β-actin upper primer 5'-ATG GAT GAT GAT GAT ATC GCC GCG-3’ and β-actin lower primer 5’-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG GCC-3’ (Biogenesis, Poole, UK). Twenty microlitres of the PCR product was subjected to electrophoresis on a 2% agarose gel and bands visualised by staining with ethidium bromide under UV light. Gels were subjected to densitometric analysis using a Gel Doc 2000 scanner (Bio-Rad, Herts, UK), and band intensity normalised to β-actin to control for variations in loading then normalised to the appropriate control conditions (100%).

2.18. Analysis Of PPAR-γ Protein Expression Using Western Blot

Modulation of PPAR-γ in THP-1 monocytes in response to mesangial cell matrix was examined by Dr. Ravinder Chana using Western blot analysis. Monocytes seeded into 24 well plates at a density of 1.5 x 10^6 cells/well and incubated with matrix (500 μg/ml) for various times up to 48 hours. Supplemented RPMI media containing either PMA or BSA served as a positive and negative control respectively. After stimulation, cells were collected, washed twice with cold PBS and lysed in Laemmli buffer (60 mmol/L Tris, pH 6.8, 10% glycerol, 2% sodium deoxycholate, 100 mmol/L dithiothreitol and 0.01% bromophenol blue). Cell lysates were heated at 100°C for 5 minutes and subjected to 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Herts, UK). Proteins were transferred on to Protran nitrocellulose membranes (Schleicher and Schuell BioSciences GmbH, Dassel, Germany). PPAR-γ was detected using a monoclonal antibody (SC7273; SantaCruz Biotech, Cambridge, UK) at 1:1000 dilution

and a secondary horseradish peroxidase-linked anti-mouse IgG antibody (A4416; Sigma) at 1:2000. Bound antibodies were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Bucks, UK). For densitometric analysis, bands from Western blots were scanned and quantified using Scion Image version 4.0.2.

2.19. Modified Lowry Assay For Lipoprotein And Cell Membrane Protein Estimation

Protein concentration was estimated using a modified Lowry assay as described by Markwell. (Markwell, Haas et al. 1978).

2.19.1. Materials

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

2) Reagent A: consisting of 2% Na2CO3, 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) 1 N Folin-Ciocalteu solution (F-C) (Sigma Ltd. Dorset UK)

2.19.2. Lowry Assay

BSA stock was diluted in dH_2O to obtain standards as follows: 0, 5, 10, 20, 40, 60, 80 and 100µg/ml. Next 3ml of reagent C (solution A: solution B at 100:1) were added to 1 ml of sample containing 10-100µg of protein and incubated at room temp for 10 min. Next 0.3 ml of 1 N F-C reagent was added and vortex mixed then incubated for 45 min at room temperature. Finally, absorbance was read at 660nm using a spectrophotometer and the content of sample protein calculated using the BSA standard curve.

2.20. Preparation Of Human Low Density Lipoprotein (LDL)

LDL was isolated from plasma by sequential ultracentrifugation as described by Havel (Havel, Eder et al. 1955).

2.20.1. Materials

1) 0.2M disodium EDTA (pH 7.4) (Merck Ltd. Lutterworth, Leics, UK)

2) 2.5% sodium azide (Sigma, Dorset, UK)

3) Benzyl penicillin 600 U/ml

4) Streptomycin sulphate 100 mg/ml

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

6) 0.3M Sodium chloride (Merck Ltd. Lutterworth, Leics, UK)

7) Sodium bromide (Merck Ltd. Lutterworth, Leics, UK)

8) Visking tubing

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

2.20.2. LDL isolation

4ml of 0.2M disodium EDTA was mixed with 1ml of 2.5% sodium azide, 0.125ml of benzyl penicillin (600 U/ml), 0.125ml streptomycin sulphate (100mg/ml), 0.125ml of kallikrein inactivator aprotinin (200 U/ml) and 7ml of 0.3M sodium chloride. 1ml of this preservative solution was added to universal tubes to prevent enzymatic degradation of lipoprotein particles.

Venous blood (180ml) was then collected from normo-lipidaemic healthy volunteers who had undergone an overnight fast (20ml of blood was added into each universal container which had been pre filled with 1ml of the preservative solution). The plasma was separated by centrifugation at 3000g for 10 minutes. LDL (density range 1.019-1.063g/ml) was isolated by ultracentrifugation in a Beckman L8-55 M 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 1.019g/ml using formula 1 to calculate the amount of NaBr to be added and centrifuged at 40000rpm for 20 hours at 4°C to remove chylomicrons (CMs), very low-density lipoprotein (VLDL) and intermediate density lipoprotein (IDL).

Formula 1

$$\text{Amount of NaBr (g) to be added} = \frac{vi \quad (df - di)}{1 - [V \times df]}$$

vi	The initial volume
d*i*	The initial density of plasma
d*f*	Final density required
V	Partial specific volume of NaBr (0.235)

Once the tubes had been removed from the rotor, they were placed on ice, the tube caps removed and the supernatant containing the VLDL and IDL aspirated and discarded using a 19 gauge needle attached to a 10ml syringe. The infranatants were pooled, mixed thoroughly and adjusted to a density of 1.063g/ml by adding NaBr using formula 1. The tubes were then re-capped and centrifuged at 40000rpm at 4°C for a further 20 hours to obtain LDL, which could be visualised as an orange layer at the top of the tube. LDL was harvested using a 19 gauge needle attached to a 10ml syringe, concentrated and purified by centrifugation at 40000rpm at 4°C for a further 20 hours. The 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 hours, changing dialysate twice. The EDTA acts as a chelating agent to sequester metal ions. Twenty four hours prior to the start of an oxidation experiment, a portion of the

LDL was re-dialysed as described above but omitting the EDTA. LDL protein concentration was measured using a Lowry assay as modified by Markwell et al (see above) (Markwell, Haas et al. 1978).

2.20.3. Agarose gel electrophoresis of lipoproteins

Native and oxidised LDL samples were separated by electrophoresis according to their net charge at pH 8.6 using low voltage electrophoresis (Noble 1968). Separated lipoproteins were stained using Fat Red 7B. The electrophoretic mobility of each sample was compared to that of a native LDL standard and the results expressed as relative electrophoretic mobility (REM).

2.20.3.1. Materials

1) Barbitone buffer (0.05 mol/L pH 8.6) Sodium barbital 8.85g/L, barbital 1.3g/L, sodium chloride 0.5g/L, sodium EDTA 0.35g/L. All chemicals are Analar grade (BDH Ltd, Dorset, UK)

2) Pre-prepared agarose gels (10g/L) catalogue number 470100 (Ciba-Corning, UK)

3) Fat Red 7B stain (Sigma, Dorset, UK)

4) Methanol (Rathburn chemicals, UK)

5) Distilled water

2.20.3.2. Preparation of reagents

Barbitone buffer (2L) was prepared, pH adjusted to 8.6 and stored at 4°C for up to 4 months. A stock solution of Fat Red 7B stain (0.225g/l) was prepared by stirring 0.045g of the stain in 100ml methanol in a conical flask for approximately 6 hours. Immediately prior to use, a working stain solution was prepared by adding 2ml of distilled water to 10ml of stain in a clean dry measuring cylinder. A de-stain was prepared by mixing methanol with water (2:1).

2.20.3.3. Agarose gel electrophoresis

The agarose film was gently peeled away from its backing plate and placed on a level surface. The wells were filled with approximately 0.8µl of sample or reference control. Both the anode and cathode chambers of the Ciba-Corning electrophoresis tank were filled with 100ml barbitone buffer and the gel placed in the holder and then lowered into the buffer. The sample origin was placed on the cathode side as the samples migrated towards the anode.

The electrophoresis chamber was plugged into the power pack and electrophorised at a voltage of 100mV with a current of 4mA for 40 minutes. The progress of the electrophoresis was followed by the movement of the tracking dye (bromophenol blue). When electrophoresis was complete, the gel was removed from the chamber and dried in an oven at 55°C for 20 minutes. The gel was then placed in a

staining tray and stained for 1-4 minutes with freshly prepared working stain, in order to visualise the bands. The gel was then de-stained in methanol:water (2:1 v/v) and the backing on the gel was wiped and dried in an oven for 20 minutes.

2.21. LDL ACETYLATION

LDL was acetylated by incubation with saturated sodium acetate solution at a ratio of 1:2 and stirred continuously for 30 minutes at 4°C. Aliquots of acetic anhydride (1.5 µl/mg LDL) were added to the mixture over 90 minutes. The Ac-LDL was then dialysed against PBS containing 0.01% EDTA, pH 7.4, using a PD10 Column (Amersham). Freshly isolated native LDL and Ac-LDL were then passed through a 0.2 µm filter and protein concentrations were measured using a modified Lowry method (Lowry OH 1951). Acetylation of LDL was confirmed by assessing the changes in mobility of modified lipoprotein using agarose gel electrophoresis.

2.22. LDL OXIDATION BY MESANGIAL CELL MATRIX

Relative electrophoretic mobility was used to assess oxidation of LDL by matrix (Wheeler, Chana et al. 1994). Matrix (500 µg/ml) and native LDL (250 µg/ml) were co-incubated in the absence of cells. As a positive control, native LDL (250 µg/ml) was incubated in the presence of $CuSO_4$ (10 µM). Native LDL co-incubated with BSA (500 µg/ml) and native LDL alone served as negative controls. The effect of the antioxidants EDTA (100 µM) and butylated hydroxytoluene (BHT, 20 µM) on matrix co-incubated

with LDL was also assessed. Following incubation for 24 hours at 37°C, 5% CO_2, the protein fraction was adjusted to 50 µg/ml with PBS and 5 µl of each sample was loaded on to a 0.5% agarose gel (Paragon Lipogel, Beckman, Austria) and subjected to electrophoresis. Bands were visualised by staining gels according to the manufacturer's instructions.

2.23. ANALYSIS OF SCAVENGER RECEPTOR-A ACTIVITY

Based on previous experiments, THP-1 monocytic cells (1.5 x 10^6 cells/ml) were resuspended in supplemented RPMI medium and incubated with 500 µg/ml of solubilized matrix or with 500 µg/ml BSA protein (negative protein control) or 125nm PMA for 48 hours at 37°C. Cells were then exposed for a further 3 hours to 10 µg/ml Ac-LDL labelled with 1,1'-dioctadecyl-3,3,3'3'-tetramethyllindocarbocynanine perchlorate (Dil-labelled Ac-LDL, Biogenesis, Poole, UK) in the presence or absence of an excess of unlabelled Ac-LDL (250 µg/ml) to confirm the specificity of receptor-mediated uptake. After incubation, the cells were recovered by centrifugation at 350 x g for 5 minutes, washed three times with PBS and fixed in 5% formalin solution in PBS. Ac-LDL binding and uptake was assessed by flow cytometry (EPICS XL-MCL; Beckman Coulter, Bucks, UK). Forward and side scatter gates were established to exclude dead cells and cell debris from the analysis. Fluorescence signals from the accumulated Dil associated with the cells were detected at 555-600 nm by a photomultiplier, and then converted to digital format and processed. The mean

fluorescence intensity (MFI) of 5 x 10^3 cells were analysed in each sample. Auto-fluorescence signals generated by unlabelled cells were used as negative controls in each experiment. The MFI of the Dil-labelled cells was calculated by subtracting the auto-fluorescence intensity from the observed MFI of labelled cells. Each experiment was carried out in duplicate, on four separate occasions with different preparations of cells and Ac-LDL. The average of the duplicate determinations was used for statistical analysis.

2.24. Morphological Examination Of Functional Scavenger Receptors

THP-1 monocytes were incubated in chamber slides (0.5 x 10^6 cells/ml) with solubilized matrix (500 μg/ml). PMA (125 nM) and BSA (500 μg/ml) served as positive and negative controls respectively. Polyinosinic acid (poly I) (100μg/ml) was also added as an inhibitor of the scavenger receptor. After 120 hours incubation at 37°C, cells were further incubated with 50 μg/ml Ac-LDL for 48 hours. Cells were then washed with PBS, fixed for 30 minutes with 5% formalin solution in PBS, stained with Oil Red O for 30 minutes and counter-stained with haematoxylin for another 5 minutes. Lipid inclusion was assessed by observing at least 8 fields under a light microscope.

2.25. Staining Of Human Kidney Biopsy Material For Macrophage Activation Markers

Ethical approval to use human kidney biopsy material was obtained. Sections of formalin-fixed paraffin embedded kidney tissue were dewaxed and treated with

hydrogen peroxide to block endogenous peroxidase. Four kidney samples from patients with non-inflammatory conditions (two with ischaemic nephropathy, one with thin membrane nephropathy and one with myoglobinuria) and five from patients with inflammatory diseases (three with pauci-immune necrotizing/vasculitic glomerulonephritis, one with anti-glomerular basement membrane antibody disease and one with lupus nephritis) were submitted to Mr. James Gaya who then conducted staining for macrophage activation markers. Sections were then heated in TRIS-EDTA buffer (pH 9.0) before being stained with either a mouse monoclonal antibody to CD68 diluted 1:200 (PG-M1 antibody, Dako, Cambridge UK), a mouse monoclonal antibody to PPARγ diluted 1:100 (Santa Cruz), or a goat antibody to scavenger receptor diluted 1:500 (Abcam, Cambridge UK). In the case of the anti-CD68 antibody, samples were pre-treated with trypsin for 10 minutes. After incubation with the first stage antibody for 1 hour, an Envision kit (Dako) was used for the second stage in the case of the mouse antibodies and a peroxidase-conjugated rabbit anti-sheep antibody in the case of the goat antibody. Hydrogen peroxide and diaminobenzidine were used as substrates and sections were counterstained with haematoxylin.

2.26. DATA ANALYSIS

Groups of data in all experiments were evaluated for significance using a Mann-Whitney unpaired non-parametric two tailed test or where indicated by paired student's t-test. Results are expressed as mean + SEM unless otherwise stated and $p < 0.05$ was considered significant.

CHAPTER 3

MONOCYTE ADHESION TO MESANGIAL MATRIX MODULATES CYTOKINE AND METALLOPROTEINASE PRODUCTION

3.1. INTRODUCTION

Blocking signalling pathways that determine monocyte/macrophage function represents an attractive target for therapeutic intervention. Such strategies have been evaluated in animal models of acute glomerulonephritis. For example monoclonal antibodies directed against leukocyte functional associated molecule-1 (LFA-1) and its endothelial ligand, intercellular adhesion molecule-1 (ICAM-1) were found to block monocyte trafficking and inhibit glomerular injury in rats given nephrotoxic serum (Kawasaki, Yaoita et al. 1993). In the same model, blocking antibodies directed against another monocyte integrin, very late antigen-4 (VLA-4), also attenuated renal injury but without affecting the number of infiltrating cells, whilst blockade of vascular cell adhesion molecule-1 (VCAM-1), a major ligand of VLA-4, had no effect (Allen, McHale et al. 1999). These results suggest that binding of VLA-4 to a ligand other than VCAM-1 within the glomerulus may be important in modulating monocyte function in the context of glomerular injury. Previous studies by our group demonstrated that adhesion of monocytes to activated mesangial cells was mediated, at least in part, by cell-associated fibronectin (Chana and Wheeler 1999). It was postulated that this matrix component may be an important VLA-4 ligand. The following experiments were designed to test the hypothesis that interactions with mesangial matrix modulates the secretory function of monocyte/macrophages. The results indicate that matrix may promote mononuclear cell accumulation within the mesangium, and that interactions between monocytes (in part mediated by VLA-4) and matrix components (particularly fibronectin) promote secretion of monocyte products that may modify disease outcome.

3.3. RESULTS

3.3.1. Matrix composition

Staining studies demonstrated that after solubilization of mesangial cells, no nuclear material remained and that fibronectin was present in the residual matrix. Electron microscopy confirmed an absence of cellular debris (Figure 3.1A). In keeping with previous published work, pre-stimulation of mesangial cells with TGF-β alone and in combination with TNFα led to increased fibronectin production (Pawluczyk and Harris 1998). At a concentration of 10 ng/ml, exposure to TGF-β increased cell-associated fibronectin to (mean ± SEM)) 127.8% ± 10.1% ($p < 0.05$) compared to control cells grown in serum-free medium (100%). The cell-associated fibronectin was further increased to 256.7% (3.9%, $p < 0.001$) when a combination of 10 ng/ml of TNFα and 10 ng/ml TGF-β was added suggesting that these cytokines had a synergistic effect on matrix synthesis. TNFα alone had little effect on cell-associated fibronectin production (114.5% + 4.1%, p = NS). When matrix is assessed for cytokine entrapment, it was found to contain very low concentrations of TGF-β (<0.05 pg/μg), and virtually undetectable amounts of TNFα (<0.02 pg/μg), IL-1β (<0.003 pg/μg) and IL-6 (<0.003 pg/μg).

3.3.2. Monocyte characteristics

U937 and peripheral blood-derived monocytes recovered after 24 hours incubation on tissue culture plastic plates possessed both early (CD 69) and late (HLA-DR) monocyte surface markers. There was no change in the expression of either cell marker, even after the cells were cultured on matrix or plastic for up to 72 hours.

3.3.3. Adherence of monocytes to mesangial cell matrix

Both U-937 and peripheral blood-derived monocytes adhered strongly to matrix synthesized by mesangial cells grown under standard conditions, following the removal of the mesangial cells (Figure 3.1B). Binding occurred predominantly to the matrix itself, with minimal adhesion to the uncoated plastic plate determined by subtracting background staining to plastic alone. Using U937 cells it was demonstrated that pre-stimulation of mesangial cells with TGF-β and TNFα led to increased monocyte binding to the residual matrix (Figure 3.2). Compared to control matrix (i.e. that produced by mesangial cells exposed to serum free medium alone, 100% binding), binding of U937 cells to matrix synthesized by cells pre-stimulated with TGF-β and TNFα was increased by 127.5% ± 17.7%, ($p < 0.05$) and 123.6% ± 9.7% ($p < 0.05$) respectively. When a combination of the two cytokines was used, binding increased to 188.0% ± 5.5% ($p < 0.001$) compared to control matrix.

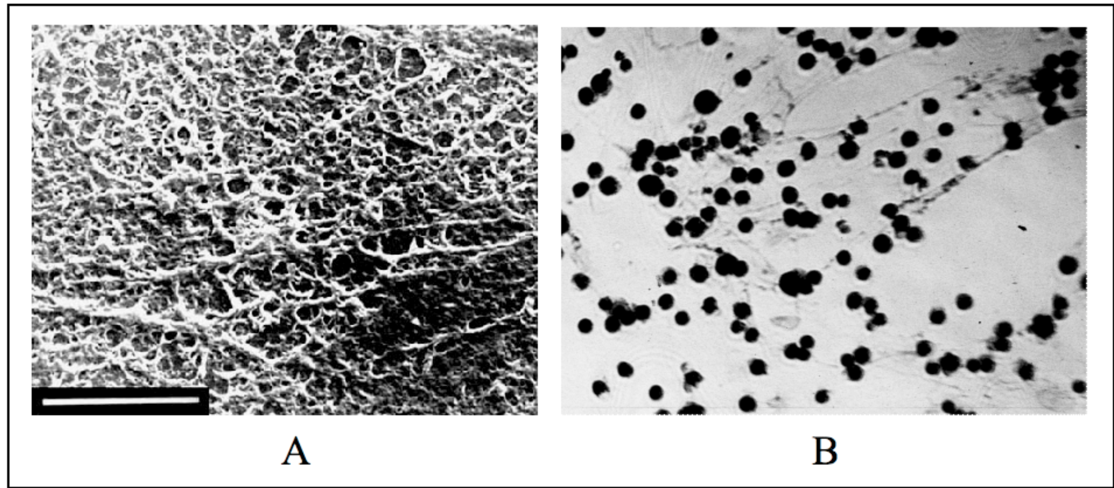


Figure 3.1.

(A) Electron micrograph of mesangial matrix. The cell layer was removed from the underlying matrix by adding 2.5mM NH_4OH and 0.1% Triton X-100. The matrix was then fixed and prepared for electron microscopy as described in the methods section.

Bar = 1 μm, original magnification X 5000.

(B) U-937 monocytes adherent to cell matrix. U-937 monocytes were incubated with matrix for one hour. After washing, adherent monocytes were fixed and stained with crystal violet as described in the methods section and visualised under a light microscope. Original magnification X 100.

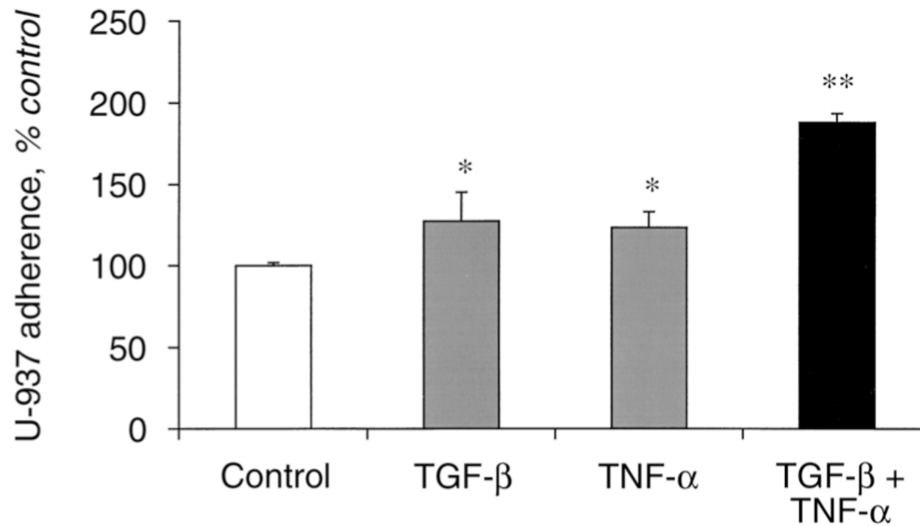


Figure 3.2. Adhesion of U-937 monocytes to matrix synthesized by mesangial cells prestimulated with TGF-β, TNFα and TGF-β/TNFα. Monocytes were incubated for one hour with matrix after removal of mesangial cells. Adherent monocytes were fixed, stained, solubilized and quantified by colorimetry. The mean (SEM) of triplicate absorbance readings, corrected for background staining of matrix, is expressed as a percentage of control (matrix arising from unstimulated cells). The results are representative of those obtained in 4 experiments. * $p < 0.05$, ** $p < 0.001$ vs. control.

3.3.4. Inhibition of monocyte adhesion to matrix

Antibodies directed against VLA-4 and VLA-5 integrins reduced U937 monocyte binding to mesangial matrix when compared to control (no antibody, 100%). Using non-prestimulated mesangial cells, anti-VLA-5 antibody reduced monocyte binding to 78.4% ± 6.0%, ($p < 0.005$) (Figure 3.3A) when compared to control matrix (no antibody, 100%). In contrast, binding to matrix synthesized by TGF-β/TNFα pre-stimulated mesangial cells was reduced by anti-VLA-5 antibody to 44.8% ± 2.5% ($p < 0.0001$) (Figure 3.3B). Once again, a reduction in binding was observed when matrix from non-prestimulated mesangial cells was primed with anti-VLA-4 antibody, but not with anti-fibronectin antibody (Figure 3.3A). Anti-VLA-4 and anti-fibronectin antibodies also reduced monocyte adhesion to matrix produced by TGF-β/TNFα pre-stimulated mesangial cells to 54.9% ± 4.9% ($p < 0.0001$) and 56.6% ± 5.6% ($p < 0.0001$), respectively, compared to control (Figure 3.3B). In a comparative experiment, binding of peripheral blood-derived monocytes to mesangial matrix synthesised by non-prestimulated mesangial cells was inhibited by 61.5 ± 4.3% and 58.5 ± 3.6% by anti-VLA-4 and anti-VLA-5 antibodies respectively.

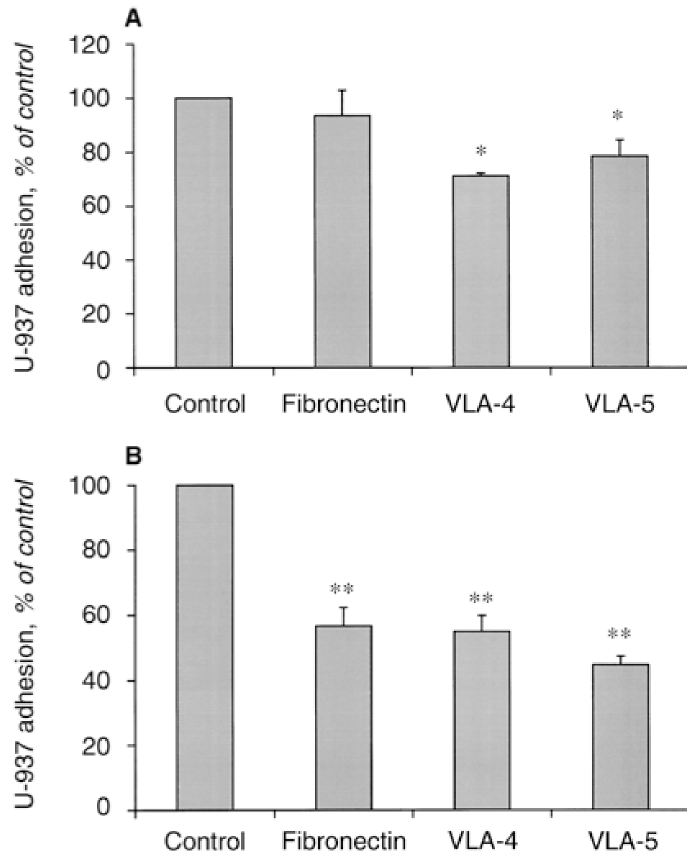


Figure 3.3. Inhibition of U-937 monocyte adhesion to matrix produced by unstimulated mesangial cells (A) or mesangial cells prestimulated with TGF-β/TNFα (B). The matrix was pre-incubated with anti-fibronectin (25 μg/ml) antibody for 2 hours before a suspension of U-937 monocytes was added. Alternatively monocytes were pre-incubated with anti-VLA-4 (10 μg/ml) and anti-VLA-5 (5 μg/ml) antibodies for 1 hour before being added to matrix. After washing, adherent monocytes were fixed, stained with crystal violet, solubilized and quantified by densitometry. The results are expressed as mean (SEM) absorbance, corrected for the background staining of matrix, and expressed as a percentage of control (100%). These results are a representative of 4 experiments; each conducted in triplicate at optimal antibody concentrations. * $p < 0.0001$ vs. control.

3.3.5. Adhesion to mesangial matrix stimulates monocyte cytokine secretion

Adhesion of peripheral blood-derived monocytes to mesangial matrix led to an increase in the production of IL-1β by 14-fold when compared to control monocytes incubated in plastic wells (7.74 + 1.25 pg/ml vs. 0.55 + 0.23 pg/ml, $p < 0.001$) (Figure 3.4). In contrast, there was a 226-fold increase following LPS stimulation (124.7 + 15.26 pg/ml, $p < 0.001$). Similar results were obtained when the production of IL-6 and TNFα was examined. There were approximately 37-fold and 6-fold increases in IL-6 and TNFα production by monocytes bound to matrix when compared to control cells incubated in plastic wells. These increases in cytokine levels cannot be attributed to the change in monocyte cell number. Proliferation studies using ^{3}H-thymidine showed similar radiolabel incorporation into U-937 and peripheral blood-derived monocytes incubated on either matrix or plastic culture plates.

Like whole matrix, individual matrix proteins stimulated cytokine production but differed in terms of the magnitude of this effect. For example, there was approximately a 19-fold increase in IL-1β when peripheral blood-derived monocytes were incubated with fibronectin (100μg/ml, $p < 0.005$) when compared to control (monocytes incubated in plastic wells) (Figure 3.5A). In contrast, collagen type IV and laminin increased cytokine production by approximately 14- and 5-fold respectively. This compared to a 9-fold increase when solubilized whole matrix was added at the same concentration. These proteins also stimulated monocyte IL-6 and TNFα secretion (Figure 3.5B and 3.5C) with a similar pattern of response.

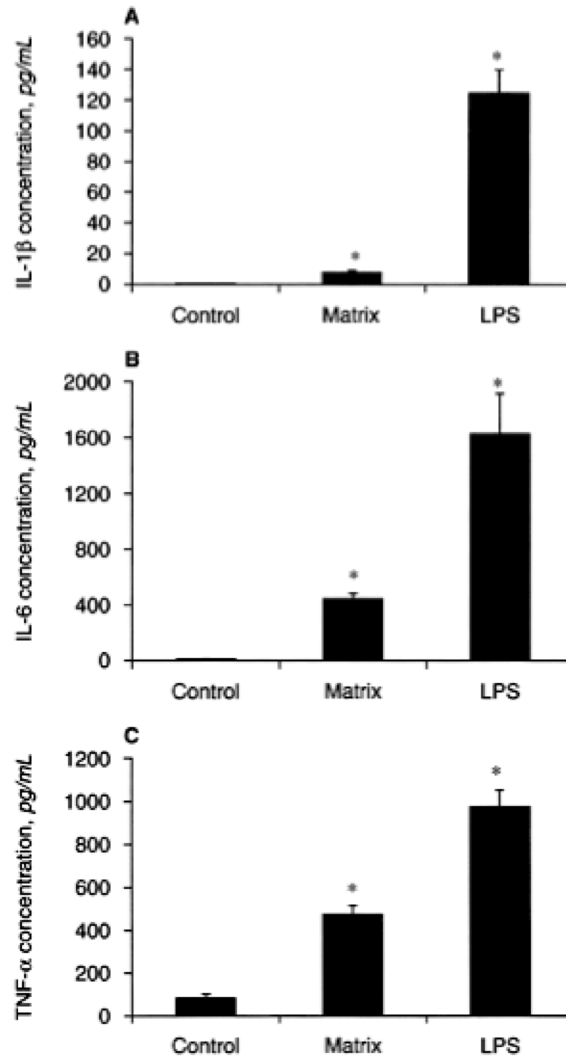


Figure 3.4. IL-1β (A), IL-6 (B) and TNFα (C) production by peripheral blood-derived monocytes cultured in the presence of mesangial cell matrix. Peripheral blood-derived monocytes (0.5 x 10^6 cells/ml) were incubated with mesangial cell matrix for 24 hours. For positive and negative controls, monocytes were incubated in plastic wells with or without 10 μg/ml LPS respectively. The resulting conditioned medium was collected, spun and cytokines measured by ELISA. Results are mean + SEM of 4 experiments, each performed in quadruplicate. * $p < 0.001$ vs. control.

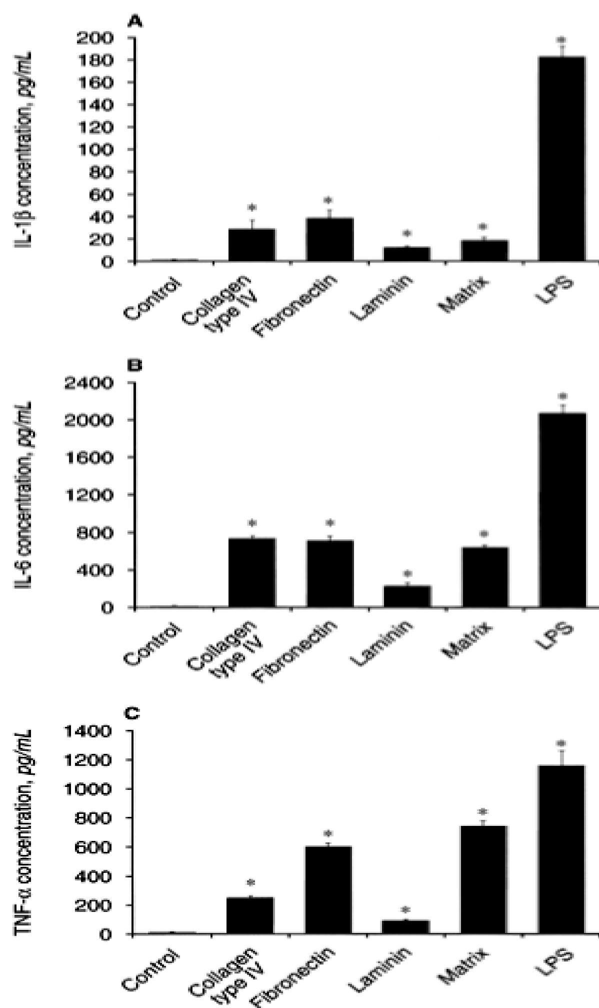


Figure 3.5. IL-1β (A) and IL-6 (B) and TNFα (C) production by monocytes cultured in the presence of soluble matrix and its protein components. PBMCs (0.5 x 10^6 cells/well) were incubated with solubilized mesangial cell matrix (100 μg/well) or its protein components (100 μg/well) for 24 hours. For positive and negative controls, monocytes were incubated in plastic wells with or without 10 μg/ml LPS respectively. The resulting conditioned medium was collected, spun and cytokines measured by ELISA. Results (mean + SD) are representative of 4 experiments, each performed in quadruplicate. *p <0.005 vs. control.

3.3.6. Effects of antibodies on cytokine production

The secretion of cytokines by peripheral blood-derived monocytes was mimicked by incubation with anti-integrin antibodies. For example, incubation of monocytes in plastic culture plates with anti-VLA-4 (10 μg/ml) and anti-VLA-5 (5 μg/ml) antibodies increased IL-1β production compared to control monocytes in medium alone (Table 3.1). Similarly, TNFα secretion was increased by both antibodies when compared to control.

Table 3.1. Production of cytokines by human macrophages exposed to anti-VLA-4 (10 μg/ml) and anti-VLA-5 (5 μg/ml) antibodies for 24 hours.

Antibody	IL-1β (pg/ml)	p value	TNFα (pg/ml)	p value
Control	2.0 + 2.4	-	64.2 + 6.0	-
anti-VLA-4	45.4 + 12.0	<0.005	2649.6 + 424.2	<0.0001
anti-VLA-5	93.3 + 13.5	<0.0001 <0.05*	3551.7 + 458.4	<0.0001 <0.005*

Cytokines were measured in the conditioned medium by ELISA. Results are mean +SEM of 4 experiments, each performed in triplicate or quadruplicate. Statistical analysis was performed using a paired student's t-test. p vs. control. *p vs. anti VLA-4.

3.3.7. Mesangial cell matrix stimulation of MMP release

Incubation with mesangial matrix resulted in increased peripheral blood-derived monocyte MMP-9 secretion (Figure 3.6A). Densitometric analysis showed a significant 2.4-fold rise in MMP-9 activity compared to control (Figure 3.6B). In contrast, matrix had no effect on the release of monocyte MMP-2 (data not shown). Addition of matrix proteins also led to increased activity of monocyte MMP-9. The response to fibronectin was dose-dependent (Figure 3.7).

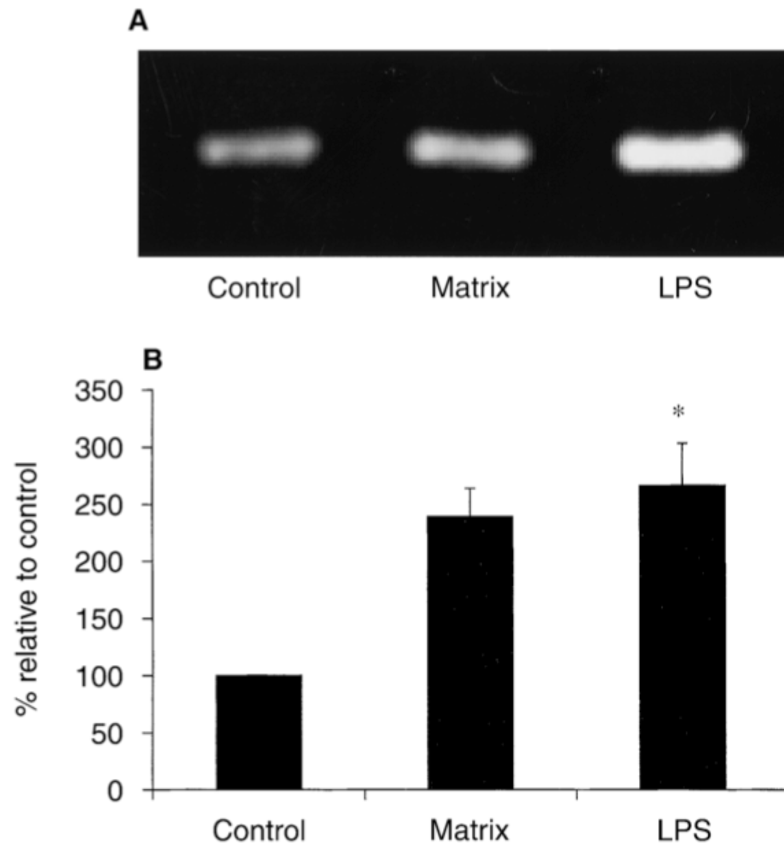


Figure 3.6. Matrix metalloproteinase (MMP) released by monocytes cultured in the presence of mesangial cell matrix (A). Peripheral blood-derived monocytes (0.5 x 10^6 cells/ml) were incubated with mesangial cell matrix for 24 hours in the absence of foetal calf serum. For controls, monocytes were incubated in plastic wells with or without 10 μg/ml LPS. The resulting conditioned medium was collected, spun and analyzed by zymography. Lane 1, control; Lane 2, cell matrix; Lane 3, LPS. Results are representative of 5 experiments. Densitometric evaluation of zymograms **(B)** shows the relative change in metalloproteinase secretion compared to control (100%).

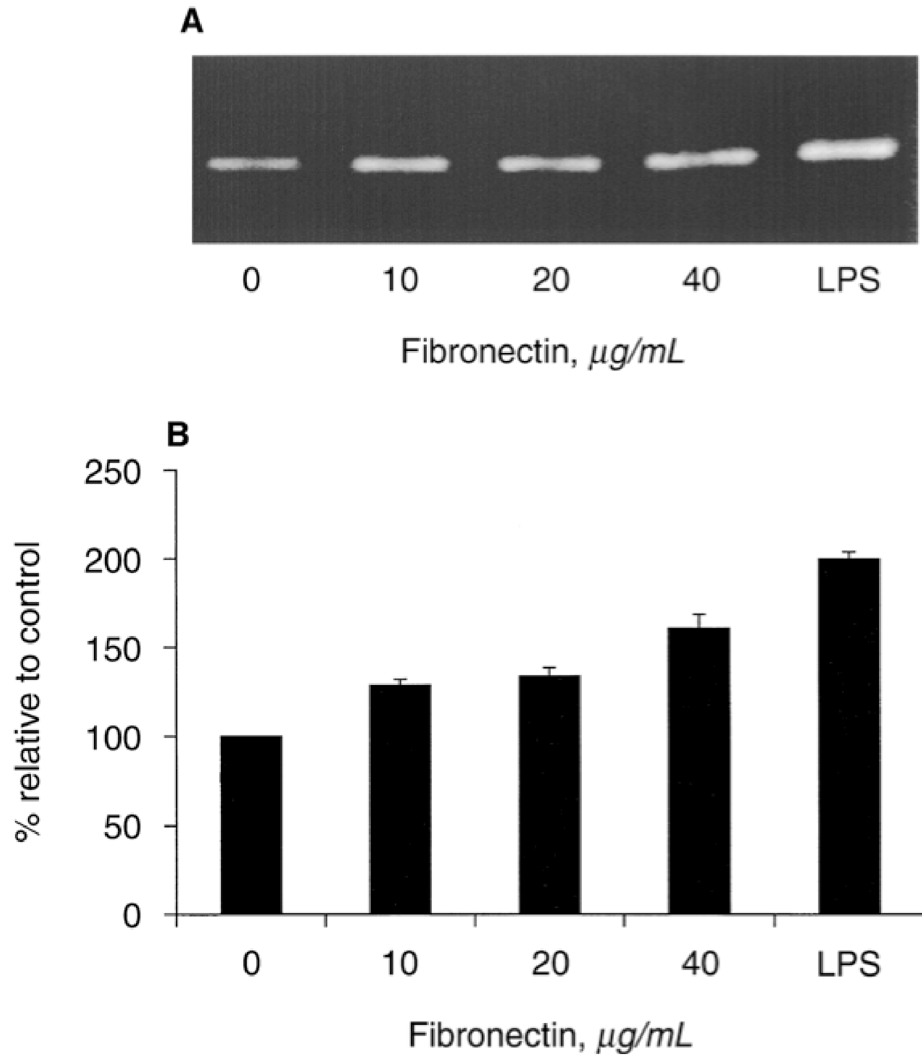


Figure 3.7. Matrix metalloproteinase (MMP) release by monocytes cultured in the presence of fibronectin (A). Peripheral blood-derived monocytes (0.5 x 10^6 cells/ml) were co-incubated with increasing concentrations of fibronectin for 24 hours in the absence of foetal calf serum. The resulting conditioned medium was subjected to zymography. Results are representative of 2 experiments. Densitometric evaluation of zymograms **(B)** shows the relative change in metalloproteinase secretion compared to control (100%).

3.3.8. Effect of matrix and soluble proteins on monocyte TIMP secretion

Matrix did not stimulate peripheral blood-derived monocyte TIMP I or TIMP II secretion (Table 3.2), nor was there any effect seen with addition of fibronectin (data not shown). The effect of anti-VLA-4 and anti-VLA-5 antibodies on monocyte secretion of metalloproteinases or their inhibitors was not investigated.

Table 3.2. Release of tissue inhibitors of metalloproteinases by human macrophages incubated on plastic culture plates (control), with mesangial cell matrix, and LPS for 24 hours.

	TIMP I (ng/ml)	TIMP II (ng/ml)
Control	16.7 + 3.8	848.1 + 126.0
Matrix	14.3 + 1.3	725.5 + 102.1
LPS	22.5 + 1.4	630.3 + 99.3

TIMP I and II were measured in the conditioned medium by ELISA as described in the methods section. Results are mean + SEM of 4 experiments (TIMP I) and 3 experiments (TIMP II) each performed in quadruplicate.

3.4. DISCUSSION

This study demonstrates that monocytes specifically bind to mesangial matrix and to its component proteins including fibronectin and to a lesser extent collagen type IV and laminin. Monocyte binding to whole matrix is mediated, at least in part by fibronectin. An increase in monocyte binding to mesangial matrix was shown to accompany the enhanced synthesis and secretion of fibronectin induced by cytokine pre-stimulation of mesangial cells. Incubation of monocytes with mesangial matrix and individual matrix proteins led to the secretion of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and to the activation of metalloproteinase (MMP-9). The release of MMP-9 was not associated with a change in production of tissue inhibitors of metalloproteinase indicating a net breakdown of matrix, thus exposing an increased potential for the binding of infiltrating monocytes. By adding radiolabelled thymidine to monocytes incubated with mesangial matrix it was possible to demonstrate that this increased secretion of cytokines and metalloproteinase was not associated with cell proliferation. These results therefore suggest that accumulation of mesangial matrix, particularly fibronectin, promotes monocyte entrapment and that binding to matrix proteins specifically stimulates monocyte secretion of inflammatory cytokines and matrix degrading metalloproteinase. Such interactions may have important implications in the pathogenesis of renal injury, particularly because the mechanisms by which macrophages are activated is known to determine their functional characteristics (Song, Ouyang et al. 2000).

Binding of monocytes to fibronectin is likely to be mediated by integrin receptors which are composed of the α and β subunits (Hynes 1992). Among these integrins, α4β1 (VLA-4) which binds to the CS1 and CS-5 regions of the IIICS domain of the fibronectin (Humphries, Komoriya et al. 1987; Mould, Komoriya et al. 1991) and α5β1 (VLA-5) which binds to the RGD sequence (Brown and Goodwin 1988; Brown, Phillips et al. 1989) are found on activated monocytes (Ferreira, Garcia-Pardo et al. 1990; Hemler, Elices et al. 1990). The possible involvement of VLA-4, and VLA-5 was therefore investigated. Anti-VLA-4 and VLA-5 antibodies caused a marked inhibition of U937 and peripheral blood-derived monocyte adhesion to whole matrix, suggesting that both cells shared common binding mechanisms involving both integrins. However, neither antibody completely blocked the adhesion process thereby indicating that other integrins and matrix components including collagen and laminin may participate in this interaction. The demonstration of a common binding mechanism allowed us to use U937 cells, which were more readily available than peripheral blood-derived monocytes, for binding studies although the latter were employed to examine the effects of binding on secretory function.

Chronic glomerular diseases are characterised by the accumulation of extracellular matrix composed of proteins including collagens, laminin and fibronectin along with proteoglycans and glycosaminoglycans. In the healthy glomerulus, these components not only provide structural support for glomerular cells but also influence their behaviour (Border, Okuda et al. 1989). Fibronectin, one of the most abundant mesangial matrix proteins, has been shown to have chemotactic properties for

monocytes, macrophages and fibroblast, and to be mitogenic to fibroblasts and mesangial cells (Ruoslahti 1988). Since these results demonstrate that individual matrix components differ in their ability to bind and activate macrophages, it follows that the nature of disease-related changes in matrix composition might in turn influence the extent of macrophage accumulation within the diseased glomerulus and the secretory characteristics of these infiltrating cells. Such interactions could determine the outcome of an acute or chronic inflammatory process.

Several animal and human studies suggest that monocytes/macrophages play a critical role in the initiation and progression of renal diseases. For example, in rats with remnant kidneys, macrophage accumulation was shown to strongly correlate with the progression of focal sclerosis suggesting that these cells may play a role in the scarring process (van Goor, Fidler et al. 1991). In humans, monocyte accumulation is seen in most forms of glomerulonephritis, including those associated with progressive fibrosis (Magil and Cohen 1989; Li, Hancock et al. 1990). Whilst monocyte infiltration may have beneficial functions, for example, promotion of the resolution of inflammation by apoptosis of infiltrating cells (Duffield, Erwig et al. 2000; Huynh, Fadok et al. 2002), several monocyte/macrophage secretory products may have a detrimental influence on the function of adjacent mesangial cells. These results demonstrate that binding of monocytes to whole matrix and matrix proteins, particularly fibronectin, enhances secretion of proinflammatory cytokines and matrix degrading metalloproteinases. These findings are in agreement with those of other investigators who have reported enhanced monocyte secretion of IL-1, IL-6, IL-8 and TNFα upon exposure of cells to matrix

proteins including fibronectin (Haskill, Johnson et al. 1988; Heinel, Singleton et al. 1995; Mahnke, Bhardwaj et al. 1995; Takizawa, Nishinarita et al. 1995). For example, Takizawa et al demonstrated that addition of fibronectin to monocytes isolated from human plasma stimulated production of IL-1, IL-6 and TNFα (Takizawa, Nishinarita et al. 1995). There have also been reports of matrix proteins modulating secretion of metalloproteinases by various cells including fibroblast (Huhtala, Humphries et al. 1995) and keratinocytes (Larjava, Lyons et al. 1993). Studies by Martin et al have demonstrated that specific matrix components enhance secretion of MMP-2 and MMP-9 by human mesangial cells and that membrane type metalloproteinase MTMMP, which is selectively induced by fibronectin, is important in this process (Martin, Eynstone et al. 2001). The present study extends these findings by demonstrating that matrix produced by glomerular cells may also modulate the accumulation and activation of infiltrating inflammatory cells. Matrix-mediated effects may help to explain the changes in metalloproteinase to inhibitor ratios observed by Mené et al in co-culture experiments involving mesangial cells and monocytes (Mene, Caenazzo et al. 2001).

Since binding of both U937 and peripheral blood-derived monocytes to mesangial matrix components involved the integrins VLA-4 and VLA-5, an investigation into whether these molecules might be involved in signal transduction was carried out. Stimulation of peripheral blood-derived monocytes with either anti-VLA4 or anti-VLA5 antibodies mimicked the effects of matrix on cytokine production, as well as blocking monocyte binding to matrix. Studies in other cell types have shown similar effects, for example, activation of fibroblasts by a crosslinking anti-ICAM-1 antibody

(Clayton, Evans et al. 1998) and of fibrosarcoma cells by antibodies to α5, α6 and β1 integrin subunits (Stanton, Gavrilovic et al. 1998) was associated with activation. Whilst the down stream events were not investigated in this study, in other experimental settings the binding of integrin components to matrix induces activation of phospholipases (Cybulsky, Carbonetto et al. 1993), kinase signalling pathways (Malik and Parsons 1996) and the AP-1 transcription factor (Yamada, Nikaido et al. 1991). Thus it is reasonable to propose that infiltrating monocytes may be activated by interactions with matrix components via integrin receptors. Monocyte responses may be influenced by pre-programming as has been observed following exposure to a variety of cytokines (Erwig, Kluth et al. 1998; Erwig, Stewart et al. 2000; Song, Ouyang et al. 2000). Furthermore accumulation and disease-specific modification of matrix components may alter monocyte/macrophage behavior and thereby potentially influence disease outcome. For example, these results would suggest that accumulation of fibronectin enhances metalloproteinase production, without increased inhibitor activity, a situation that is likely to promote matrix degradation. Inhibition of monocyte responses by blockade of these signalling pathways represents a potential target for therapeutic intervention in human glomerular disease and has proved effective in recent animal studies (Allen, McHale et al. 1999).

In summary, these results demonstrate that mesangial matrix plays a key role in the immobilization and activation of monocytes within the glomerulus. Since matrix proteins differ in their ability to modulate monocyte secretory functions, changes in matrix composition or organization in glomerular disease may influence the behavior of

infiltrating cells and thereby the outcome of the disease process. Better understanding of the potential importance of these processes may help in the design of treatment strategies for chronic glomerular diseases.

CHAPTER 4

MESANGIAL MATRIX-ACTIVATED MONOCYTES EXPRESS FUNCTIONAL SCAVENGER RECEPTORS AND ACCUMULATE INTRACELLULAR LIPID

4.1. INTRODUCTION

Transendothelial migration of monocytes into the glomerular mesangium is a recognized early feature of glomerular injury in man and in experimental models of kidney disease (Brady 1993). These cells play a central role in orchestrating tissue inflammation and may be critical in determining whether the final outcome of an acute inflammatory glomerular lesion is complete resolution or permanent scarring (Duffield 2003). Interactions between monocytes and extracellular structures encountered during the process of transmigration may play a critical role in determining the phenotype and therefore the behaviour of the activated tissue macrophage. As described in section 1.3, extracellular matrix is a highly ordered network of fibrous proteins and associated glycoproteins embedded in a hydrated ground substance of glycosaminoglycans and proteoglycans. It is recognised that matrix not only provides a structural framework, but also influences cellular behaviour. For example, integrin-mediated adhesion of monocytes to extracellular matrix may regulate expression of numerous inflammatory and immune response genes (de Fougerolles and Koteliansky 2002). The importance of this process is demonstrated by disease states thought to arise from dysregulation of matrix-integrin interactions (Campbell, Senior et al. 1987).

In the previous chapter, it has been demonstrated that exposure of human monocytes to both intact glomerular matrix (and to its individual components) enhanced the production of a range of inflammatory cytokines and matrix-degrading metalloproteinases. However, these experiments did not conclusively demonstrate that such interactions induced monocyte to macrophage differentiation. The experiments

described in this chapter were designed to test the hypothesis that activation by mesangial matrix converts monocytes to a macrophage phenotype. The expression of three macrophage specific markers were studied: a) the peroxisomal proliferator-activated receptor-γ (PPAR–γ), a nuclear receptor that acts as a transcriptional mediator for genes involved in lipid metabolism and adipogenesis (Moore, Rosen et al. 2001), b) CD36, a class B scavenger receptor and c) scavenger receptor class-A. Both these scavenger receptors are located in the plasma membrane of the macrophage and are involved in the cellular uptake of modified lipoproteins (Brown, Basu et al. 1980). Since unregulated uptake of modified lipoproteins is a characteristic of the tissue macrophage, the capacity of matrix-activated monocytes to accumulate intracellular lipid when exposed to acetylated low density lipoprotein (Ac-LDL), a scavenger receptor ligand was also tested. To further examine the role of matrix in foam cell formation, an assessment of the capacity of matrix to modify LDL in the absence of cells to produce oxidised LDL (ox-LDL), a naturally occurring scavenger receptor ligand identified in diseased glomeruli, was conducted. To confirm the relevance of these observations to human glomerular disease, human kidney biopsy sections from patients with inflammatory and non-inflammatory glomerular disease were stained for macrophage activation markers. These results demonstrate that mesangial cell matrix has the potential both to induce monocyte to macrophage maturation and to oxidise LDL, thereby indicating a likely modulatory role in glomerular inflammation and foam cell formation. It was also possible to demonstrate activated macrophages in glomeruli derived from patients with inflammatory glomerular disease.

4.3. RESULTS

4.3.1. PPAR-γ expression by matrix-activated monocytes

Whilst no PPAR-γ mRNA was detected by RT-PCR analysis of total RNA extracted from freshly isolated THP-1 monocytes, message was detectable within 24 hours when cells were incubated with soluble mesangial matrix (500 μg/ml). Expression was maximal at 48 hours, persisting over at least 5 days and was comparable to that observed when cells were stimulated with PMA over a similar time period under identical experimental conditions (Figure 4.1). Increased expression of PPAR-γ protein within 24 hours of exposure of matrix stimulation was confirmed by Western analysis, with levels of expression being similar to those observed following PMA stimulation (Figure 4.2). No further increase in expression was observed when incubation was extended beyond 48 hours to 7 days.

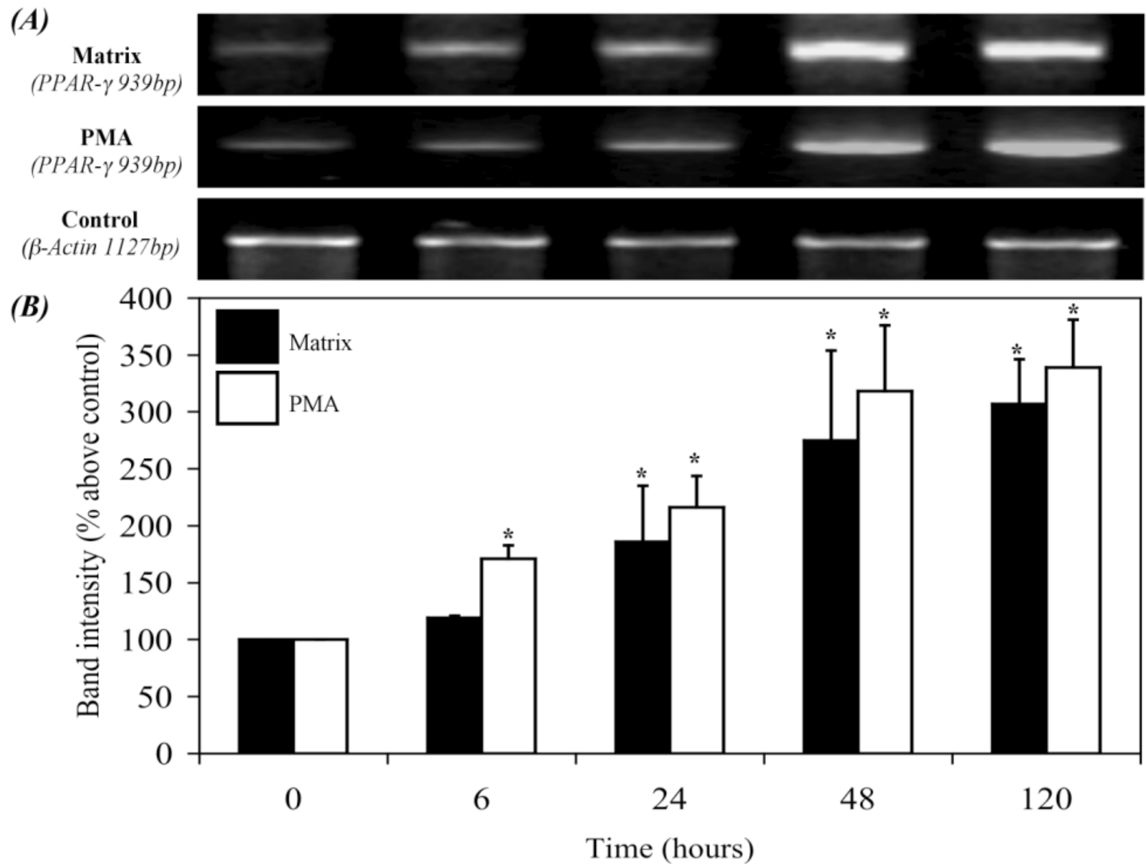


Figure 4.1. Time-dependent expression of PPAR-γ mRNA by THP-1 monocytes.

Monocytes were incubated with mesangial cell matrix (500 μg/mL) or PMA (125 nM) for up to 120 hours. **A)** PPAR-γ mRNA expression was examined by RT-PCR. **B)** Histogram showing analysis of mean ± SEM density of bands of PPAR-γ mRNA from 4 experiments, normalised by subtracting BSA protein control and comparison with β-actin mRNA.

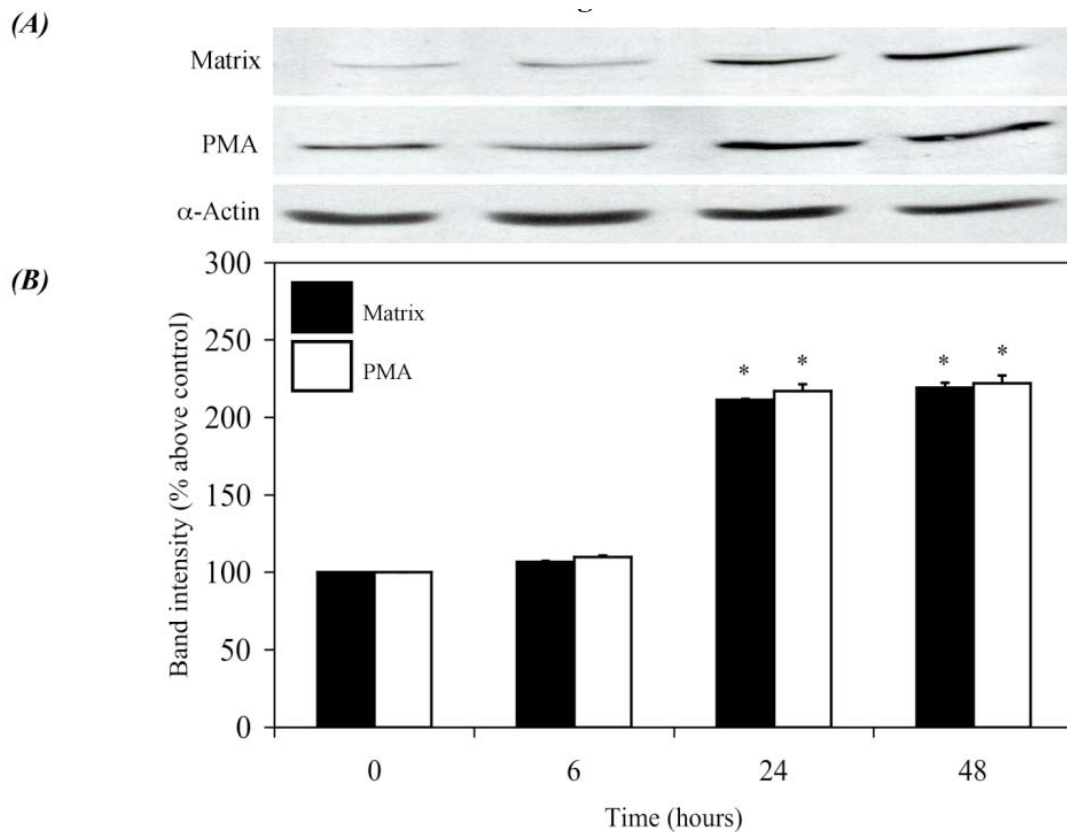


Figure 4.2. Western analysis of PPAR-γ protein expression by THP-1 monocytes. Monocytes were incubated with 500 μg/mL mesangial cell matrix or PMA (125nM) for 0, 6, 24 and 48 hours. Cells were lysed and subjected to SDS-PAGE and proteins transferred to nitrocellulose membrane. **A)** Western blot showing PPAR-γ detected using an anti-PPAR-γ antibody. **B)** Histogram showing mean ± SEM density of bands of PPAR-γ protein from quadruplicate wells, normalised by comparison with α-actin protein, and expressed as percentage of control (0 hours). *p<0.005 vs. Control.

4.3.2. CD36 expression by matrix-activated monocytes

Enhanced expression of CD36 mRNA was detected by RT-PCR analysis of total RNA extracted from THP-1 monocytes exposed to soluble mesangial matrix (500 μg/ml). An increase in message was detected after 48 hours of incubation, and was comparable with that observed when cells were stimulated with PMA for the same time period under identical experimental conditions (Figure 4.3). No further increase in expression was observed when incubation was extended beyond 120 hours to 7 days (data not shown).

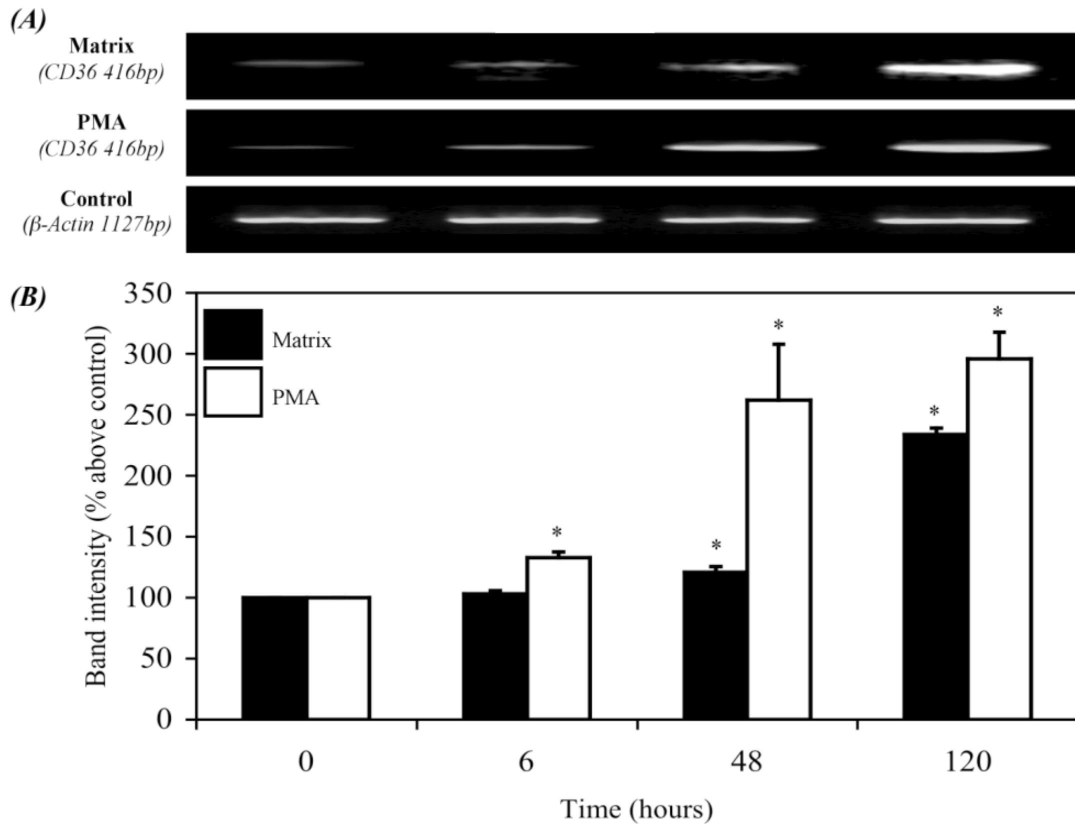


Figure 4.3. Time-dependent expression of CD36 mRNA in response to mesangial matrix.

Mesangial cell matrix (500 µg/mL) or PMA (125 nM) were incubated with monocytes for 0, 6, 48 and 120 hours. **A)** RT-PCR analysis of CD36 mRNA expression. **B)** Histogram showing analysis of mean ± SEM of CD36 mRNA bands from 4 experiments, normalised by subtracting BSA protein control and comparison with β-actin mRNA. Results are expressed as a percentage of control (0 hours). *$p<0.005$ vs. control.

4.3.3. Scavenger receptor expression by matrix-activated monocytes

Scavenger receptor-A mRNA expression increased in a concentration-dependent manner when THP-1 monocytes were incubated with increasing concentrations of soluble matrix protein for 48 hours with a maximal response at 100 μg/ml (Figure 4.4). A time-dependent increase was observed with addition of 500 μg/ml matrix protein increasing up to 120 hours incubation (Figure 4.5), with no further change up to 7 days (data not shown). No expression was observed under baseline conditions prior to stimulation, neither did equivalent concentrations of BSA induce detectable scavenger receptor-A message, suggesting that the observed effect was specific.

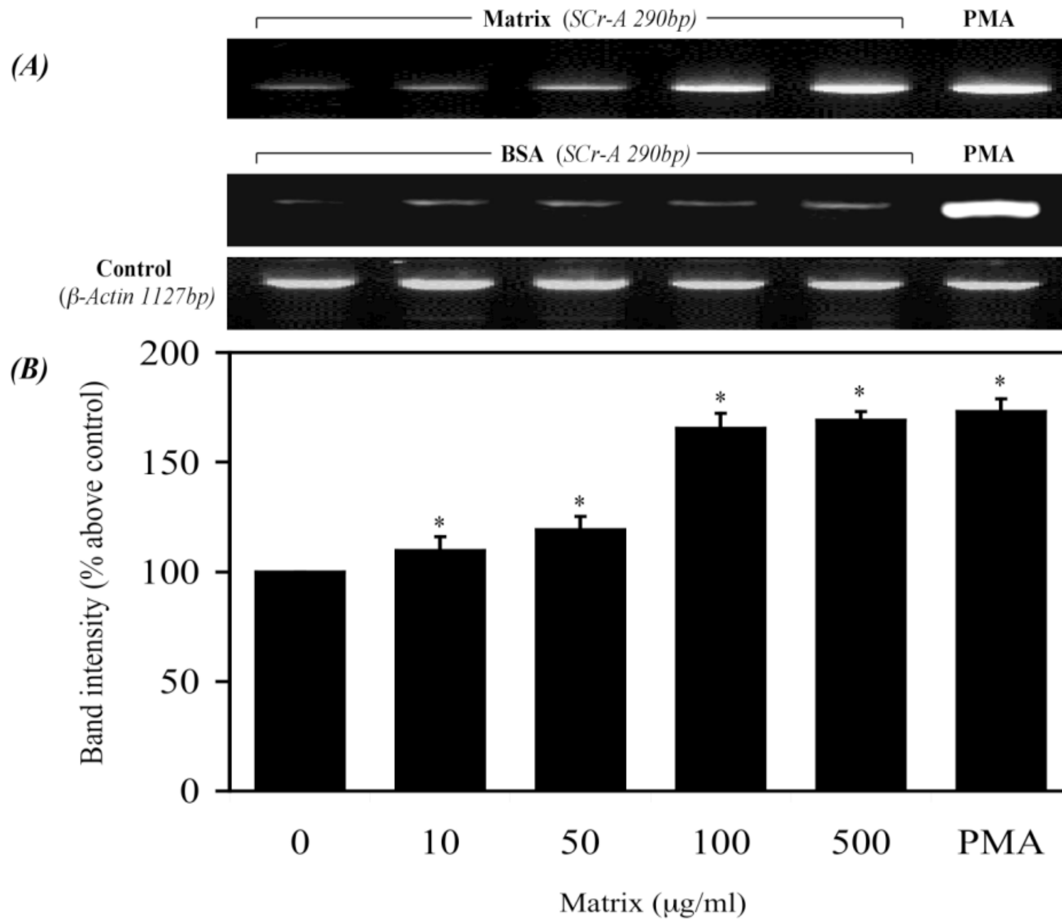


Figure 4.4. Scavenger receptor A mRNA expression by THP-1 monocytes incubated in the presence of increasing mesangial matrix for 48 hours.

Monocytes were incubated with increasing mesangial cell matrix concentrations of 0-500 μg/mL or PMA (125 nM) for 48 hours. **A)** Scavenger receptor A mRNA expression examined by RT-PCR. **B)** Histogram of mean ± SEM density of scavenger receptor A mRNA bands from 4 experiments, normalised by comparison with β-actin mRNA, and expressed as percentage of results obtained when equal amounts of BSA were added. *p<0.005 vs. equivalent BSA control.

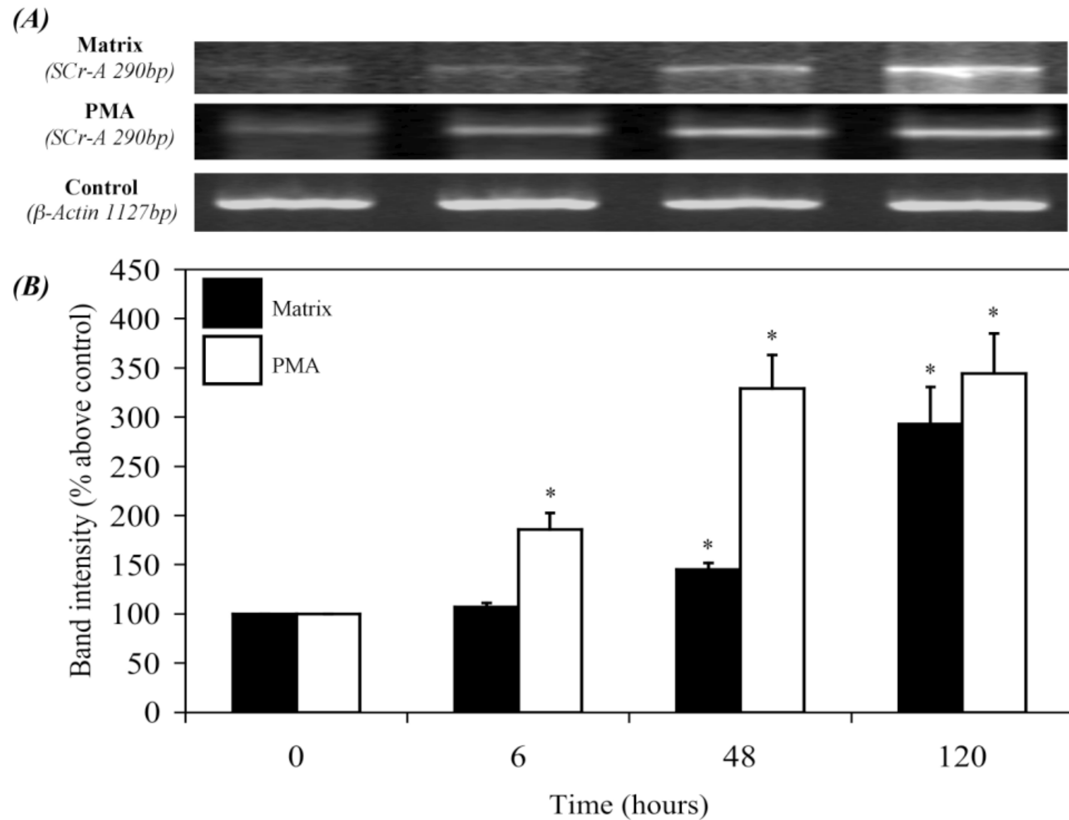


Figure 4.5. Time-dependent expression of scavenger receptor A mRNA in response to mesangial matrix.

Mesangial cell matrix (500 μg/mL) or PMA (125 nM) were incubated with monocytes for 0, 6, 48 and 120 hours. **A)** RT-PCR analysis of scavenger receptor A mRNA expression. **B)** Histogram of mean ± SEM density of scavenger receptor A mRNA bands from 4 experiments, normalised by subtracting BSA protein control and comparison with β-actin mRNA. Expressed as a percentage of control (0 hours). $*p<0.005$ vs. control.

4.3.4. Uptake of modified lipoproteins by matrix-activated monocytes

The presence of functional scavenger receptors was confirmed using flow cytometry in which incubation of matrix-activated monocytes with Dil-labelled Ac-LDL led to an increase in mean fluorescence intensity (MFI) (Figure 4.6). This effect was largely reversed by addition of an excess of unlabelled ligand. The MFI of THP-1 cells incubated with Dil-labelled Ac-LDL after exposure to matrix increased to 373±34.8% ($p<0.005$) as compared to cells exposed to BSA (100%). PMA pre-stimulation of monocytes, increased MFI to 423±55.5% ($p<0.005$). These increases in MFI induced by matrix-and PMA activation were inhibited by the addition of excess unlabelled Ac-LDL to 134±12.1% ($p<0.001$ vs. no excess of unlabelled lipoprotein) and to 170±16.1% ($p<0.001$) respectively, suggesting specific binding of Dil-labelled Ac-LDL to scavenger receptors.

Incubation of monocytes with unlabelled Ac-LDL following stimulation by exposure to matrix for 120 hours led to intracellular accumulation of Oil Red O- stained lipid droplets (Figure 4.7A). Lipid uptake did not occur following BSA stimulation (Figure 4.7B). Prior exposure to PMA was also associated with intracellular lipid deposition but no intracellular lipid staining was observed when poly I was added with Ac-LDL following activation of monocytes by matrix or PMA.

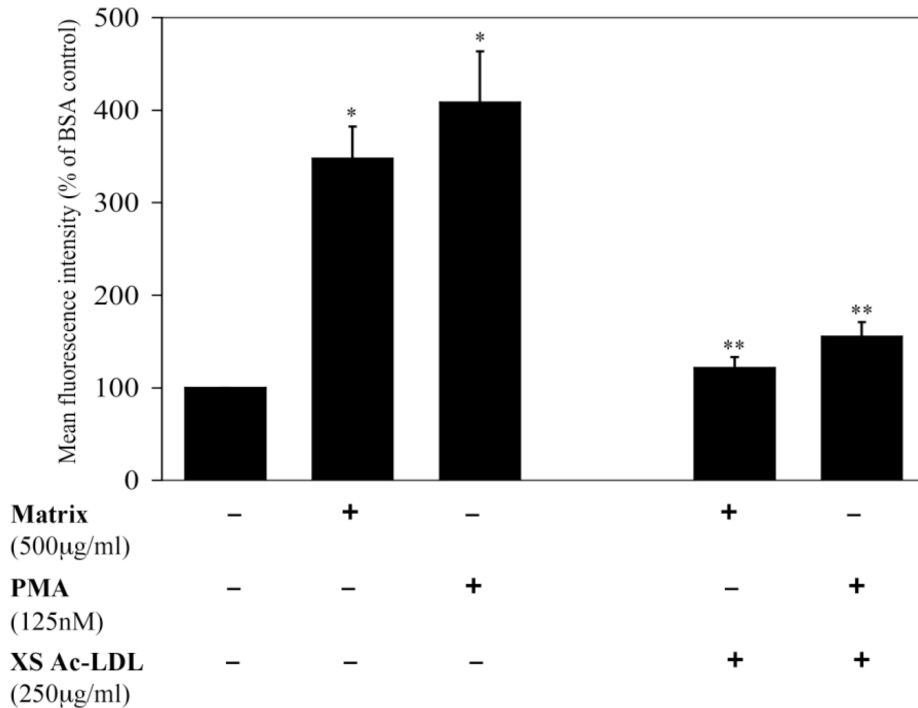


Figure 4.6. Effects of matrix on Ac-LDL uptake by monocyte/macrophages.

THP-1 monocytes were incubated with matrix (500 µg/mL) for 48 hours. BSA (500 µg/mL) and PMA (125 nM) served as negative and positive controls, respectively. Monocytes were recovered and incubated for a further 3 hours with 10 µg/mL DiI-labelled Ac-LDL in the presence or absence of an excess (XS) of unlabelled Ac-LDL (250 µg/mL). The mean fluorescence intensity (MFI) was calculated by subtracting the auto-fluorescence intensity from the observed fluorescence intensity of labeled cells. The histogram represents mean ± SEM MFI calculated from 4 experiments under the conditions shown, carried out in duplicate and expressed as percentage above BSA control (100%). *$p<0.005$ vs. BSA control, **$p<0.001$ vs. no excess unlabelled Ac-LDL.

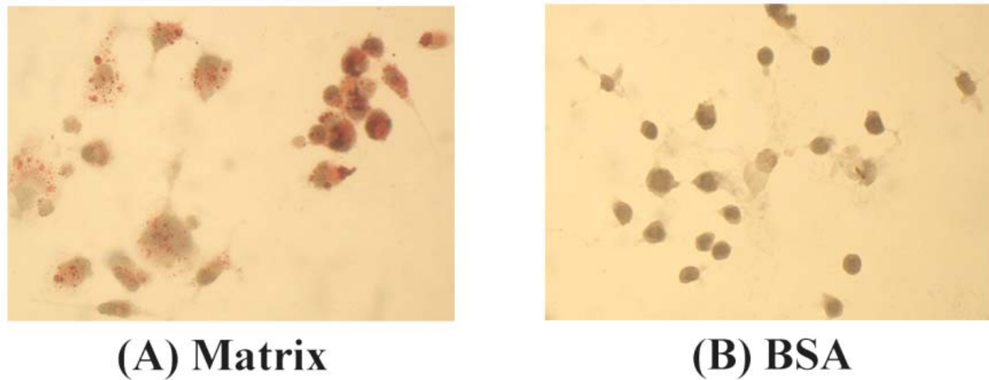


Figure 4.7. Visualisation of Ac-LDL uptake by THP-1 monocytes following exposure to mesangial matrix.

THP-1 monocytes were incubated with **A)** mesangial matrix (500 μg/mL) for 120 hours, or (500 μg/mL) for 120 hours, or **B)** BSA (500 μg/mL, negative control). The cells were then incubated with 50 μg/mL Ac-LDL for 48 hours at 37°C, fixed and examined for lipid inclusions by Oil Red O staining. The results shown are typical of those observed in 3 separate experiments.

4.3.5. Oxidation of LDL by mesangial cell matrix

Incubation of LDL with mesangial cell matrix in the absence of cells led to enhanced electrophoretic mobility of recovered lipoprotein on agarose gel (Figure 4.8). A similar shift in mobility was seen when LDL was exposed to copper sulphate, a powerful oxidising agent, but was blocked when the antioxidants EDTA (100 μM) and BHT (20 μM) were added, suggesting that matrix induces LDL oxidation. In contrast, incubation with BSA did not change the electrophoretic mobility of the lipoprotein.

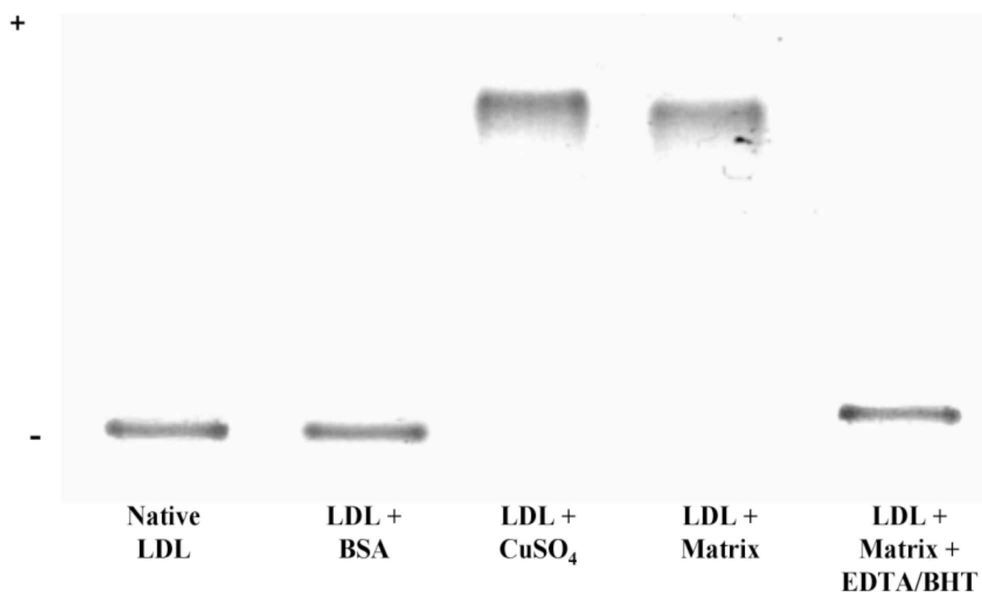
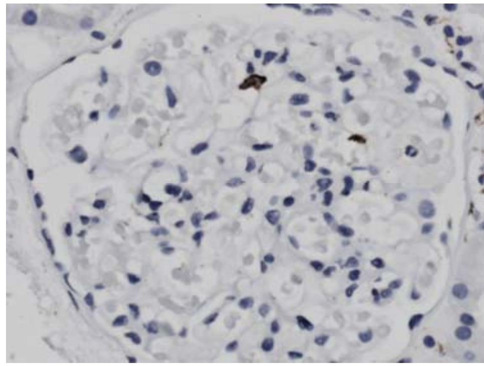


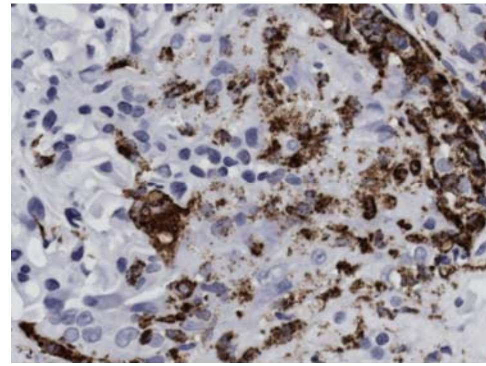
Figure 4.8. Agarose gel electrophoresis demonstrating oxidation of LDL by mesangial matrix.

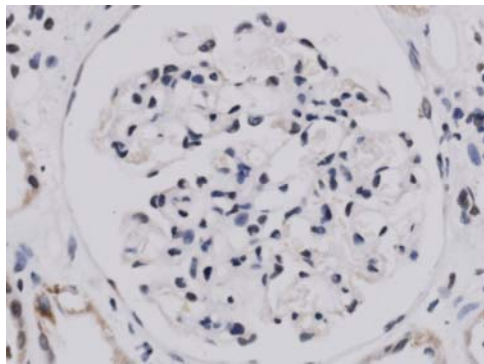
Lane 1: Freshly isolated native LDL (negative control), Lane 2: LDL incubated with BSA protein (500μg/mL), Lane 3: LDL incubated with $CuSO_4$ (10 μM, positive control), Lane 4: Matrix (500μg/mL) incubated with LDL, Lane 5: Matrix incubated with LDL and with the antioxidants EDTA (100 μM) and BHT (20 μM). LDL incubated with mesangial matrix had a mobility similar to that observed with the positive control ($CuSO_4$). This effect was abolished by the addition of EDTA and BHT, indicating that matrix promotes LDL oxidation.

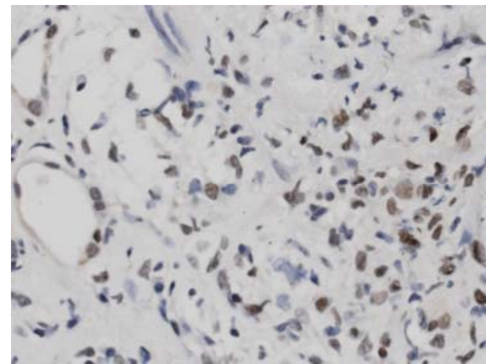
4.3.6. Identification of macrophage activation markers in human kidney biopsy material

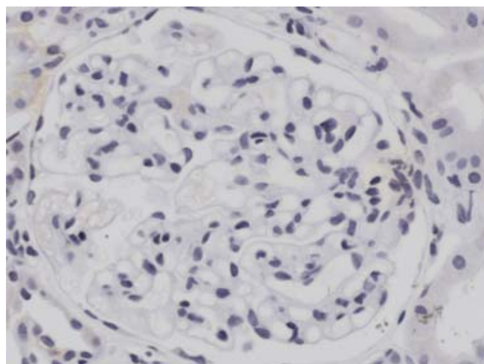
CD-68 positive cells were extremely difficult to identify in the normal kidney section and there was no staining for PPAR-γ or scavenger receptor. In contrast, all three markers were readily detected in the inflamed kidney, predominantly within the glomeruli (Figure 4.9). CD68 and scavenger receptor were located in a cytoplasmic distribution and PPAR-γ within nuclei in keeping with the cellular location of these markers.


Non-inflamed: CD68


Inflamed: CD68


Non-inflamed: PPAR-γ


Inflamed: PPAR-γ


Non-inflamed: Scavenger receptor

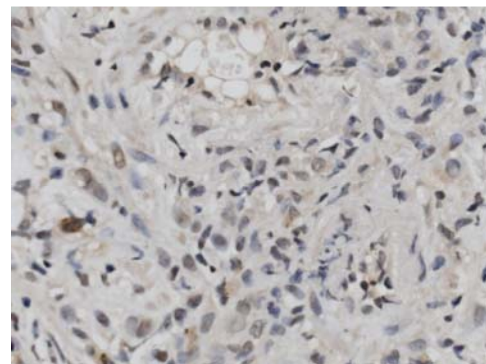

Inflamed: Scavenger receptor

Figure 4.9. Staining of human kidney sections for macrophage activation markers. Sections of non-inflamed and inflamed human kidney were stained for the macrophage antigen CD-68 and for the activation markers PPAR-γ and Scavenger receptor.

4.4. DISCUSSION

These results demonstrate that exposure of monocytes to mesangial cell matrix in vitro induces expression of PPAR-γ, CD36 and scavenger receptor-A and promotes phagocytic activity, characteristics usually associated with a macrophage phenotype. This would suggest that conversion of infiltrating monocytes to mature tissue macrophages within the glomerular mesangium in vivo may not necessarily depend on the presence of mesangial cells, but may result from direct interactions with matrix components. While circulating monocytes are relatively inert, the activated tissue macrophage may take on a destructive role, inducing cell death by apoptosis and degrading extracellular matrix. Alternatively, these cells may facilitate repair by inducing cell proliferation and secretion of replacement matrix components (Duffield 2003). Macrophages also play a key role in the phagocytosis of cellular debris, lipids and denatured proteins in inflamed tissue, a process that may result in the formation of foam cells. Foam cells are characteristically seen at sites of tissue injury, for example in the arterial intima in atherosclerosis, and are recognised in the kidney in damaged glomeruli (Moorhead 1991). Given the pivotal role of the monocyte/macrophage in modulating tissue injury and the diverse biological activities of these cells, these findings may have important implications in the context of glomerular disease as supported by the demonstration of both PPAR-γ and scavenger receptor expression in inflamed human kidney sections. Thus, matrix-mediated activation may influence the behaviour of monocytes that infiltrate the glomerular mesangium and thereby potentially modify the outcome of an inflammatory process.

It is recognised that the phenotypic state adopted by a tissue macrophage is influenced by the activation signals that naïve monocytes receive and that broadly, two distinct activated phenotypes can be identified. The classical activation pathway results from exposure to Th-1 type cytokines such as TNF-α, IL-1β and IL-6 and results in a macrophage with pro-inflammatory properties, capable of further generation of pro-inflammatory cytokines and the degradation of normal and abnormal matrix components (Erwig, Kluth et al. 2001). Classically activated cells also possess the ability to take up modified lipoproteins, potentially resulting in the formation of lipid-laden foam cells. In contrast, the alternative activation pathway induced by Th-2 type cytokines such as IL-4 and IL-13 produces a macrophage that generates anti-inflammatory cytokines, suppresses the synthesis of pro-inflammatory cytokines and is resistant to re-activation, thus being responsible for co-ordinating resolution of an inflammatory process (Duffield 2003). Taken together with the previous chapter, which demonstrated that incubation of monocytes with mesangial cell matrix stimulates secretion of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α as well as matrix-degrading metalloproteinases, it seems reasonable to conclude that mesangial cell matrix activates macrophages via the classical pathway.

In late 2005 the Th-1 type and Th-2 paradigm was further simplified. Monocytes primed by Th-1 type cytokines, which promote differentiation into proatherogenic ‘Classical’ macrophages were termed M1 macrophages, while those primed by Th-2 type cytokines, which lead to an “Alternative” anti-inflammatory macrophage phenotype, were termed M2 macrophages (Mantovani, Sica et al. 2005). Functional

polarization of macrophages into M1 or M2 cells was deemed as more operationally useful and provided a simplified conceptual framework describing the plasticity of mononuclear phagocytes (Mantovani, Sica et al. 2004). Genetic approaches have begun to shed new light on mechanisms underlying macrophage differentiation and attempts to dissect the actual in vivo significance of their polarization are currently being investigated (Rauh, Ho et al. 2005; Biswas and Mantovani 2010).

These findings are highly relevant to the fate of monocytes that undergo transmigration to become tissue macrophages, but not to cells that undergo reverse-transmigration since they adopt the phenotype of an immature or mature dendritic cell (depending on the absence or presence of inflammatory stimuli respectively) (Randolph, Beaulieu et al. 1998).

Other macrophage-specific markers which have been previously explored by my colleagues included CD69 and the HLA-DR antigens (Chana, Martin et al. 2003). However it was discovered that both markers were expressed at low levels on THP-1 monocytes with no significant up-regulation occurring following stimulation with PMA, an accepted and potent activator of monocytes. Other investigators have observed that HLA-DR expression varies with the source of the macrophage, such that 15% of peritoneal macrophages express the antigen compared to 50% of spleen and thymus-derived cells (Lewis, Norris et al. 1990). Another potential candidate was Mac-1, a member of the β2 integrin family also known as CD11b/CD18, however, this cell surface adhesion receptor proved not to be specific to macrophages and was also expressed at low levels by freshly isolated monocytes as demonstrated by other workers

(Miller, Bainton et al. 1987). PPAR-γ expression proved to be a more useful indicator since this intracellular receptor showed very low levels of expression in monocytes, but is strongly induced during their differentiation into mature macrophages, suggesting that it may be involved in the differentiation process. In addition, PPAR-γ has been implicated in the modulation of several macrophage functions including the regulation of pro-inflammatory activities and stimulation of ox-LDL uptake further strengthening the use of this factor as a macrophage marker (Moore, Fitzgerald et al. 2001). PPAR-γ is also abundantly expressed in lesions such as atherosclerotic plaques where formation of foam cells is observed (Tontonoz, Nagy et al. 1998). It should be emphasised that PPAR-γ was used simply as a macrophage marker in these studies and that ligand-induced activation of this receptor was not examined. It seems likely that other signalling pathways are activated by matrix-monocyte interactions, particularly since PPAR-γ activation does not explain the increase in cytokine production that has previously been reported (Chana, Martin et al. 2003).

Scavenger receptor-A is a macrophage-specific cell surface protein that specifically binds and internalises oxidised and chemically modified LDL particles, similar to the class B scavenger receptor; CD36, which also bind modified forms of LDL (Brown, Basu et al. 1980). Scavenger receptor expression is restricted to macrophages thereby providing a reliable marker for the purpose of these studies. Scavenger receptor-A has been implicated in mediating a variety macrophage functions, including intracellular signalling, endocytosis, adhesion and phagocytosis. Unlike uptake of native LDL via the LDL receptor, which is tightly controlled, scavenger receptor-A mediated

uptake of modified lipoprotein is not regulated by intracellular cholesterol levels and can therefore potentially lead to intracellular cholesterol accumulation and the formation of foam cells (Gough, Greaves et al. 1999). The class B scavenger receptor CD36 has also been implicated in the process of lipid accumulation in macrophages and serves as an adhesion receptor on macrophages for matrix components such as collagen and thrombospondin (Tandon, Kralisz et al. 1989).

In keeping with the changes in lipoprotein receptor expression observed, matrix-activated monocytes accumulated intracellular lipid when incubated with Ac-LDL, a synthetic scavenger receptor ligand, as demonstrated by intracellular Oil Red O staining. This phagocytic capacity was further confirmed by flow cytometry of matrix-activated monocytes exposed to Dil-labelled Ac-LDL. Uptake of Ac-LDL was shown to be specific, since it could be inhibited by addition of an excess of unlabelled acetylated lipoprotein, thus confirming receptor involvement. Induction of phagocytic activity was also observed following PMA-mediated activation, but not when an irrelevant protein (BSA) was added.

Lipoproteins, including LDL, infiltrate the normal mesangium and are found deposited in diseased glomeruli (Wheeler and Chana 1993). Having previously shown that mesangial cells oxidise LDL in vitro (Wheeler, Chana et al. 1994), It has been demonstrated here that exposure of LDL to mesangial matrix has a similar effect. Thus, not only does matrix exposure induce a phagocytic macrophage phenotype in monocytes, but also converts LDL to an appropriate scavenger receptor ligand thereby potentially contributing to the development of foam cells. The mechanisms by which matrix promotes LDL oxidation were not explored but may involve entrapment of

lipoprotein by glycosaminoglycans, thereby rendering particles more susceptible to the effects of reactive oxidative species (Abuja 2002).

In conclusion, mesangial matrix has the capacity to convert monocytes to macrophages displaying characteristics associated with a classically activated phenotype. By inducing macrophage scavenger receptor expression and converting LDL to an oxidised product, matrix may also play a key role in the formation of foam cells within the glomerular mesangium. The impact of changes in matrix composition on these interactions and the potential for such changes to modify the outcome of an inflammatory process within the glomerulus warrant further investigation.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1. Research Questions Addressed In This Thesis

Infiltration of mononuclear cells into the mesangium and their differentiation are recognized as an early event in many diseases that ultimately lead to chronic kidney failure. A variety of factors are likely to play key roles in orchestrating monocyte influx and in determining the phenotype that the infiltrating monocytes ultimately adopt. In particular, monocyte interactions with components of mesangial matrix may play a key role in these processes.

The work presented in this thesis set out to dissect the nature of the interaction between monocytes and mesangial cell matrix and the resulting changes in monocyte phenotype. A representative matrix protein component, namely Fibronectin, which is up-regulated when mesangial cells are exposed to TGF-β and TNF-α (Chana and Wheeler 1999) was used to conduct matrix binding and blocking studies to examine the extent to which matrix exposure modified cytokine, MMP and TIMP production by monocytes. Whole matrix, as well as other matrix protein components namely Collagen type IV and Laminin were also examined for their effects on cytokine production.

To address the extent of monocyte differentiation into macrophages upon exposure to mesangial cell matrix, one challenge was the identification of a suitably robust marker of monocyte to macrophage conversion. After a thorough literature search three reliable macrophage specific markers were studied: a) PPAR–γ, b) CD36 and c) Scavenger receptor class-A.

Finally since LDL may play a central role in the pathogenesis of glomerulosclerosis it was assessed whether this lipoprotein might become oxidized in

the mesangium by exposure to matrix, thereby becoming a ligand for uptake via macrophage scavenger receptors and promoting foam cell formation.

5.2. LIMITATIONS OF THE EXPERIMENTAL WORK

5.2.1. Monocyte Binding Studies

From earlier experiments by our group (Chana and Wheeler 1999) it has demonstrated that exposure of mesangial cells to LDL stimulates the production of the monocyte chemoattractant MCP-1, indicating that the presence of LDL within the injured glomerular mesangium might promote monocyte accumulation. It was also demonstrated that LDL increases the synthesis of the matrix component fibronectin by mesangial cells, thus providing an additional factor by which monocytes might be retained. In the experiments described in this thesis, this work was taken forward by examining ligand-integrin interactions involving fibronectin and monocytes. One criticism is that most of these experiments were based on a monocyte cell line. However, although U937 monocytes were used in the earlier experiments, these data were strengthened by using the same blocking antibodies in experiments using whole matrix and human PBMCs, and also found a decrease in monocyte adhesion to matrix through the VLA-4 and VLA-5 integrin receptors. When examining the resulting secretory behaviour of monocytes upon binding to whole matrix and individual matrix proteins PBMCs were also used in some of these experiments. It can therefore be accepted that the U937 cell line provided an appropriate model on which to base these experiments.

Ideally it would have been possible to extract individual component proteins of mesangial matrix. However this would have required huge amounts of matrix and a complex extraction process which would not have guaranteed pure protein components. The other option was to qualitatively and quantitatively define the components of mesangial cell matrix. Although attempts to do this were investigated, it was not a feasible option given the focus of these investigations. Instead, a decision to use an equal volume of the three major matrix proteins was taken, namely Collagen Type IV, Fibronectin and Laminin and make a comparison with whole matrix when looking at changes in inflammatory cytokine secretory behaviour and activation of MMP-9 and TIMPs by PBMCs.

Since experiments involving cytokine and MMP secretion and TIMP activation were performed at a single time-point of 24 hours, the possibility that at later time points, matrix activated macrophages lose their pro-inflammatory capacity, and take on the alternatively activated anti-inflammatory phenotype, cannot be excluded. In vivo, both cell phenotypes are likely to be present in inflamed tissue and the balance between them may be critical in determining the extent of subsequent fibrosis. It is also possible that a classically-activated macrophage programmed by exposure to matrix does not respond to the signals usually associated with the alternative activation pathway, potentially resulting in an uncontrolled inflammatory response with limited subsequent tissue repair (Duffield 2003). Further time-points up to 7 days may prove useful in answering this question.

5.2.2. Monocyte Activation Studies

When designing these experiments one challenge was the identification of a suitably robust marker of monocyte to macrophage conversion. The three macrophage specific markers chosen were a) PPAR-γ, b) CD36 and c) Scavenger receptor class-A. MAC-1 was considered but was abandoned due to there being low levels of expression on freshly isolated monocytes, as also demonstrated by others (Bainton, Miller et al. 1987; Cifarelli, Libman et al. 2007; Yakubenko, Belevych et al. 2008).

The observed changes in macrophage specific markers following matrix interaction were reproduced using the THP-1 monocyte cell line supplied by two companies (ECACC and ATCC) both of which claim that these cells differentiate into macrophages. In addition, a number of published studies appear to support the differentiation of this cell line (Tsuchiya, Yamabe et al. 1980; Kritharides, Christian et al. 1998; Kim, Studer et al. 2008). Taking into account the available literature and discussions with experts working in the field of inflammation, it can be accepted that these cells provided a reasonable model for these experiments.

To further validate the model, studies comparing the response of human peripheral blood monocytes and THP-1 cells to mesangial matrix assessing the production of inflammatory cytokines and MMP-9 were performed. These studies show almost identical patterns of induction suggesting that the two cell types show similar responsiveness. Furthermore, previous work by our group has demonstrated similarities

between monocyte cell lines and cells isolated from peripheral blood in terms of surface markers and binding characteristics (Wheeler, Chana et al. 1994).

5.2.3. Disease Specific Matrix Modification

Matrix elaborated by healthy mesangial cells were used, however this did not take account of the fact that disease-specific matrix modification may influence monocyte activation, since there is strong evidence to suggest that monocyte-macrophage differentiation is influenced by the nature of the matrix proteins (Laouar, Collart et al. 1999) For example, matrix glycation that occurs in diabetes mellitus influences the balance between matrix synthesis and degradation by mesangial cells, promoting accumulation (Schleicher and Olgemoller 1992) and may also affect monocyte to macrophage differentiation. Indeed many studies have shown that non-enzymatically glycated matrix occurring in diabetes influences monocyte to macrophage differentiation (Jacob, Shastry et al. 2001; Min, Lyons et al. 2009). A key matrix protein, collagen type 1 when non-enzymatically glycated was shown to accelerate monocyte to macrophage differentiation, leading to foam cell formation upon interaction with oxidised LDL (Jacob, Shastry et al. 2001). Since mesangial cell matrix may be particularly prone to glycation as a result of its prolonged lifespan, an extension to this work would be to investigate various matrix modifications on monocyte differentiation such as non-enzymatic glycation.

5.2.4. Mesangial Cell Matrix Sequesters Cytokines and Growth Factors

The possibility that these results are at least in part explained by retention of low concentrations of cytokines within the matrix material cannot be ruled out. More detailed experiments investigating the ability of matrix to sequester growth factors and cytokines would be the next logical step in investigating the effects on monocyte behaviour. For example investigating the effects of matrix impregnated with TGF-β, matrix has been shown to sequester this growth factor and it has been shown to play an important role in matrix production. (Gambaro and Baggio 1998).

5.2.5. Scavenger receptor: Protein Level Expression

Experiments were limited to examining the expression of scavenger receptors at the message level without going on to provide protein expression data as was the case with PPAR-γ expression. However, up-regulation of functional scavenger receptors in the lipoprotein uptake studies was demonstrated and it is unlikely that further extending these studies to examine scavenger receptor expression at the protein level would have changed the conclusions.

5.3. Implications Of Major Findings

The work presented in this thesis help us to better understand how matrix components contribute to changes in monocyte phenotype. Chapter 3 demonstrates that mesangial matrix plays a key role in the immobilization and activation of monocytes

within the glomerulus. These results would also suggest that accumulation of fibronectin enhances metalloproteinase production, without increased inhibitor activity, a situation that is likely to promote matrix degradation.

In Chapter 4, initial findings were further strengthened by demonstrating that mesangial matrix has the capacity to convert monocytes to macrophages displaying characteristics associated with a classically activated phenotype. Since lipoproteins, including LDL, infiltrate the normal mesangium and are found deposited in diseased glomeruli (Wheeler and Chana 1993) mesangial cell matrix may play a key role in the formation of foam cells within the glomerular mesangium through its capacity to oxidise LDL as well as stimulate monocyte to macrophage differentiation.

Thus it has been demonstrated that interactions between mesangial matrix components and infiltrating monocytes may play a key role in the progression of glomerular injury.

5.4. POTENTIAL THERAPEUTIC IMPLICATIONS

Interactions between adhesion molecules and infiltrating macrophages have been successfully blocked and could serve as targets for therapeutic interventions (Adler and Brady 1999; Allen, McHale et al. 1999; Chana and Wheeler 1999; Cook, Khan et al. 2002). The humanized version of this anti-$\alpha4$ monoclonal antibody known as Natalizumab has been used successfully in multicenter double-blind controlled studies in Crohn's disease and multiple sclerosis (Ghosh, Goldin et al. 2003; Miller, Khan et al. 2003; Miller, Soon et al. 2007; Targan, Feagan et al. 2007). Despite there being adverse

reactions in some patients to this drug it was given approval by the FDA as its clinical benefits outweighed the risks involved (Ransohoff 2010; Steiner 2010). It would be interesting to see the effects of this drug on patients suffering from glomerulosclerosis.

The beta-2 integrins include CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1/Complement receptor 3). A humanized monoclonal antibody to CD18 known as Efalizumab, which blocks CD11a/CD18 and CD11b/CD18 has been reported to reduce infiltrating leukocytes and improve vasculitic ulcers in patients with systemic vasculitis (Lockwood, Elliott et al. 1999), however ten years on Efalizumab has been withdrawn from the market as it was associated in some cases with fatal brain infections (Major 2010). Inhibition of monocyte responses by blockade of these signalling pathways represents a potential target for therapeutic intervention in human glomerular disease.

5.5. CONCLUSION

In summary, enhanced monocyte adhesion to mesangial cell matrix results in monocyte retention, activation and differentiation within the glomerular mesangium. In the presence of LDL, macrophages are likely to accumulate lipid to form foam cells. Inhibition of monocyte-matrix interactions represents a potential therapeutic intervention that may prove protective in the setting of kidney disease. .

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