THE ROLE OF THE MACROPHAGE IN PROGRESSIVE GLOMERULOSCLEROSIS

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ABSTRACT

Glomerulosclerosis is the final outcome of a number of causes of glomerular injury during which the structures of the glomerulus are obliterated by extracellular matrix. Accumulating evidence has focused on macrophages as playing a pivotal role in the pathogenesis of this process. This thesis was undertaken to delineate the role played by macrophages at both the cellular and molecular levels on the initiation of the sclerotic process, to characterise the factor(s), and to elucidate the potential interactions involved.

Macrophages were shown to promote potent pro-sclerotic effects in mesangial cells, by inducing them to express the protein and message for the matrix protein fibronectin. In addition, the genes for the matrix proteins laminin and collagen IV, the tissue inhibitor of matrix metalloproteinases TIMP-1, the pro-fibrotic growth factors TGFβ and PDGF, and the specific macrophage chemoattractant MCP-1 were also upregulated.

$TGF\beta_1$, PDGF, TNFα and IL-1β individually, could not reproduce the effects of macrophage conditioned medium (MPCM) on fibronectin production. However, in combination, as may be found *in vivo*, they were able to synergistically upregulate the production of this matrix protein to levels comparable with those seen with MPCM. Although these cytokines could not be detected in MPCM *per se*, constitutive secretion of TGFβ and PDGF was upregulated in mesangial cells in response to MPCM. However,experiments with neutralising antibodies and a ligand:receptor binding antagonist demonstrated that these growth factors only play a minor role in macrophage stimulated rat mesangial cell fibronectin production.

Characterisation studies suggested that the macrophage-derived factor(s) responsible for the accumulation of fibronectin was a protein of the order of 12.5 kD, heat stable and susceptible to some degree of proteolytic digestion.

The data presented in this thesis provides the most compelling evidence to date for a direct role of macrophages in the pathogenesis of glomerulosclerosis.

To Lala and Tomaszek

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ABBREVIATIONS

AII	Angiotensin II
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
$[Ca^{2+}]_i$	Calcium concentration (intracellular)
cDNA	Complimentary DNA
CSF-1	Colony stimulating factor-1
DNA	Deoxyribonucleic acid
ED-A	Extra domain-A
EDTA	Ethylenediaminetetraacetic acid
EFAD	Essential fatty acid deficient
ELISA	Enzyme linked immunosorbent assay
Fc	Fragment-crystalisable
FCS	Foetal calf serum
GBM	Glomerular basement membrane
HBSS	Hanks' balanced salt solution
HEPES	N-[2-hydroxyethyl]-N'-[2-ethanesulphonic acid]
HRP	Horseradish peroxidase
Ia	Immune associated
Ig	Immunoglobulin
ICAM-1	Intercellular adhesion molecule-1
LFA-1	Lymphocyte function associated antigen-1
IFNγ	Interferon gamma
IGF	Insulin-like growth factor
IL-1ß	Interleukin-1ß
IL-1ßra	Interleukin-1 receptor antagonist
IL-6	Interleukin 6
IL-10	Interleukin-10
IP-10	Interferon-inducible protein-10
kD	Kilo Dalton
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MCP-1	Macrophage chemotactic factor-1
M-CSF	Monocyte/macrophage colony stimulating factor
met	Methionine
MHC	Major histocompatability
MOPS	3-[N-Morpholino]propanesulphonic acid
MPCM	Macrophage conditioned medium
NADH	Nicotinamide adenine dinucleotide (reduced)
OD	Optical density
OPD	1,2-phenlenediamine dihydrochloride
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAN	Puromycin aminonucleoside
PBS	Phosphate buffered saline
PCA	Perchloric acid
PDGF	Platelet derived growth factor
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Saline-sodium-citrate
SSPE	Saline-sodium-phosphate-EDTA
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TIMP-1	Tissue inhibitor of metalloproteinases-1
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFß	Transforming growth factor ß
TNFα	Tumour necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1

Prologue

End stage renal failure is a cause of significant mortality and morbidity and as a consequence has large economic implications for providers of healthcare. An understanding of the processes of renal scarring is essential if effective treatments are to be developed.

The progression of chronic renal failure and associated renal scarring remain a challenge to investigators in the renal field, although recent advances have been made in our understanding of some of the mechanisms underlying both glomerulosclerosis [Johnson R, 1994] and tubulointerstitial fibrosis [Kuncio GS *et al*, 1991]. Current thinking is that the pathogenesis of the two processes share some common mechanisms, although they are thought not to be identical. Generally speaking, in the glomerulus, sclerosis occurs with progressive accumulation of extracellular matrix components that reduce filtration surface area, whilst in the interstitium, early inflammatory changes accompany glomerulonephritis with later development of fibrosis and tubular atrophy. Whether glomeulosclerosis leads to tubular atrophy as a result of 'redundancy' or whether the interstitium is the primary site of injury with glomerular loss being secondary to this event are topics of current debate.

Progressive glomerulosclerosis and renal insufficiency are seldom observed in the absence of tubulointerstitial scarring, although renal failure can occur in primary diseases of the interstitium in which the glomeruli are intact [Neilson EG, 1989]. Furthermore, studies by Risdon and co-workers [1968] demonstrated that the severity of chronic tubulointerstitial disease is the single best histologic correlate of the decline in renal function and long term prognosis. However, since the tubulointerstitium makes up approx 95% of the kidney, this is perhaps not surprising.

Glomerulosclerosis is still considered to be the histological hallmark of end-stage renal failure and is likely to be the pathological correlate of the progressive decline in glomerular filtration rate that is seen in many renal diseases. This thesis will therefore, focus on glomerulosclerosis and particularly the role played by macrophages in progression towards it.

CHAPTER ONE

Introduction

1.1 The glomerulus

In 1666 Marcello Malpighi first described the glomerulus as an important part of the mammalian kidney. The glomerulus is the vascular component of the nephron whose physiological function is to filter blood, producing a protein free plasma filtrate whilst retaining all cellular components and plasma proteins in the circulation. Blood carried in the afferent arteriole enters the glomerulus via the vascular pole or hilus, where the local hydrostatic pressure of the blood causes the glomerular filtrate to be forced across the capillary wall which is lined by endothelial cells. This cell layer is interrupted by fenestrations or porous openings [Jorgensen F, 1961] which are themselves covered by thin, highly permeable diaphragms of a protein-polysaccharide film and which present no significant barrier to the passage of large molecules. The endothelial cells sit on the glomerular basement membrane - the only continuous barrier to the blood. This membrane consists of arrays of fibrils embedded in amorphous matrix, the main component of which is a procollagen-like material associated with glycoproteins [Kefalides NA, 1978]. The basement membrane contains pores and is negatively charged due to the presence of glycosaminoglycans rich in heparan sulphate [Kanwar YS and Farquar MG, 1979a., Kanwar YS and Farquar MG, 1979b]. It has been suggested that the glycosaminoglycans play a role in establishing the permeability properties of the basement membrane to plasma proteins [Kanwar YS *et al,* 1980., Rosenweig LJ and Kanwar YS, 1982]. Other matrix proteins found in the basement membrane include laminin [Madri JA *et al*, 1980., Scheinman JI *et al*, 1980a., Courtoy PJ *et al,* 1982] and small amounts of fibronectin [Courtoy PJ *et al,* 1980., Courtoy PJ *et al,* 1982]. On the external surface of the basement membrane lie the visceral epithelial cells or podocytes which are the largest of the glomerular cell population. These cells make contact with the external surface of the

basement membrane via individual foot processes which extend out from the main cell body. Gaps between the foot processes form filtration slits or slit pores which, like in the endothelium, are covered by thin membranes or diaphragms [Latta H, 1970., Rodewald R and Karnovsky MJ, 1974].

Having crossed the epithelial cell layer the ultrafiltrate reaches the urinary space of the Bownmans capsule, the outer wall of which is composed of squamous parietal epithelial cells sitting on their basement membrane. This fluid is then funnelled into the proximal tubule of the nephron. The ultrafiltrate is subsequently extensively modified by the various nephron segments to produce urine.

1.2 The Mesangium

The central, connective tissue-like space of the capillary tuft first was described by Zimmerman in 1933. This space is now known as the mesangium. The mesangium serves as the structural support for the complex capillary network which forms the glomerular tuft. It also plays a regulatory role in modulating the filtration surface of the glomerular basement membrane. This highly specialised intercapillary tissue is composed predominantly of contractile mesangial cells expressing myofibroblast-like properties [Schlondorf D, 1987] and a small number of resident phagocytes or macrophages [Schreiner GF and Unanue ER, 1984b], embedded in extracellular matrix. In order to maintain the normal structure and function of the mesangium, the proliferative and secretory activities of mesangial cells must be tightly regulated. A continuous flow of molecules from the systemic circulation perfuses into the mesangial area via the fenestrations of the glomerular endothelium. These molecules may be endocytosed by

mesangial cells or accumulate in the matrix. If the perfusate contains cytokines, growth factors or immune complexes these may activate mesangial cells to proliferate or to alter their secretory phenotype. The secreted products could then interact with other glomerular cells in a paracrine fashion. During acute injury, the mesangium is susceptible to the effects of an additional influx of inflammatory cells. These may subsequently disturb the complex events controlling the maintenance of the mesangium including cell proliferation and secretion. Expansion of the mesangium due to hypercellularity as a result of proliferation or infiltration and the augmented accumulation of extracellular matrix are the histological hallmarks of most types of progressive glomerular diseases [Klahr S *et al*, 1988].

The relationship between mesangial cells, infiltrating macrophages and matrix production is central to this thesis, therefore these topics will be discussed in greater detail.

1.3 Mesangial cells

Mesangial cells represent approximately one third of the total glomerular cell population [Olivetti G *et al,* 1977]. They are found embedded in the amorphous extracellular matrix located between the capillaries of the glomerular tuft. A system of tethers anchor the mesangial cells to the basement membrane of the capillary loops [Sakai T and Kriz W, 1987]. The cells can be recognised by their irregular shape as a result of multiple elongated cytoplasmic processes which extend around the capillary lumen and between the basement membrane and the overlying endothelium. Their cytoplasm contains a regular dense nucleus, a well developed rough endoplasmic and golgi apparatus, large numbers of mitochondria and occasional cytoplasmic granules [Latta H, 1973]. Mesangial cells also

possess an organised network of myosin filaments and microtubules [Andrews PM and Coffey AK, 1983]. Actin bundles span the entire length of the mesangial cell processes establishing contact with the glomerular basement membrane. These cytoskeletal elements form the contractile apparatus of the cell and allow the mesangial cells to exert mechanical traction on the glomerular basement membrane and endothelial capillary lining so that minor changes in mesangial cell volume will affect both the filtering surface area of the glomerulus and glomerular haemodynamics. Mesangial cell contraction can also be stimulated by a variety of vasoactive agents including angiotensin II, vasopressin, noradrenaline, thromboxane, leukotrienes and endothelin [Schlondorff D, 1987., Kreisberg J *et al,* 1985., Ausiello DA *et al*, 1980., Badr KF *et al,* 1989]. Receptors for angiotensin II, vasopressin and endothelin have been demonstrated on the mesangial cell surface [Schlondorff D, 1987., Simonsen MS *et al*, 1989b]. In contrast agents such as prostaglandin E_2 (PGE_2), atrial peptides and dopamine cause mesangial cell relaxation [Schlondorff D, 1987] thus providing a counter-regulatory mechanism to the effects of vasoconstrictors.

Mesangial cell express Fc receptors on their surface which enables them to endocytose immune complexes [Schlondorff D, 1987]. Binding and cross-linking of the Fc receptors by IgG is required to stimulate increases in intracellular calcium ($[Ca^{2+}]_i$) and prostanoid synthesis or to reduce mesangial cross-sectional area [Knauss TC *et al*, 1989]. Other investigators have reported that phagocytosis of opsonized zymosan by cultured mesangial cells is associated with the production of prostaglandins, reactive oxygen species and lipoxygenase products [Baud L *et al,* 1983]. Mesangaial cells have also been shown to ingest neutrophils undergoing apoptosis [Savill J *et al,* 1992]. Phagocytosed material is

thought to be cleared from the mesangium by cell to cell transport to the extra glomerular mesangial region at the vascular pole of the glomerular tuft [Elema JD *et al*, 1976]. Much of the phagocytic capability of the mesangium however, is associated with the resident macrophages [Schreiner GF *et al,* 1981., Schreiner GF and Cotran RS, 1982].

Mesangial cells have an extensive secretory profile releasing a variety of growth factors and cytokines including platelet-derived growth factor (PDGF), interleukin-1 (IL-1), interleukin-6 (IL-6), insulin-like growth factor (IGF) and transforming growth factorß (TGFß). Receptors for these cytokines have also been detected on the mesangial cell membrane thus enabling these cytokines to function as autocrine growth factors maintaining normal mesangial cell proliferation and turnover [Shultz PJ *et al*, 1988., Horii Y *et al*, 1989., Lovett DH *et al*, 1986., Conti FG *et al*, 1988]. Other secretory products include numerous vasoactive substances such as prostaglandins [Sraer J *et al*, 1979], reactive oxygen species [Baud L and Ardaillou R, 1986], proteinases and proteinase inhibitors [Lovett DH *et al*, 1983b., Lacave R *et al,* 1989., Davies M *et al*, 1988].

During the course of progression to glomerulosclerosis mesangial cells have been shown to undergo phenotypic modulation and functional changes [Johnson RJ, 1994]. Most notably mesangial cells aquire smooth muscle cell characteristics including the expression of αsmooth muscle actin as well as intermediate filament proteins such as vimentin and desmin. The expression of α-smooth muscle actin has been particularly associated with mesangial cell proliferation during the course of experimental models of anti-Thy-1 nephritis [Johnson RJ *et al*, 1991] and nephrotoxic serum nephritis [Zhang G *et al*, 1995]. The mechanisms involved in the induction of α-smooth muscle actin during the course of

glomerular injury remain to be fully elucidated although growth factors such as TGFß and PDGF and matrix proteins such as fibronectin are thought to play a role [Desmouliere A and Gabbiani G, 1994].

Our understanding of the properties and functions of mesangial cells have been largely derived from cell culture studies. Mesangial cells in culture retain many of the morphological and ultrastructural characteristics of mesangial cells *in vivo*. They have a characteristic stellate or fusiform morphology and grow in irregular bundles of intertwined cells [Mahieu PR *et al*, 1980]. With increasing age the cultures form ridges of multilayered cells and eventually nodular protrusions or hillocks become visible [Sterzel RB *et al*, 1986]. However, fundamental differences also exist: *in vivo* mesangial cells grow in a three dimensional configuration surrounded by matrix and exposed only to plasma. They proliferate very slowly with a life span of 50-100 days [Pabst R and Sterzel RB, 1983] and are, for the most part, in a state of quiescence. Cultured mesangial cells, on the other hand, are usually grown on a two dimentional surface and are exposed to non autologous serum. They proliferate with a doubling time of approximately 1.5 days [Simonsen MS and Dunn MJ, 1986] although they can be rendered quiescent by growing the cells in low concentrations of serum over 3-4 days. Some investigators suggest that mesangial cells in culture may actually mimic the state of glomerular inflammation *in vivo* rather than normal quiescence [Lovett DH and Sterzel RB, 1986a., Striker GE and Striker LJ, 1985]. As with all cell culture systems data from cultured mesangial cells must be carefully interpreted.

1.4 Mesangial macrophages

Macrophages are the other resident cell type in the mesangium. They comprise between 5 and 15% of the total number of cells present in the normal glomerulus [Schreiner GF *et al*, 1981., Schreiner GF and Cotran RS, 1982]. These resident macrophages are bone marrow derived mononuclear leukocytes analogous to the Kupffer cells of the liver or the alveolar macrophages of the lung. They express leukocyte common antigen (LC), a membrane glycoprotein characteristic of haematopoietic cells, on their surface [Schreiner GF and Unanue ER, 1984a]. A proportion of these cells additionally express Ia (immune associated) antigens, membrane proteins encoded in the I region of the major histocompatability complex (MHC) [Schreiner GF *et al*, 1981]. Resident macrophages also express Fc and C3 receptors and are capable of phagocytosis both *in vivo* and *in vitro* [Schreiner GF *et al,* 1981]. The function of these resident macrophages is as yet unknown, although they are thought to play a role in normal glomerular physiology and/or participate in routine immune surveillance. Under certain circumstances these normally quiescent cells are capable of becoming highly activated. Macrophage activation is regarded as a complex and mulitistep process modulated via a number of extracellular signals [Adams DO and Hamilton TA, 1992]. Infiltrating macrophages particularly, are an heterogeneous population with respect to their degree of activation. The expression of membrane antigens such as class II MHC (Ia antigens) [Schreiner GF *et al*, 1984a., Cook HT *et al*, 1989] and secretion of mediators such as reactive oxygen species [Cook HT *et al*, 1989., Boyce NW *et al,* 1989], cytokines [Matsumo K and Hatano M, 1989., Tipping PG *et al,* 1991a., Tipping PG *et al*, 1991b], eicosanoids [Cook HT *et al,* 1989], plasminogen activator [Tipping PG *et al*, 1988] and nitric oxide [Cook HT and Sullivan R, 1991] have been associated as markers of macrophage activation. Over 100 products are so far known to be

secreted by macrophages in various states of activation [Nathan CF, 1987], although not all macrophage products are simultaneously secreted by a single macrophage at any one time. The composition or secretory phenotype will depend on local environmental conditions.

The turnover time for resident cells is approximately 4 days [Schreiner GF and Unanue ER, 1984b., Gurner A *et al,* 1986], although infiltrating macrophages may persist for a number of weeks if not months [Van Furth R, 1980].

1.5 Mesangial matrix

It was originally thought that the mesangial matrix was inert and static and served only to provide the resident glomerular cells with support and stability. However, this has now been found not to be the case. The extracellular matrix is a dynamic structure and has a profound physiological effect upon intrinsic glomerular cells both under normal and pathophysiological conditions [Hay ED, 1983., Hewitt T and Martin GR, 1984]. The cells synthesise the matrix components and control their polymerisation and assembly whilst the matrix itself dictates the organisation and function of the cells [Hay ED, 1983., Hewitt T and Martin GR, 1984., Ruoslahti E *et al*, 1985] - they are in effect mutually dependent on one another. The matrix is strong yet flexible and provides support for the glomerular capillaries and allows for the formation of channels for filtering and processing macromolecules. The mesangial matrix is biochemically well characterised, although its effects on mesangial cell function are yet to be fully determined. It consists mainly of glycoproteins embedded in a hydrated polysaccharide gel of glycosaminoglycans arranged in a lattice structure [Grond J and Elema JD, 1985]. Like other matrices the mesangial matrix contains collagens, adhesive glycoproteins and glycosaminoglycans.

1.5.1 Collagens

The characteristic feature of collagen molecules is the triple-stranded helical structure [Parry DAD, 1988]. These collagen chains, termed α chains, are organised around one another in a regular superhelix to form a cord-like collagen molecule. Collagens contain large quantities of proline and glycine residues, which contribute to the formation of the triple helix. About 20 distinct collagen α chains have been identified each encoded by separate genes [Martin GR *et al*, 1985]. They assemble in various combinations, giving rise to a number of different collagen types [Burgeson RE, 1988]. The most abundant collagens found in the mesangium are types IV and V [Scheinman JI *et al,* 1980b]. Collagen IV is well designed for mesangial function since it appears as a continuous sheet forming open networks with tensile strength, high elasticity and an open, largely non fibrillar structure to aid the passage of fluids [Bornstein P and Savage H, 1980., Hewitt T and Martin GR, 1984., Martin GR and Timpl R, 1987., Timpl R, 1986]. Collagens I and III are present in fibrils and large vessels surounding the glomerulus. Under normal circumstances these interstitial collagens are not detectable in the glomerulus. However, they are known to be expressed in the mesangium and other sclerotic lesions in a variety of glomerular diseases [Oomura A *et al*, 1989., Yoshioka K *et al,* 1989., Yoshioka K *et al,* 1990]. The interstitial collagens are inelastic and highly ordered forming crosslinked bundles that make the matrix inflexible and less permeable [Hay ED, 1983., Hewitt T and Martin GR, 1984]. Paradoxically, mesangial cells in culture constitutively secrete collagens I and III mimicking the pathological state. This anomaly may be an artifact of tissue culture [Sterzel RB *et al,* 1986].

1.5.2 Glycosaminoglycans/Proteoglycans

The mesangial matrix is a rich source of proteoglycans, long unbranched polysaccharide chains of repeating, often sulphated, disaccharides composed of uronic acid and either N-acetyl-glucosamine or N-acetyl-galactosamine [Toole BP, 1981], of which heparan sulphate and chondroitin sulphate predominate [Brown DM et al, 1981., Farquar MG, 1981., Houser MT *et al*, 1982., Kanwar YS *et al*, 1983a., Kanwar YS *et al*, 1983b]. With the exeption of heparan sulphate, all glycosaminoglycans are covalently linked to a core protein in the form of proteoglycans [Ruoslahti E, 1988a]. Their ability to bind water and form hydrated gels provides the matrix with turgor and resistance to compressive forces and hence they regulate the visco-elastic properties of the matrix [Hewitt T and Martin GR, 1984., Hook M *et al*, 1984., Timpl R, 1986]. Although their precise biological roles are uncertain the cell associated proteoglycans are thought to act as receptors for plasma macromolecules. Through their interactions with collagen and glycoproteins they stabilise the marix and play a role in growth control [Hook M *et al*, 1984]. Their high negative charge allows them to function as molecular sieves and cation exchangers regulating selective transport of macromolecules [Kanwar YS *et al,* 1983a., Kanwar YS *et al,* 1983b].

1.5.3 Glycoproteins

The most important function of glycoproteins is to mediate cell adhesion to neighbouring cells and matrix and to stabilise the supramolecular organisation of mesangial matrix [Courtoy PJ *et al*, 1980]. Three major adhesive glycoproteins are located in the mesangium: fibronectin, laminin and thrombospondin.

Fibronectin is a large 450kD glycoprotein occurring as a soluble protein in plasma and as a cell associated protein of most mesenchmal cell types [Yamada KM and Olden K, 1978]. It is the major component of the mesangial matrix [Courtoy PJ *et al*, 1980., Courtoy PJ *et al,* 1982., Linder EA *et al,* 1980., Stenman S and Vaheri A, 1978., Weiss MA *et al*, 1979]. It consists of two disulphide linked subunits (A and B chains) each having a molecular weight of around 200 kD [Hynes RO, 1990., Mosher DF, 1989., Akiyama SK and Yamada KM, 1987., Ruoslahti E, 1988b]. Both subunits are transcribed from a single gene. However, as a result of alternate splicing in three regions of the primary transcript multiple mRNAs exist and these contribute to the polymorphism and diversity of function of the fibronectin protein [Hynes RO, 1985., Zardi L *et al,* 1987]. TGFß for example, preferentially increases the expression of one isoform of fibronectin called fibronectin ED-A (extra domain-A) in human cultured fibroblasts [Balza E *et al*, 1988] and in human tubular epithelial cells [Viedt C *et al*, 1995]. Fibronectin has a multi domain structure typical of large extracellular matrix proteins. Each domain presents high affinity binding sites for other components of extracellular matrix, among them collagen, heparan sulphate and fibrin [Hynes RO, 1990., Ruoslahti E, 1988b., Buck CA and Horowitz AF, 1987]. These domains or interaction sites for other matrix proteins play an important role in matrix assembly [M^{c}Donald JA, 1988]. At least two cell binding domains exist and provide binding sites for specific cell surface receptors [Hynes RO, 1990., Ruoslahti E, 1988]. The cell binding domains of fibronectin contain a tripeptide sequence formed from the amino acids arginine, glycine and asparagine (RGD). This sequence is specifically recognised by receptors or intergrins on the mesangial cell surface [Ruoslahti E and Pierschbacher MD, 1987., Simonsen MS *et al*, 1989a]. These mesangial cell receptors mediate interactions of the mesangial cytoskeleton and extracelluar matrix

[Yamada KM, 1991]. With limited proteolysis these domains can be cleaved into separate functionally independent entities [Hynes RO, 1990., Ruoslahti E, 1988b]. The structure of fibronectin therefore, confers a number of functional properties upon the molecule. Other properties of fibronectin include promoting localisation and uptake of circulating immune complexes [Cosio FG and Bakaletz AP, 1987], stimulating proliferation in quiescent mesangial cells [Simonsen MS *et al,* 1989a] and controlling cell differentiation and migration of embryonic and tumour cells [reviewed Hynes RO and Yamada KM, 1982]. It is of interest to note that fibronectin is absent from the surface of transformed cells which may, in part, explain the altered behaviour of these cells [Hayman EG, *et al,* 1981]. Immunohistochemical studies on tissue sections have shown that fibronectin is localised intracellularly within the stacked Golgi cisternae, suggesting secretion by mesangial cells *in vivo* [Courtoy PJ *et al,* 1980]. Fibronectin is especially concentrated between mesangial cells and endothelial cells where it modulates the attachment of these cells to one another and to the mesangial matrix [Courtoy PJ *et al,* 1980., Courtoy PJ *et al,* 1982]. In most pathophysiological conditions of the glomerulus the magnitude and distribution of fibronectin are found to be increased [Ikeya *et al,* 1985,. Petterson EA and Colvin RB, 1978].

Laminin is a 850 kD sialoglycoprotein found in low concentrations in the mesangium [Courtoy PJ *et al,* 1982]. It is however, the major adhesive protein in the basement membrane [Rohde H *et al,* 1979]. It consists of three disulphide-linked polypeptide chains which, like fibronectin, express high affinity binding sites for matrix components and cell surface receptors [Martin GR and Timpl R, 1987].

Thrombospondin is a 450kD trimer of three nearly identical subunits [Wight TN *et al*, 1985]. Mesangial cells synthesise and secrete this matrix protein. Intracellularly it is localised in the rough endoplasmic reticulum. Immunoelectron microscopy studies have shown it to be evenly distributed across the plasma membrane and cytoplasmic extensions [Raugi GJ and Lovett DH, 1987]. The influence of disease on its distribution are still largely unknown.

1.6 Matrix degradation

The steady state levels of mesangial matrix are strictly controlled by a balance between matrix synthesis and degradation. Matrix breakdown usually depends on the synthesis and secretion of various proteinases. These enzymes fall into four major classes referred to as aspartic, cysteine, serine and metallo-type proteinases. The metalloproteinases are considered to be the major physiological regulators of matrix degradation [Birkedal-Hansen H *et al*, 1993]. These enzymes are further divided into three major groups, collagenases, gelatinases and stromelysins the latter of which have the broadest substrate specificity. Collectively the metalloproteinases are capable of degrading all the major components of extracellular matrix. These enzymes may be secreted constitutively or induced by a variety of hormones, growth factors and cytokines [Salo T *et al*, 1991., Katsura M *et al*, 1989] with the effect of any particular factor being dependent on cell type and the presence of synergistic or antagonistic factors. For example, rat mesangial cells, coincubated with elicited rat peritoneal macrophages or crude macrophage conditioned media have been shown to upregulate gelatinase activity [Martin J *et al*, 1986]. In the same series of experiments IL-1, purified from the conditioned medium was also able to induce gelatinase activity, albeit to a lesser degree than the conditioned medium suggesting, that the levels

of the proteinase were being controlled via multiple factor interactions. Metalloproteinases, and by definition matrix degradation, can also be controlled via the secretion of specific tissue inhibitors of matrix metalloproteinases, the TIMPs. These inhibitors are also differentially regulated by a variety of factors [Shingu M *et al,* 1993, Doherty AJP *et al,* 1985]. The net amount of matrix degradation will therefore, depend upon on the relative activities of specific proteases and protease inhibitors.

1.7 Glomerulosclerosis

Expansion of the mesangium and deposition of extracellular matrix are the histological hallmarks of progressive glomerulosclerosis [Klahr S *et al,* 1988]. It is generally accepted that once a critical number of nephrons have been damaged, the scarring process with progressive loss of renal function occurs even when the initial cause of injury is no longer present [Klahr S *et al,* 1988]. Despite much investigation the pathogenesis of glomerulosclerosis remains to be fully understood. It is clear however, that the aetiology of progressive renal scarring is complex and multifactorial with haemodynamic [Brenner BM *et al,* 1982], metabolic [Diamond JR and Karnovsky MJ, 1988., Keane WF *et al,* 1991], immunologic [Matsumoto K *et al,* 1982., Matsumoto K *et al,* 1983] and genetic [Weening JJ *et al,* 1986] factors all being involved. Regardless of aetiology, the histopathology of the "end stage" kidney is the same. It is characterised by obliteration of the glomerular capillary tuft, localised areas of hypercellularity due to intrinsic cell proliferation and inflammatory cell infiltration and the deposition of extracellular matrix. The composition of deposited matrix may vary between diseases but it generally contains increased amounts of matrix proteins normally present in the glomerulus such as type IV collagen, fibronectin, laminin and heparan sulphate proteoglycan as well as those which

are only produced under pathological conditions such as the interstitial collagens types I and III [Abrass CK *et al*, 1988., Adler S *et al*, 1986., Downer G *et al*, 1988., Falk RJ *et al*, 1983., Striker LM *et al*, 1984]. This suggests that the different aetiologies ultimately feed into a common pathway which results in the replacent of the nephron with a fibrous scar. Some of the causative factors that lead to glomerulosclerosis are described below.

1.7.1 Role of glomerular hypertension

The partial nephrectomy model has been extensively studied to assess the role of glomerular hypertension in promoting glomerulosclerosis. Following a critical loss of renal mass a compensatory increase in single nephron glomerular filtration rate occurs in the remaining glomeruli. This increase is mediated, in part, by increased single nephron plasma flow rates and intracapillary hydraulic pressure [Hostetter TH *et al*, 1981]. These compensatory responses appear to be maladaptive since proteinuria and further glomerular damage ensue. Manoeuvres aimed at reducing glomerular hypertension such as angiotensin converting enzyme (ACE) inhibitors [Anderson S *et al*, 1986., Meyer TW *et al*, 1985] or low protein diets [Brenner BM, 1985] have been shown to markedly decrease the rate of development of glomerulosclerosis and reduce the degree of proteinuria. This has led to the hypothesis that that glomerular capillary hypertension and glomerulosclerosis are causally linked [Anderson S *et al*, 1985].

1.7.2 Role of glomerular hypertrophy

The participation of other factors contributing to glomerulosclerosis arose from experimental observations describing glomerulosclerosis in the absence of glomerular hyperfiltration. Evidence from serial micropuncture studies on glomeruli from remnant

kidneys found that there was no correlation between glomerular capillary pressure and glomerulosclerosis [Yoshida Y *et al*, 1988]. Moreover, treatment of remnant rats with anti-hypertensive triple drug therapy markedly reduced glomerulosclerosis without an effect on glomerular hypertension [Yoshida Y, 1989a]. It has thus been argued that compensatory glomerular hypertrophy, rather than hyperperfusion or altered haemodynamic parameters, may be the more important causative factor in progression to sclerosis [Fogo A and Ichikawa I, 1989., Yoshida Y *et al,* 1989b]. Glomerular hypertrophy is now known to precede the development of sclerosis in renal ablation, diabetic nephropathy, following glucocorticoid exposure and after placement on a high protein diet [Klahr S *et al*, 1988].

1.7.3 Role of epithelial cells

Glomerulosclerosis can also preceded by alterations in glomerular epithelial cells [Hostetter TH *et al*, 1981., Olson JL *et al*, 1982., Okuda S *et al*, 1986., Glasser RJ *et al*, 1977., Nakamura T *et al*, 1986]. *In vitro* studies by Castellot and co-workers demonstrated that cultured glomerular epithelial cells secrete a heparin-like molecule which is capable of inhibiting mesangial cell proliferation [Castellot JJ *et al*, 1985]. Heparan sulphate (a heparin-like molecule) is known to be a natural secretory product of intact epithelium [Foidart JB *et al*, 1980] and as such may exert a suppressive regulatory control over mesangial cell activation. Since most forms of glomerulosclerosis are associated with both epithelial injury as well as mesangial cell activation [Olson J, Heptinstall R, 1989], it is conceivable that some degree of mesangial cell proliferation results from a loss of regulatory control as a consequence of epithelial cell damage [Schreiner GF, 1990].

1.7.4 Role of pro-coagulant activity

Intra-glomerular thrombus formation is a factor thought to play a role in the development of glomerulosclerosis. Aggregation and activation of platelets at sites of glomerular capillary endothelial cell injury may cause the release of platelet products such as platelet activating factor (PAF) and PDGF which could stimulate mesangial cell proliferation and matrix protein secretion [Bonventre JV and Weber PC, 1988].

Thrombin has been reported to stimulate TIMP-1 and collagen I synthesis in human mesangial cells [Kaizuka M *et al*, 1995]. α-Thrombin has also been shown to stimulate the production and gene transcription of monocyte chemotactic protein (MCP-1), an important mediator of monocyte recruitment [Grandalino G *et al*, 1994].

The anti-coagulants heparin and warfarin have also been reported to inhibit glomerulosclerosis in rats with partial renal ablation [Klahr S *et al*, 1986., Olsen J and Heptinstall R, 1989]. However, since nonanticoagulant heparin also showed similar effects in PAN nephrotic rats and in the remnant kidney model [Diamond JR and Karnovsky MJ, 1986., Purkerson ML *et al*, 1988] the data supported an effect of heparin independent of its anti-coagulant capacity.

1.7.5 The role of hyperlipidaemia

In 1982 Moorhead and associates hypothesised that abnormal lipid metabolism may play a role in the progression of initial glomerular injury to glomerulosclerosis. Diet induced hypercholesterolaemia of otherwise normal animals results in the development of moderate glomerulosclerosis - the degree of injury correlating with circulating lipid levels [Keane WF *et al*, 1991]. Moreover, superimposition of hypercholesterolaemia on pre-existing glomerular disease such as PAN nephrosis [Hirano T *et al*, 1992., Diamond JR and

Karnovsky MJ, 1987], renal ablation [Rayner HC *et al*, 1991., Kasiske BL *et al*, 1990] or hypertension [O'Donnell MP *et al*, 1992., Tolins JP *et al*, 1992] increases the magnitude and severity of glomerulosclerosis. In contrast pharmacologically lowering lipid levels has been shown to ameliorate the amount of renal injury in several experimental models of renal disease [Kasiske BL *et al*, 1988a., Kasiske BL *et al*, 1988b].

With the exception of certain lipid disorders such as lecithin-cholesterol acyltransferase deficiency [Norum KR *et al*, 1989] or lipoprotein glomerulopathy [Oikawa S *et al*, 1991., Saito T *et al*, 1993] the occurence of renal damage in the majority of patients with hyperlipidaemia is uncommon, suggesting that additional factors are required in humans for lipid-mediated renal injury. Lipids may, however, serve as modulators of renal damage.

The mechanism by which hyperlipdaemia exerts its detrimental effects on mesangial structure or function is not as yet fully understood. Low density lipoproteins (LDL) may bind to glycosaminoglycans of the GBM or mesangial matrix and neutralise the anionic sites of the filtration barrier [Simpson LO, 1981]. LDL entering the mesangium via the endothelial fenestrations may have direct effects on mesangial cells by promoting a series of cellular events conducive to the scarring process; *in vitro* LDL have been shown to stimulate mesangial cells to upregulate fibronectin and collagen synthesis [Rovin BH and Tan LC, 1993., Keane WF *et al,* 1992] as well as inducing the production of MCP-1 and PDGF [Rovin BH and Tan LC, 1993., Grone E *et al*, 1992]. Other investigators have demonsrated that LDL can induce mesangial cell proliferation or, when oxidised, may be more cytotoxic than LDL [Moorhead J *et al*, 1989., Wheeler DC *et al*, 1994].

It has now become increasingly evident that macrophages play an important role in modulating lipid-induced glomerular injury. Interactions between macrophages and lipoproteins within the glomerulus may parallel those occuring in the arterial cell wall in the process of atherosclerosis [Diamond JR, Karnovsky MJ, 1988]. Evidence exists that diet induced hypercholesterolaemia can alter several variables of macrophage function in such a way that would contribute to lesion formation in atherosclerosis [Rogers KA *et al,* 1986]. These changes include increased adhesion to endothelial cell monolayers and increased secretion of smooth muscle chemoattractants and mitogenic factors. Other investigators have demonstrated that peritoneal macrophages from PAN nephrotic and diet induced hypercholesterolaemic rats were elicited in greater numbers, exhibited a greater degree of phagocytosis and showed a trend towards increased production of thromboxane B_2 than control rats. All of these parameters were synergistically increased in macrophages from nephrotic rats on a high cholesterol diet [Diamond JR *et al,* 1989a]. It is plausble therefore, that such alterations of macrophage function in response to hyperlipidaemia could enhance their ability to modulate the progression to glomerulosclerosis. Macrophages take up oxidised LDL via scavanger receptors on their surface and become foam cells [Schonholzer KW *et al*, 1992]. Foam cells, like their parent macrophages, can release growth factors and cytokines inducing mesangial matrix synthesis and proliferation [Schreiner GF, 1991]. The degree of mesangial proliferation has been shown to correlate with the number of infiltrating macrophages in PAN nephrosis, a condition which is associated with secondary hyperlipdaemia [Diamond JR *et al*, 1992].

1.8 The macrophage in glomerular injury

An influx of macrophages into the mesangium is seen in response to renal injury. Accumulation of phagocytic cells was first demonstrated in patients with crescentic glomerulonephritis [Atkins RC *et al*, 1976], although evidence now suggests that glomerular macrophage infiltration occurs in most forms of glomerulonephritis [Hooke DH *et al,* 1987., Ferrario F *et al,* 1985., Markovic-Lipkowski J *et al,* 1990., Kobayashi M *et al*, 1991]. A variety of factors have been implicated in the recruitment of macrophages into the glomerulus. It is thought that following the initial insult chemotactic or activating substances such MCP-1 [Leonard EJ and Yoshimura T, 1990] or lipid derived chemotactic molecules [Rovin BH *et al*, 1990a] are released or secreted by intrinsic glomerular cells which directly attract an influx of monocytes. Margination of the leukocytes into the tissue space then involves the co-ordinated expression of, and interaction between endothelial leukocyte adhesion molecules and specific ligands expressed on the activated leukocytes [Springer TA, 1990., Mackay CR and Imhof BA, 1993]. Adhesion molecules, particularly ICAM-1 and VCAM-1 play a vital role in leukocyte migration from the systemic circulation into the mesangium [Tamatani T *et al*, 1991]. The functional importance of ICAM-1/LFA-1 interactions has been demonstrated in studies wherein treatment of nephritic WKY rats with monoclonal antibodies against rat ICAM-1 or LFA-1, prevented the development of proteinuria, monocyte influx and crescent formation [Kawasaki K *et al*, 1993., Nishikawa H *et al*, 1993]. *In vitro* studies have further demonstrated that anti-ICAM-1 antibodies were able to inhibit the binding of monocytes to cytokine stimulated mesangial cells [Brady HR *et al*, 1992]. Cytokines and particularly TNFα and IL-1ß have been shown, at least *in vitro,* to be potent stimulators of the adhesion process via the induction of leukocyte intergrins or their endothelial cell ligands [Denton MD *et al,* 1991].

Accumulating evidence, largely from animal studies, has focused on the important pathogenetic role played by infiltrating macrophages in the progression of renal injury. In PAN nephrosis, for example, a macrophage infiltrate is observed at day 11 following administration of the toxin, coinciding with peak proteinuria [Schreiner GF *et al*, 1984a]. A glomerular macrophage influx is also an early response to renal ablation, a lesion known to result in glomerulosclerosis [Harris KPG *et al,* 1993., Van Goor H *et al,* 1991., Van Goor H *et al*, 1992., Floege J *et al*, 1992a]. In the uninephrectomy model for example, an infiltrate occurs within 1-2 weeks of surgery, before the observation of histological changes by light microscopy, the detection of microalbuminuria or the development of renal scarring [Harris KPG *et al,* 1993].

Further evidence in support of macrophage-associated injury has come from studies using an essential fatty acid deficient (EFAD) diet. The first observation in this context was made in an experimental murine model of lupus nephritis. Animals placed on an EFAD diet were protected from renal failure, despite the persistance of immune complexes and complement in the glomerulus [Hurd ER *et al*, 1981]. Schreiner's group hypothesised that the beneficial effects of an EFAD diet were due to a significant depletion of infiltrating macrophages [Lefkowith J and Schreiner GF, 1987., Schreiner GF *et al*, 1988]. An EFAD diet has subsequently been shown to be protective in PAN nephrosis [Diamond JR *et al*, 1989b] and in anti-GBM nephritis [Schreiner GF *et al*, 1989]. In all cases there was a significant reduction of glomerular macrophages whilst effects on neutrophils or other aspects of immune function were unchanged. Supplementation of the EFAD diet with linoleic acid resulted in repopulation of the glomerulus with macrophages [Lefkowith J and Schreiner GF, 1987]. Interestingly, release of a lipid chemotactic factor was also found to be inhibited under conditions of this diet [Rovin BH *et al*, 1990b].

Whole body X-irradiation is another macrophage depleting manoeuvre which has been shown to attenuate subsequent progression to glomerulosclerosis in several experimental models of renal injury [Diamond JR and Pesek-Diamond I, 1991a., Schreiner GF *et al*, 1978., Van Goor H *et al*, 1992]. In addition, specific macrophage depletion using anti-macrophage serum has been shown to abrogate injury in acute serum sickness and passive autologous nephrotoxic nephritis in rabbits [Holdsworth SR *et al*, 1981., Lavelle KJ *et al*, 1981]. Macrophage repletion studies in the nephritic rabbits have been shown to partially restore injury in animals whose macrophages had been previously depleted with nitrogen mustard [Holdsworth SR and Neale TJ, 1984].

Since macrophages are a rich source of cytokines and growth factors it is possible that these peptides mediate some of the adverse effects of macrophages. In PAN nephrosis for example, the macrophage infiltrate occurs in temporal association with an increased glomerular TGFß and fibronectin gene expression [Ding G *et al*, 1993]. Furthermore, immunohistochemical labelling studies have demonstrated that the TGFß protein staining corresponds locally with ED1 positive macrophages [Ding G *et al*, 1993]. When dietary hypercholesterolaemia was superimposed on the PAN nephrotic rats their underlying injury was aggravated in parallel with a further increase in the magnitude of the macrophage infiltrate and a concomitant upregulation of glomerular TGFß gene expression [Ding G *et al,* 1993]. More recently it has been demonstrated that TGFß message is expressed by glomerular macrophages isolated from PAN nephrotic or diet induced hypercholesterolaemic rats. Similarly, peritoneal macrophages from the PAN nephrotic animals also expressed significantly more TGFß mRNA than did macrophages from normal rats thus identifying the macrophage as a potential source of the profibrogenic cytokine

[Ding G *et al*, 1994]. In PAN nephrosis then, not only are there more glomerular macrophages but these macrophages appear to be in a more activated state.

To date, the conventional view is that macrophage accumulation during glomerulonephritis results almost exclusively from the recruitment of circulating blood monocytes [Van Furth R, 1988]. Some investigators have suggested that due to the low proliferative rate of inflammatory macrophages, proliferation of these cells is unlikely to account for the expanded macrophage population [Auger MJ and Ross JA, 1992]. However, in the light of recent animal studies the consensus is now changing. Ren KJ *et al,* [1991] reported a significant increase in the rate of glomerular macrophage proliferation in association with the onset of proteinuria in experimental immune complex nephritis. These investigators further demonstrated that glomerular macrophages from rats with proliferative nephritis also divided more vigorously than macrophages from normal rats in short term culture. Similarly, Lan HY *et al,* [1995] reported that following an initial influx as much as 62% of the total macrophage population (glomerular and interstitial) could be accounted for by proliferating macrophages. Significant increases in the number of proliferating macrophages within the kidney have also been reported during acute allograft rejection [Kerr PG *et al*, 1994] and in anti-Thy-1 disease [Johnson RJ *et al*, 1995].

The fate of the expanded population of macrophages is uncertain, they could die *in situ* or migrate to a different location. There is some evidence to suggest that they migrate into the periglomerular interstitium [Lan HY *et al*, 1991]. Other authors have demonstrated that during experimental glomerulonephritis there is significant trafficking of the infiltrate to the kidney lymph nodes. This may provide a mechanism whereby nephritogenic antigens,

released as a result of the local inflammatory response, may be presented to T and B lymphocytes within the lymph nodes, resulting in the amplification of the immune response in glomerulonephritis [Lan HY, 1993a].

Van Goor H *et al* [1991] carried out a comprehensive statistical study examining a variety of glomerular changes associated with the progression of focal sclerosis in the remnant kidney model. These authors identified serum cholesterol and protein excretion as the major clinical determinants and mesangial cell proliferation and glomerular macrophage influx as the major structural variables that correlated with increased susceptablity to focal sclerosis.

Macrophage infiltration then, is strongly correlated with renal injury and makes a significant contribution to the marked alterations in mesangial structure that result in the development of glomerulosclerosis in both immune and non immune models of glomerular injury. However, the precise mechanisms whereby the increased number of glomerular macrophages results in the induction of the sclerotic process are yet to be fully understood. Macrophages (both resident and inflammatory) are a rich source of peptide growth factors and cytokines and it is plausible that these secretory products go on to modulate extracellular matrix protein production in, and proliferation of, resident glomerular cells as well as altering other functional parameters such as modifying glomerular basement membrane permeability and regulating blood flow. In order to understand the precise mechanisms involved in this process it is necessary to identify the factors which sustain glomerular injury and perhaps more importantly to define the nature of the interactions among such factors that facilitate the progression towards glomerular destruction.

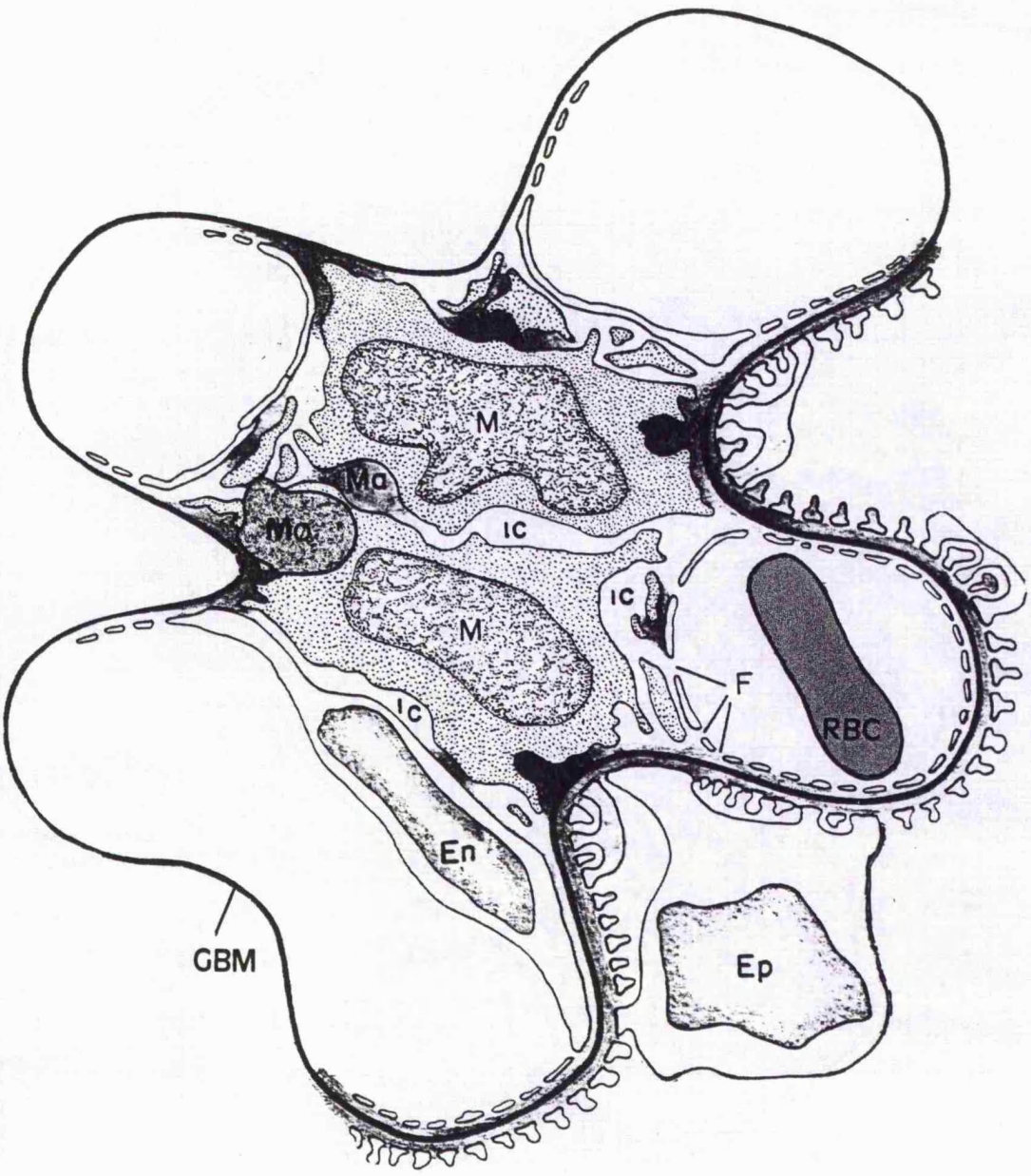


Fig 1.1 Schematic representation of the glomerulus.

Mesangial cells (M), Mesangial matrix (Ma), Macrophage (Mϕ), Endothelial cells (En), Epithelial cells (Ep), Intercellular channels (IC), Fenestrations (F), Red blood cells (RBC), Glomerular basement membrane (GBM). Modified from Latta H, 1973.

1.9 Mediators of glomeruar injury

1.9.1 Cytokines

Cytokines are a group of inducible, low molecular weight peptides (5-80kD) that play a critical role in a wide variety of biological processes including hypertrophy, proliferation, modulation of matrix synthesis and degradation, immune inflammatory responses, development and differation and regulation of vascular tone. A growing body of evidence suggests that cytokines are involved in the mediation of pathologic abnormalities, both structural and functional, that occur in glomerulonephritis and progressive forms of glomerular injury. During the course of glomerular injury the intrinsic glomerular cells become the target for these peptide mediators released via the systemic circulation or locally by infiltrating inflammatory cells. Once activated the glomerular cells may then assume an effector function secreting peptide mediators and thus modifying the outcome of glomerular injury. The major cytokines that have been detected in the injured glomerulus will now be described.

1.9.1.1 TGFβ

A growing body of evidence suggests that TGFß, a 25kD homodimeric peptide, plays a pivotal role in the pathogenesis of glomerular disease both in humans and experimental animals. TGFß is secreted by most cells and released by degranulating platelets, which contain high concentrations of this growth factor, at sites of injury [Derynck R *et al,* 1985]. It is secreted in a biologically inactive or latent form consisting of a non-covalently bound complex of mature TGFß and a portion of the TGFß precursor remaining after proteolytic cleavage of the N-terminal signal peptide [Wakefield LM *et al*, 1989]. This precursor fragment is denoted the latency-associated peptide or LAP. Before TGFß can

bind to its receptor and exert its biologiacl effects it has to be dissociated from this complex in a process of "activation". In vitro, this is usually achieved by acidification although the physiological relevance of this method is questionable. Purified TGFß can also be activated by plasmin which acts by cleaving within the amino terminal region of TGFß destabilising the latent complex and releasing active TGFß [Lyons RM *et al,* 1990]. Latent TGFß is disulphide-linked to a 120-190kD glycoprotien termed the latent TGFß binding protein (LTBP), which has been demonstrated to enhance secretion of latent TGFß, and to participate in latent TGFß activation [Flaumenhaft R *et al*, 1993., Miyazono K *et al, 1991].* The *in vivo* method for activation has not, as yet, been completely determined, but given that most cells secrete latent protein the activation process represents an important control step for regulating the effects of TGFß. Active TGFß is not found in appreciable amounts in serum, where it exists in an inactive form complexed to α2-macroglobulin and from which it can be dissociated by heparin [McCaffrey TA *et al*, 1989].

The actions of TGFß are mediated via binding to cell surface receptors of which three have been identified, types I II and III. Only types I and II have been identified in renal tissue [MacKay K *et al*, 1990]. Type III receptor has been identified as a chondroitin/heparan sulphate proteoglycan (betaglycan) [Boyd FT and Massague J, 1989]. The type II receptor is a serine threonine kinase. Receptor types I and II are involved in the mediation of signal transduction where they interact with one another; receptor I requires receptor II to bind TGFß while receptor II requires receptor I to transduce the signal [Boyd FT and Massague J, 1989., Laiho M *et al*, 1991].

TGFß can induce its own secretion by glomerular cells [Kaname S *et al*, 1992] and acts as a potent chemoattractant of leukocytes which themselves secrete more of this peptide. TGFß acts as a multifunctional regulator of cell proliferation; at low concentrations it

behaves as a mitogen, whilst at high concentrations it acts as an inhibitor of cell proliferation. TGFß expression has been implicated in the process of hypertrophy. Glomerular and tubular hypertrophy in rats with experimental type I diabetes mellitus and angiotensin II (AII) induced hypertrophy of vascular smooth muscle cells have been attributed to the autocrine effects of TGFß [Shankland SJ *et al*, 1994., Gibbons GH *et al*, 1992]. Moreover, Choi ME *et al* [1993] reported that cultured mesangial cells also hypertrophy in response to the growth factor.

One of the most important actions of TGFß is that of modulation of extracellular matrix deposition and in this capacity it has the greatest diversity of action of all other growth factors. It stimulates the secretion of a variety of matrix proteins including fibronectin, collagens, proteoglycans (particularly the chondroitin/heparan sulphate proteoglycans decorin and biglycan), tenascin, osteopontin, osteonectin, thrombospondin [reviewed in Roberts AB *et al*, 1990a]. Accumulation of matrix also occurs via modulation of matrix degradation. TGFß increases the levels of protease inhibitors such as plasminogen activator inhibitor [Laiho M *et al*, 1986] and tissue inhibitor of metalloproteinases (TIMP) [Docherty AJP *et al*, 1985] and decreases the secretion of proteases such as transin [Matrisian LM *et al*, 1986] and serine proteinase (plasminogen activator) [Laiho M *et al*, 1986]. TGFß also modulates cell surface matrix receptors or intergrins which allow cell adhesion to matrix and particularly to fibronectin [Kagami S *et al*, 1993]. Cummulatively these effects lead to the deposition and accumulation of matrix and the formation of scar tissue. The pathologic consequences of the excessive action of TGFß within the kidney have been coined as the "dark side" of tissue repair [Border WA and Ruoslahti E, 1992b].

1.9.1.2 PDGF

Platelet-derived growth factor (PDGF) is one of the most potent mesangial cell mitogens [Jaffer F *et al*, 1989., Shultz PJ *et al*, 1988., Silver BJ *et al,* 1989]. Biologically active PDGF exists as a 30kD disulphide linked dimer composed of two polypeptide chains designated A and B encoded by two seperate genes [Ross R *et al,* 1986., Heldin CH and Westermark B, 1990]. PDGF can therefore be found in three isoforms: heterodimeric PDGF AB or homodimeric PDGF BB and PDGF AA [Bowen-Pope DF *et al,* 1989]. Mesangial cells not only respond to PDGF but they also secrete this growth factor [Abboud HE *et al*, 1987., Shultz PJ *et al*, 1988., Silver BJ *et al*, 1989]. Multiple peptide growth factors including PDGF itself can induce PDGF A and B chain mRNA transcription resulting in the autocrine secretion of the peptide [Silver BJ *et al*, 1989]. The constitutively secreted isoform of PDGF is thought to be the AA homodimer since very high amounts of A chain message are expressed and it is also more efficiently secreted [LaRochelle WJ *et al*, 1991]. However, the AB and BB isoforms are thought to mediate the mitogenic effects of PDGF [Abboud HE *et al*, 1990]. Unlike the AA isoform the AB and BB isoforms autoinduce PDGF A chain and B chain mRNAs [Silver BJ *et al,* 1989].

The PDGF receptor consists of two distinct receptor subunits termed α and ß. The two subunits are structurally related protein tyrosine kinase receptors although molecular cloning studies have demonstrated that the subunits are encoded by two distinct genes [Hart CE *et al*, 1988., Matsui TM *et al,* 1989., Seifert RA *et al*, 1989]. The α subunits will bind both A and B chains whilst the ß subunits will only bind B chains [Hart CE *et al,* 1988]. The proposed model for PDGF receptor binding is that each chain of PDGF dimer binds an individual receptor and promotes dimerisation [Hart CE *et al*, 1988]. Following receptor dimerisation autophosphorylation in *trans* configuration occurs between the

receptors in the dimer [Kelly JD *et al*, 1991]. The receptor-ligand complex is then endocytosed with loss of receptor expression on the cell surface [Ross R, 1989]. Although mesangial cells secrete large amounts of PDGF AA very few alpha receptors are expressed on the mesangial cell surface. This probably explains why autocrine growth of these cells does not occur [LaRochelle WJ *et al*, 1991]. The PDGF ß receptor is the more abundant form in mesangial cells and as such probably acts as the major target for PDGF. PDGF α and ß receptors are also regulated by other peptides such as TGFß [Battegay EJ *et al*, 1990]. This adds another dimension to the complex effects of peptide growth factor interactions. In addition to the mitogenic effects, PDGF also acts as a potent chemoattractant for mesangial cells [Barnes JL and Hevey KA, 1990]. This property provides an important mechanism for the intraglomerular migration of mesangial cells following mesangiolysis or necrosis. PDGF has potent vasoactive properties. It acts as a vasoconstrictor [Berk BC *et al*, 1986] and in this capacity may regulate glomerular haemodynamics [Mene P *et al*, 1987]. PDGF may also regulate matrix metabolism either directly or via the release of other peptide factors [Abboud HE *et al*, 1991., Doi T *et al*, 1992]. Thus PDGF has the potential to play an important role in regulating normal mesangial cell metabolism, growth and replication and under conditions of glomerular injury may promote leukocyte infiltration proliferation and matrix production.

1.9.1.3 TNFα

TNFα is a peptide secreted primarily by activated macrophages [Old RJ, 1985., Beutler B and Cerami A, 1986]. It can be synthesised by mesangial cells in response to stimulation by LPS [Baud L *et al,* 1989], adriamycin or puromycin [Egido J *et al*, 1993] or lectins [Amore A *et* al, 1993]. It is not constitutively secreted suggesting that this peptide is only

expressed in conditions of glomerular inflammation [Baud L *et al,* 1988., Baud L *et al,* 1989]. TNFα is exists both as a 26kD membrane bound protein [Affres H *et al,* 1991] previously described on monocytes [Kriegler M *et al,* 1988], and as a soluble 17kD peptide monomer, released following cleavage of the membrane bound protein. In its biologically active form TNFα is found as a non-covalently linked trimer which binds to TNFα receptors [Smith RA and Baglioni C, 1987]. TNFα has multiple actions on mesangial cells. It stimulates mesangial cell production of prostaglandins, specifically PGE_2, $PGF_{2\alpha}$, PGI_2 [Baud L *et al,* 1988., Topley N *et al,* 1989., Floege J *et al,* 1990]. It increases mesangial cell production of reactive oxygen metabolites in amounts comparable to those produced by monocytes [Radeke HH *et al,* 1990]. It induces mesangial cell tissue factor-like procoagulant activity [Wiggins RC *et al,* 1990] as well as anti-fibrinolytic activity with the production of plasminogen activator inhibitor [Meudlers Q *et al,* 1992]. TNFα plays an important role in leukocyte recruitment; it induces mesangial cell secretion of the neutrophil chemoattractants IL-8 [Kusner DJ *et al,* 1991] and interferon-induced protein 10 (IP-10) [Gomez-Chiarri M *et al,* 1993] and the monocyte chemoattractants MCP-1 and CSF-1 [Zoja C *et al,* 1991., Satriano JA *et al,* 1993]. TNFα also promotes the adhesion of the recruited leukocytes by inducing the expression of intercellular adhesion molecules CD11/CD18 and ICAM-1 in monocytes and mesangial cells respectively [Denton MD *et al,* 1991]. The precise mechanism whereby TNFα mediates these multiple actions within the glomerulus is not as yet fully understood although binding to specific cell surface receptors is most probably the initial event. Two distinct receptors having molecular weights of 55kD and 75kD have been identified [Brockhaus M *et al,* 1990].

1.9.1.4 IL-1ß

IL-1ß is a 17kD peptide produced by a number of cell types including activated macrophages [Mizel SB and Mizel D, 1981] and mesangial cells [Lovett DH *et al,* 1986b]. It was the first cytokine found to be a mesangial cell autocrine growth factor [Lovett DH *et al,* 1986b]. The proliferative effects of macrophage supernatants on mesangial cells have been tentatively ascribed to IL-1ß [Melcion C *et al,* 1982., Lovett DH *et al,* 1983a]. However, these effects are only observed in the presence of serum [Lovett DH *et al,* 1983a] suggesting that other growth factors are required for the effect to be seen. Of interest is the finding that IL-1ß mRNA can only be detected in proliferating cells and not growth arrested cells [Lovett DH *et al,* 1988]. IL-1ß has several biologic effects which are relevant to it playing a pathophysiologic role in glomerular inflammation and glomerulosclerosis. These effects include DNA synthesis (proliferation), release of proteases [Martin J *et al,* 1986], secretion of prostaglandins [Lovett DH *et al,* 1987a] and collagen synthesis [Melcion C *et al,* 1982]. Mesangial cell secretion of IL-1ß can be induced by serum, EGF and PDGF [Lovett DH and Larsen A, 1988], by immune complexes [Matsumo K and Hatano M, 1991], and C5-C9 components of complement [Lovett DH *et al,* 1987b]. At least two distinct receptors have been reported for IL-1ß. The predominant form and the one found on mesangial cells is the 80kD type 1 receptor [Dower SK and Urdal DL, 1987]. The 67kD type 2 receptor occurs mainly on bone-marrow derived cells [Bomsztyk K *et al,* 1989].

1.9.2 Lipid-derived mediators

Under conditions of hyperlipidaemia there is an increased production of pro-inflammatory lipids. Lipoproteins or their metabolites could activate macrophages and recruit them into

the glomerulus. Some of the macrophage secretory products are themselves lipid metabolites which may stimulate over production of matrix proteins resulting in glomerulosclerosis. Platelet activating factor (PAF) is one example of a class of pro-inflammatory lipids. It can be synthesised by both mesangial cells [Lianos EA and Zanglis A, 1988] and macrophages [Nathan CF, 1987]. Actions of PAF include stimulation of platelet activation and aggregation, chemotaxis, formation of reactive oxygen species and increased vascular permeability [Badr KF, 1991] - factors which could contribute to the progression of glomerulosclerosis. Eicosanoids are another class of lipid-derived mediators. They are synthesised from unesterified fatty acids derived from cholesterol esters, triglycerides or phospholipids [Moorhead JF, 1991]. Arachidonic acid lipoxygenation results in the production of hydroxyeicosatraenoic acids, leukotrienes and lipoxins, all of which have pro-inflammatory effects, while the cyclooxygenation of arachidonic acid results in the production of prostaglandins and thromboxanes [Lianos EA, 1989]. Thromboxanes in particular have been shown to have a direct effect on the production of extracellular matrix components in cultured human mesangial cells [Bruggeman LA *et al*,1991., Bruggeman LA *et al*, 1993]. More recently a further proinflammatory lipid has been described. This is a novel non-polar lipid which is chemotactic for macrophages and has characteristics unlike those of any lipid previously described [Schreiner GF *et al*, 1992]. Production of this factor is seen in glomeruli from rats with nephrotoxic nephritis. Its production was significantly diminished in rats placed on an EFAD diet. Furthermore rats on this diet also exhibited reduced macrophage infiltration, less proteinuria and improved renal function compared to nephritic rats on a regular diet [Rovin BH *et al*, 1990b].

1.10 Aims of the study

Glomerulosclerosis is the final outcome of a number of causes of glomerular injury during which the structures of the glomerulus are obliterated by extracellular matrix. Prior to this stage the glomerulus accomodates activated or proliferating intrinsic glomerular cells and exogenously derived infiltrating cells. If the glomerulus is unable to respond appropriately to restore functional equilibrium then the inexorable road towards sclerosis is initiated. To date, the pathophysiology of this process remains incompletely understood.

The present study was therefore undertaken to establish the role played by macrophages and particularly their secretory products, both at the cellular and molecular levels, on the initiation of the sclerotic process in mesangial cells (functionally the most important cells within the glomerulus), to characterise the putative factor(s) and to delineate the potential interactions involved.

CHAPTER 2
General Materials and Methods

CELL CULTURE

2.1 Tissue Culture

All tissue culture procedures were carried out in a class II laminar flow cabinet (Gelman Sciences Ltd). The cells were incubated at 37°C in an humidified 5% CO_2, 95% air atmosphere.

2.1.1 Mesangial cell culture

Mesangial cells were cultured from the glomerular explants of adult female Wistar rat kidneys (University of Leicester breeding colony) using a technique based on a method by Foidart JB *et al* [1979].

The kidneys were asceptically excised from animals which had been killed according to schedule 1 of the Animal Scientific Procedures Act 1986 using a lethal overdose of Hypnorm/Hypnovel anaesthesia given intraperitoneally (Roche, Appendix 1). Following removal of the hilum the renal cortex was disected from the medulla and cut up into smaller pieces which were placed in Hanks' balanced salt solution (HBSS) buffered with 20mM HEPES to pH 7.4 (Gibco, Appendix 1). The cortical pieces were then passed through a series of brass/stainless steel sieves (Jencons) of decreasing pore size (500μm, 250μm and 75μm) using a piston plunger from a 5ml syringe and rinsing each sieve with HBSS-HEPES. Glomeruli retained on the 75μm sieve were collected into 10 ml HBSS-HEPES, washed by centrifugation at 1000rpm (250g) in a bench centrifuge with a swing-out bucket rotor and resuspended in 10ml HBSS-HEPES containing 750 units/ml collagenase type IV (Sigma) and 10μg/ml deoxyribonuclease type 1 (Sigma). The glomerular suspension was digested for 30min at room temperature with continual agitation. Following digestion the glomerular cores were washed twice (by centrifugation

at 250g) in HBSS-HEPES and once in RPMI 1640 (Gibco) supplemented with 20% foetal calf serum (FCS)[1], 100IU/ml penicillin, 100μg/ml streptomycin, 2mM glutamine (Gibco) and 5μg/ml bovine insulin (Sigma) (complete RPMI medium, Appendix 1). After the last wash the glomeruli were resuspended in complete RPMI medium, plated into 75cm^2 plastic tissue culture flasks (Bibby Sterilin) and placed in the incubator. Typically the glomeruli from two kidneys were resuspended into 20ml medium per flask. After 10 days of culture the explanting mesangial cells were refed with fresh complete RPMI medium, after which they were fed every 3-4 days until the cells had grown to confluence (2-3 weeks). They were then passaged by washing the cell monolayer once with HBSS-HEPES then adding 2ml 0.05% trypsin/0.53mM EDTA solution (Gibco) per 75cm^2 flask and incubating at 37°C for 10min or until the cells had lifted off the surface of the flask. The cell suspension was resuspended in approximately 40ml of complete RPMI medium and divided between two fresh flasks.

Mesangial cells for use in assays were passaged into 24 well plates (2.0cm^2/well, Linbro, ICN Biomedicals), except for cells used for RNA extraction which were passaged into 25cm^2 tissue culture flasks (Bibby Sterilin). The cells were grown to confluence and then quiesced for 72 hr by reducing the FCS concentration to 0.5% prior to use in experiments. Since it was observed that basal mesangial cell fibronectin production increased with length of time in culture all experiments were carried out on cells of passage 2 to 10.

[1]The FCS used during the course of the study was obtained from several different sources (Imperial Laboratories, Advanced Protein Products, Sigma). Every batch of FCS had to be batch tested for suitability for mesangial cell growth.

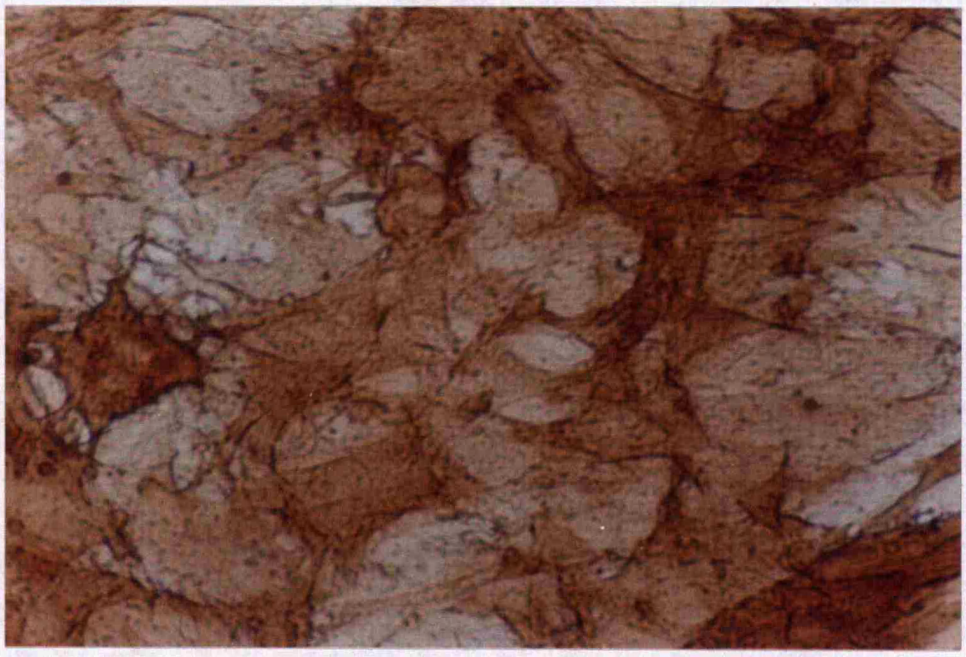
2.1.2 Characterisation of mesangial cells

Mesangial cells were characterised by their typical stellate fusiform morphology, their positive staining for the Thy-1.1 antigen (Serotech) [Bagcus WM *et al*, 1986], and their resistance to the toxic effects of D-valine (Gibco) [Gilbert SF and Migeon BR, 1975].

2.1.2.1 Staining for the Thy1.1 antigen

Mesangial cells were grown to confluence in 35mm petri dishes. The medium was removed and the cells were fixed in a mixture of acetone:formalin (40:1v/v) for 10s. After washing in PBS the dishes were incubated with a 1:20 dilution (in PBS + 0.1% BSA) of mouse anti-rat Thy-1 (MRC OX-7, Serotec) for 1hr at room temperature. Following a further wash with PBS the petris were covered with a 1:100 dilution of rabbit anti-mouse Ig horseradish peroxidase (HRP)(Dako) (in PBS + 0.1% BSA + 1% normal rat serum) for 1hr at room temperature. After washing with PBS the petris were stained for peroxidase activity with DAB (3,3'-diaminobenzidine-tetrahydrochloride). The DAB solution was made up by dissolving 1 tablet of DAB and 1 tablet of urea hydrogen peroxide in 15ml water (Sigma Fast DAB tablet set, Sigma). The anti-macrophage ED1 antibody (Serotec) was used both to exclude the presence of macrophages and as the negative contol (Fig 2.1).

A



B

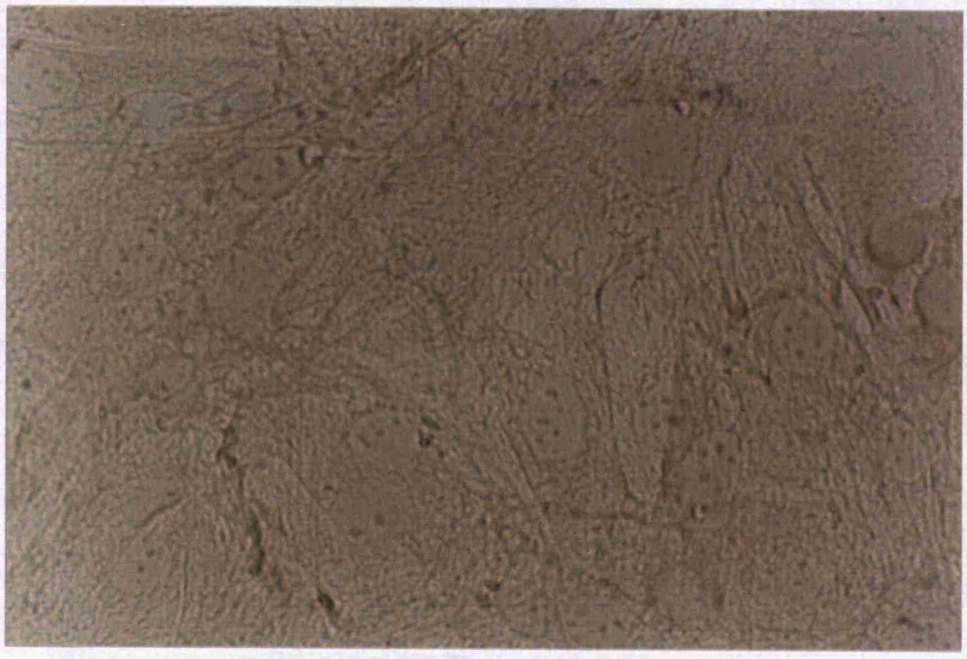


Fig 2.1 Staining for the Thy1.1 antigen on mesangial cells

A. Positive staining for the Thy1.1 antigen (40X magnification)

B. Negative control (anti-ED1 antibody) (40X magnification)

Note the stellate fusiform morphology of the mesangial cells.

2.1.2.2 Resistance to the toxic effects of D-valine

Fibroblasts lack the enzyme D-amino acid oxidase which converts D-valine into 2-ketoisovaleric acid, which is further converted to the essential amino acid L-valine. Fibroblasts will not proliferate in this medium unless the product of the D-amino acid oxidase reaction is supplied.

Mesangial cells that survived a passage and growth to confluence in MEM containing D-valine + 10% FCS (Gibco) (Appendix 1) were deemed to be true mesangial cells free from fibroblast contamination.

2.2 Isolation of peritoneal macrophages

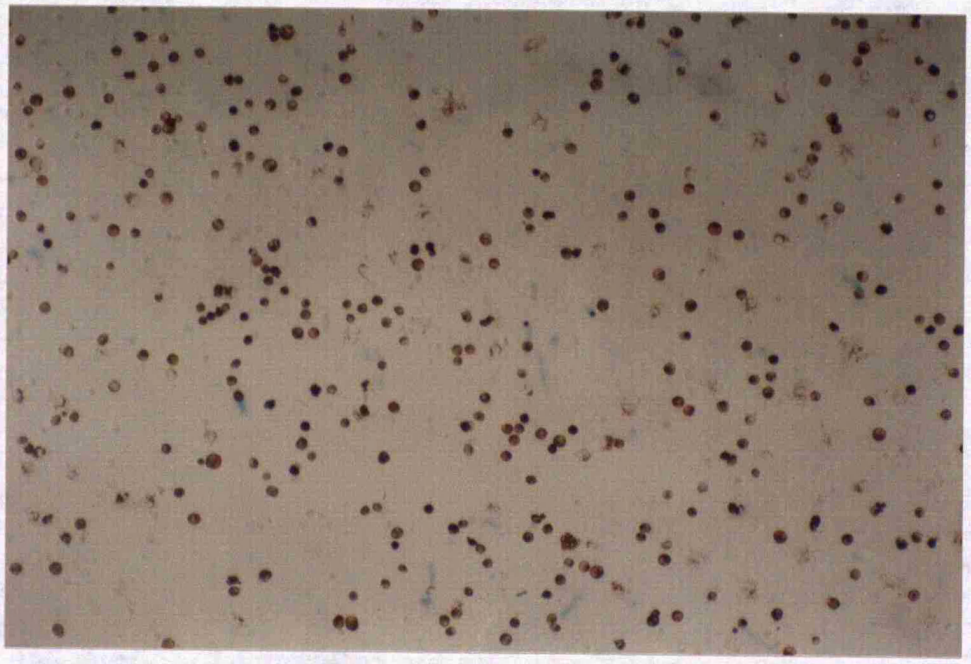
Peritoneal macrophages were obtained from female adult Wistar rats. 10ml of 3% thioglycollate broth (Sigma, Appendix 1) was injected into the peritoneal cavity. After 5 days the animals were killed with an overdose of Hypnorm/Hypnovel. The peritoneal cavity was lavaged with 20ml cold HBSS-HEPES. After 5min, during which time the abdominal region was gently kneaded, a small incision was made in the peritoneal cavity through which the peritoneal exudate was withdrawn with a pasteur pipette under sterile conditions. The exudate cells were centrifuged at 1000rpm (250g), resuspended in 5ml serum-free RPMI supplemented with 100 IU/ml penicillin and 100μg/ml streptomycin and counted using a haemocytometer slide. Immunohistochemical staining using ED1 antibody was performed to confirm that the majority of the cells obtained in this way were macrophages. The exudate cells were either co-cultured directly with mesangial cells or used to generate macrophage conditioned medium (MPCM).

2.2.1 Staining for ED1

The monoclonal antibody ED1 is widely used as a marker for rat macrophages [Dijkstra CD *et al,* 1985]. The antibody recognises a heavily glycosylated protein of 90-110kD which is expressed on cytoplasmic granules of macrophages.

Freshly isolated peritoneal exudate cells were harvested and diluted to a cell density of 50000 cells/ml. 70μl cell suspension was applied to the funnel of a cytospin cassette containing a microscope slide and filter paper. The cassettes were placed in the cytospin (Cytospin 2, Shandon) and centrifuged for 5min at 800rpm. The cells on the slides were air dried and fixed in a mixture of acetone:formalin (40:1 v/v) for 10s. After washing with PBS the slides were incubated for 2hr with a 1:100 dilution of ED1 antibody (in ascites fluid, Serotec). Following washing the cells were covered with 1:100 dilution of rabbit anti-mouse Ig HRP and incubated for 1hr at room temperature. Peroxidase activity was detected using DAB as described for mesangial cells. The anti-Thy1.1 antibody was used as a negative control (Fig 2.2).

A



B

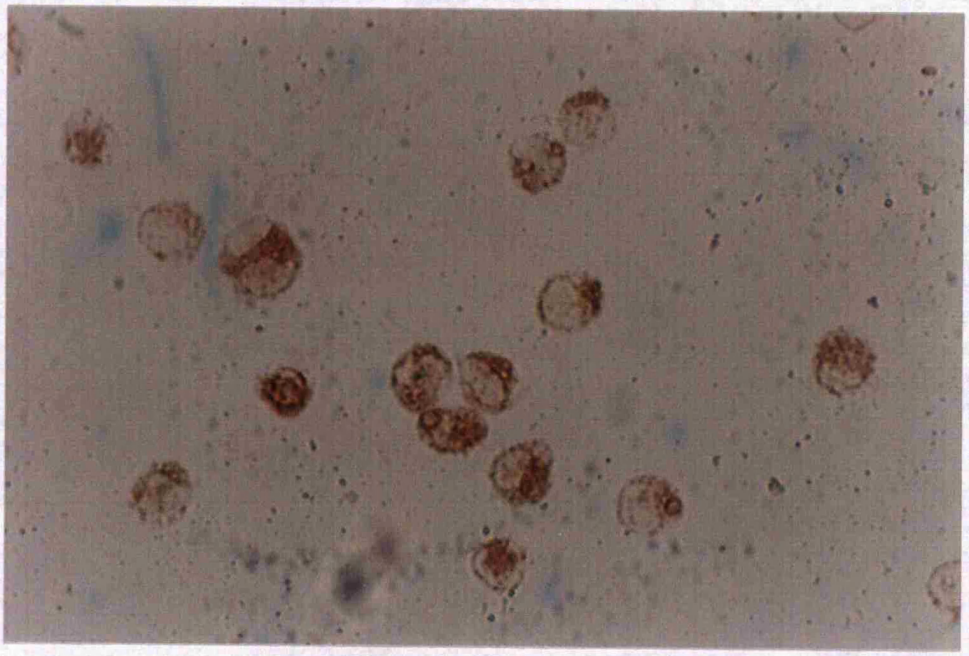


Fig 2.2 ED1 staining of peritoneal macrophages.

A. Field view showing most of the exudate cells staining positive (10X magnification)

B. Typical cytoplasmic staining of macrophages with their characteristic kidney-shaped nuclei (40X magnification).

2.3 Preparation of macrophage conditioned medium (MPCM)

Macrophage conditioned medium (MPCM) was prepared according to a modified method of Kohan DE and Schreiner GF [1988].

Peritoneal macrophages were isolated as described above (section 2.2). Following counting, the cells were plated at a density of 5 x 10^5 cells/ml, 8ml/ 25cm^2 tissue culture flask (Linbro, Bibby sterilin). After 2 hours incubation at 37°C non adherent cells were removed by washing twice with HBSS-HEPES. The macrophages were then stimulated with bacterial lipopolysaccharide (LPS) (from E.Coli 026 B6) (Sigma) at a final concentration of 1μg/ml for approximately 16 hours after which they were washed three times with HBSS-HEPES and cultured for a further 48 hours in serum-free RPMI to generate the conditioned medium. (Elicited non LPS stimulated macrophages were also used to generate conditioned medium. In this case instead of LPS an equivalent volume of HBSS-HEPES was added). The MPCM was then harvested and centrifuged for 10min at 2000rpm (800g) to remove cell debris and frozen at -20°C until required.

2.4 Culture of mesangial cells in the presence of MPCM

Unless otherwise stated confluent quiescent mesangial cells in 24 well plates were exposed to 1ml/well of a 50% solution of MPCM (0.5ml MPCM + 0.5ml RPMI + 1% FCS) such that the final concentration of FCS in the culture medium remained at 0.5%. The cultures were maintained in this medium for up to 7 days. The tissue culture supernatants from each well were harvested into 1.5ml microfuge tubes and centrifuged for 30s at 13000 rpm (11 600g) in a microcentrifuge to remove cell debris, and stored at -20°C for subsequent analysis of soluble fibronectin.

2.5 Preparation of cell lysates

After removal of tissue culture supernatants from the 24 well plates, the remaining cell monolayers were washed once with PBS. 200μl of 1.0% Nonidet P40 (BDH) in wash buffer (PBS containing 0.3M NaCl and 0.1% Tween 20, Appendix 1) was then added to each well. The cells were scraped into this solution with the piston of a 1ml syringe and incubated for approximately 30min at room temperature. The cell scrapings were harvested into 1.5ml microfuge tubes. The wells were then washed with a further 50μl of Nonidet P40 solution and washings were pooled with the original 200μl cell scrapings. The samples were then sonicated for 5s using a 50W sonicator (Jencons). Insoluble material was pelleted by centrifugation at 13000rpm (11600g) in a microcentrifuge. Sonication followed by centrifugation was repeated once more. The cell lysates were assayed for fibronectin and protein content.

ASSAYS/PROTEIN CHEMISTRY

2.6 Fibronectin ELISA

Fibronectin levels in tissue culture supernatants and cell lysates were determined using an inhibition ELISA. This ELISA was developed (by myself) from the original method of Rennard SI *et al* [1980].

2.6.1 Plate coating

100μl of 1μg/ml rat plasma fibronectin (Calbiochem) in coating buffer (0.05 M carbonate buffer pH 9.6, Appendix 1) was added to each well of a 96 well plate (Nunc Immunoplate). The plate was then sealed with an adhesive plastic cover and incubated

overnight at 4°C. The plate was washed four times with wash buffer using an automated plate washer (Denley Wellwash 4). Non-specific protein binding sites were blocked with 100μl/well 2% BSA in wash buffer (Appendix 1) for 1 hour at room temperature. The plate was then washed four times as before.

2.6.2 Standards and samples

60μl of rat plasma fibronectin standard (19-5000ng/ml) (Calbiochem), tissue culture supernatant or cell lysate was incubated with an equal volume of rabbit anti-rat fibronectin antiserum (Calbiochem) diluted 1:2000 in wash buffer at 4°C overnight.

2.6.3 Assay

50μl of the above mixture was transferred in duplicate to the wells of the pre-coated microtitre plate and incubated at room temperature for at least 2hr. After washing each well four times, 50μl/well of 1:1900 dilution HRP conjugated goat anti-rabbit IgG (Dako) was added and incubated for 1hr at room temperature. After a further four washes, 50μl/well of 0.67mg/ml 1,2 phenylenediamine dihydrochloride (OPD) in 0.03M citrate buffer pH 5.0, containing 0.012% H_2O_2 (Appendix 1) was added to develop the colour. After 5min, or when the colour reaction had reached a readily measurable intensity, the reaction was stopped with 75μl/well 1M H_2SO_4 and the absorbance read at 492nm on a Titertek Multiskan Plus automated microtitre plate reader (Flow Laboratories).
With the exception of the substrate solution all dilutions were carried out in wash buffer.

Intra assay %CV ranged from 5.0-9.7 (mean 7.5), inter assay %CV was 9.4 (calculated from 14 replicates in 5 assays).

2.6.4 Data processing

The data were analysed using a Titersoft software package (ICN Flow) which constructed a standard curve on logarithmic vs linear axes from which the unknown samples were interpolated. Any samples that did not fall on the linear range of the standard curve were repeated at a more appropriate dilution.

A typical standard curve is shown in Fig 2.3.

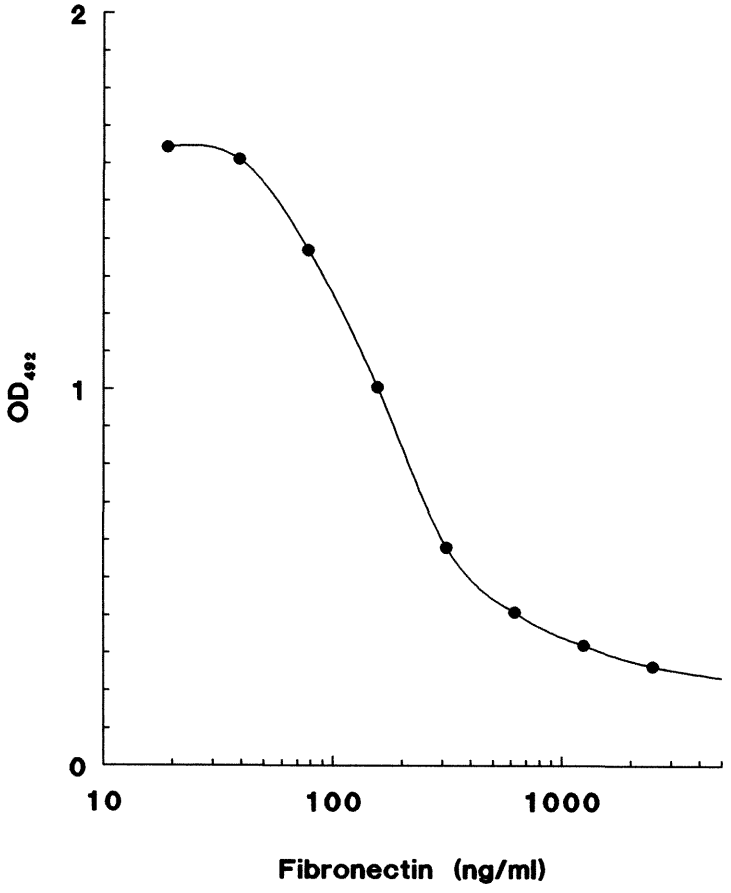


Fig 2.3 Representative standard curve from the fibronectin ELISA

2.7 DNA assay

In order to determine the DNA content of the cell monolayers secreting fibronectin following exposure to MPCM, a modification of the Burton method was utilised [Giles KW and Myers A, 1965].

After removal of the tissue culture supernatants each cell monolayer was washed four times with a 1ml aliquot of ice-cold 0.9% (w/v) NaCl and then scraped into 200μl/well 10% (w/w) perchloric acid (PCA) (BDH) using a piston plunger from a 1ml syringe and transfered into a 1.5ml microfuge tube. The wells were washed with two further 200μl aliquots and one 100μl aliquot of 10% PCA and the washings all pooled in the 1.5ml microfuge tubes. The tubes were capped, vortexed and centrifuged at 4000rpm (3000g) for 10min at 4°C in a refridgerated centrifuge (Beckman). The supernatants were discarded and the pellets of precipitated nucleic acid and protein were resuspended in 500μl of fresh 10% PCA and hydrolysed by heating at 70°C in a water bath for 20min. Following hydrolysis the tubes were chilled on ice to precipitate as much protein as possible. The tubes were then centrifuged for 10min at 4000rpm (3000g) at 4°C to spin down the protein precipitate (see protein assay). The supernatants containing the DNA were transferred to plastic test tubes.

250μl of diphenylamine reagent (1g diphenylamine/25ml glacial acetic acid) followed by 50μl of acetaldehyde solution (1.6mg/ml in water) were added to duplicate 250μl aliquots of cell hydrolysate or to 250μl calf thymus DNA standard (10-200μg/ml) which had been hydrolysed in PCA at 70°C in the same way as the samples. The samples and standards were then vortexed and incubated at 25-30°C for 16-20 hrs. 300μl of reaction solution was

transferred to the wells of a microtitre plate and the optical density was read at 595nm and 710nm on the automated plate reader. For each sample or standard the difference between the two wavelengths was calculated (light scattering at 710nm was measured to eliminate any effects arising from the slight turbidity of the samples, [Giles KW and Myers A, 1965]). A standard curve of change in absorbance vs DNA concentration was constructed using linear regression analysis from which the DNA concentration of the samples was determined.

2.8 Protein assay

For cell monolayers which had been precipitated with PCA for DNA determinations, the cell protein content was measured by the method of Lowry OH *et al,* [1951] using BSA standards.

250μl of 0.5M NaOH was added to the pellets of precipitated protein generated during the isolation of DNA from the cell monolayers. The tubes were vortexed at room temperature to dissolve the protein. Duplicate 50μl aliquots of protein sample solution or BSA standard (0-500μg/ml of globulin-free BSA (Sigma) both in 0.5M NaOH was pipetted into plastic LP3 tubes. 10μl of water was then added to each tube followed by 600μl of Lowry solution C (50 parts Lowry A + 1 part Lowry B, Appendix 1). The samples were immediately vortexed. After 10min incubation at room temperature, 60μl of Folin-Ciocalteau reagent diluted 1:2 with water was added and the solutions were immediately vortexed again. After 40min, duplicate 200μl of each reaction solution was transferred to the wells of a microtitre plate and the absorbance at 660nm was determined.

The unknown protein samples were interpolated from a standard curve constructed using

linear regression analysis.

Detergents interfere in the original Lowry protein assay. Total protein concentrations of detergent-solubilised cell monolayers were therefore determined using a commercial protein assay kit (DC Protein Assay, Bio-Rad) based on a modified method of the Lowry assay. The assay was carried out in microtitre plates according to the manufacturer's instructions using BSA standards (Bio-Rad) serially diluted in 1% Nonidet P40 in wash buffer.

Quadruplicate 5μl aliquots of standard (0.03-2mg/ml) or sample were added to the wells of a 96 well microtitre plate. 25μl of Reagent A (alkaline copper tartrate solution) containing 20μl/ml Reagent S (5-10% SDS) was then added to each well followed by 200μl of Reagent B (Folin reagent). The plate was then left to incubate at room temperature for 15min to allow the colour to develop. After 15min the absorbances were read at 750nm on the automated plate reader. The unknown protein samples were interpolated from a standard curve constructed using linear regression analysis as before.

2.9 Concentration of MPCM

Prior to processing by gel filtration chromatography or SDS-PAGE, MPCM preparations were concentrated using Centriprep 3 concentrators (Amicon) with 3 kD molecular weight cut off. Concentration of the samples was carried out according to the manufacturer's instructions.

15ml of MPCM was poured into the sample chamber of a Centriprep 3 concentrator unit. The filtrate collector assembly was then gently inserted into the concentrator unit and twist-

locked shut. The assembled concentrator unit was then centrifuged in a Beckman bench centrifuge at 4000 rpm (3000g) until the fluid levels inside and outside of the filtrate collector were in equilibrium (approx 2hr). The filtrate was decanted from the collector unit. The concentrator unit was centrifuged until the fluid levels had again equilibrated (approx 30min) and the filtrate was decanted as before. After a third 10min centrifugtion step the volume of the retentate was approximately 0.5 - 0.75ml, ie the MPCM had been concentrated 20-30 fold.

2.10 Gel Filtration Chromatography

Columns used:-

a) Superose 6 HR 10/30 (Pharmacia)

· separation range (MW) $5x10^3$-$5x10^6$

b) Superose 12 HR 10/30 (Pharmacia)

· separation range (MW) $1x10^3$-$3x10^5$

2.10.1 Column calibration

Prior to fractionation of the samples the columns were calibrated with the following molecular weight markers:

cytochrome C 12.4 kD

carbonic anhydrase 29 kD

bovine serum albumin 66 kD

alcohol dehydrogenase 150 kD

ß amylase 200 kD

blue dextran (void volume) 2000 kD

To construct a calibration curve, $\log_{10}$ molecular weight (Y) was plotted against Ve/Vo where Ve is the elution volume of the molecular weight marker and Vo is the void volume of the column.

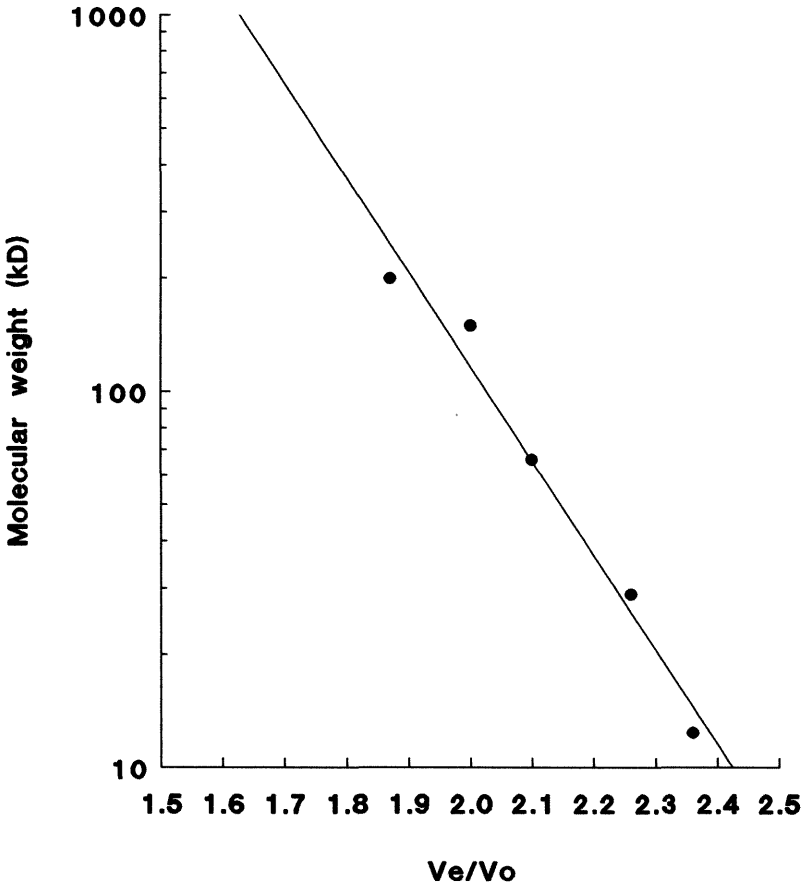


Fig 2.4 Molecular weight calibration curve for superose 6 column.

2.10.2 Sample elution

200μl of filtered, concentrated MPCM was loaded on to a superose gel filtration column equilibrated with with at least two column volumes (50ml) filtered and degassed 0.05M phosphate buffer pH 7.4 containing 0.15M NaCl (Appendix 1). The sample was eluted at 0.2ml/min and 70 x 0.4ml fractions were collected.

2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse the components of MPCM and mesangial cell tissue culture supernatants. A Bio-Rad Mini-Protean II vertical slab system was used based on the method of Laemmli UK [1970].

2.11.1 Casting the gels

0.75mm or 1.5mm thick discontinuous polyacrylamide gels with total monomer concentrations ranging from 5% to 15% were cast in glass plate sandwich assemblies according to the manufacturer's instructions.

The lower resolving gel was prepared by mixing a 30% solution of acrylamide and N'N'-bis-methylene-acrylamide (37.5:1 ratio, Bio-Rad) with 1.5M Tris-HCl buffer pH 8.8 (Appendix 1) and water to a given final concentration of acrylamide (%T). The solution was degassed under vacuum for approximately 15min after which SDS was added to a final concentration of 0.1% followed by the polymerising agents ammonium persulphate (APS) and TEMED to final concentrations of 0.05%. The gel was poured, overlayed with water and allowed to polymerise for at least 1 hr.

The upper 4% stacking gel monomer solution was prepared in a similar way this time using 0.5M Tris-HCl buffer pH 6.8 (Appendix 1). A ten tooth comb (for well formation) was immediately inserted into the monomer solution allowing approximately 1cm between the end of the comb and the beginning of the resolving gel. Following polymerisation the comb was removed and the wells washed with water.

2.11.2 Sample preparation

The samples and molecular weight markers (BioRad) were mixed with an equal volme of non-reducing sample buffer (62.5mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.02% bromophenol blue, Appendix 1) or reducing buffer (as non-reducing but including 0.5% ß-2-mercaptoethanol, Appendix 1) and heated for 5min in a boiling water bath.

2.11.3 Electrophoresis

The gels in their clamp assembly were removed from the casting stand, fixed into the cooling core and placed in the electrophoresis tank. Electrode (running) buffer (25mM Tris-HCl, 192mM glycine, 0.5% SDS pH 8.3, Appendix 1) was poured into the inner and outer chambers of the electrophoresis cell. 35μl/well or 70μl/well sample (depending on the thickness of the gel) were loaded into the wells under the electrode buffer. 10μl/well of kaleidoscopic pre-stained molecular weight markers (BioRad) were also loaded. The gels were then electrophoresed at 200V for approximately 40min until the bromophenol blue front reached the bottom of the gel.

Following electrophoresis the gels were carefully removed from between their glass plates and fixed in an aqueous solution of 40% methanol/10% acetic acid (v/v) (Appendix 1)

ready for silver staining or drying or they were placed directly into equilibration buffer prior to western blotting.

2.12 Silver staining

Because the components of MPCM are at very low concentrations they could not be detected with conventional Coomassie blue stain which detects μg levels of protein. Instead a commercial silver staining kit (Bio-Rad) based on the method of Merril CR *et al* [1981] which can detect ng levels of protein was employed. Staining was carried out according to the manufacturer's instructions (times in brackets refer to 1.5mm thick gels).

The gels were fixed in two changes of 200ml 40% methanol/10% acetic acid (v/v) for approximately 30min each (1hr) followed by 15min (30min) in 200ml 10% ethanol/5% acetic acid (v/v) (Appendix 1). The gels were then incubated in 100ml of oxidiser solution (potassium dichromate in nitric acid) for 5min (10min). They were then thoroughly washed with several changes of water until no yellow colour could be seen in the gels. 100ml of silver reagent (silver nitrate solution) was added and gels incubated for 20min (30min). The gels were then rinsed in water for 1min (2min). 100ml of developer solution (sodium carbonate and paraformaldehyde) was added for approximately 30s until a 'smokey' brown precipitate formed. This solution was poured off and replaced with fresh developer for about 5min. This was again replaced and gels incubated until the brown bands had reached the desired intensity. Development was stopped with a 5% acetic acid solution.

All incubations were carried out at room temperature on an orbital shaker in polypropylene containers which allowed free movement of the gels.

2.13 Gel drying

After staining, or prior to autoradiography, the gels were dried. They were equilibrated in 40% methanol/10% acetic acid/3% glycerol (Appendix 1) for approximately 1hr and dried on a Bio-Rad Model 543 Gel Dryer for 2-3 hr at 60-70°C on an appropriate cycle for the gel being dried.

2.14 Western blotting

Western blotting was carried out in a Bio-Rad Mini Trans-Blot system in an attempt to identify the presence of certain cyokines in MPCM.

TGFß transfer and detection was carried out according to a commercial anti-TGFß polyclonal antibody kit protocol (R&D). Transfer and detection systems for PDGF, TNFα and IL-1ß were developed following a similar protocol.

2.14.1 Electrophoretic transfer

Following electrophoresis the gel was equilibrated in electrophoretic transfer buffer (25mM Tris base, 192mM glycine, 20% methanol, 0.05% SDS, Appendix 1) for 15min as were 2 pieces of thick 3MM filter paper (Bio-Rad) and 2 scotchbrite fibre pads (Bio-Rad). A PVDF (polyvinylidene difluoride) membrane (Bio-Rad) was first soaked in 100% methanol for a few seconds and then it too was placed into transfer buffer. The gel was aligned on to the PVDF membrane and the two were sandwiched between the soaked filter papers and the fibre pads and mounted in a cassette. The cassette was placed in the electrophoresis tank in such an orientation that protein transfer occurred towards the anode. The tank was filled with transfer buffer and blotting was carried out for 2hr at 80V with cooling.

2.14.2 Immunostaining

Following blotting the membrane was stored overnight at 4°C. The membrane was then wetted with 100% methanol for 5s, rinsed in TBS (500mM NaCl, 20mM Tris-HCl, pH7.2, Appendix 1) and then blocked for 2hr at room temperature in TBS containg 1% BSA and 0.05% Tween 20 (Appendix 1). The blocked membrane was then reacted for 2hr at room temperature with 10ml of:

- rabbit anti-TGFß antibody at 1:2000 (R&D kit) or
- goat anti-PDGF antibody at 10μg/ml (R&D) or
- goat anti-TNFα antibody at 10μg/ml (R&D) or
- goat anti-IL1ß antibody at 10μg/ml (R&D)

diluted in blocking buffer. Incubations were carried out on an orbital shaker.

The membrane was then washed with four changes of TTBS (TBS + 0.05% Tween 20, Appendix 1). The blot was incubated for 1hr at room temperature with 10ml of either

- biotin-conjugated anti-rabbit IgG at 1:2000 (R&D kit) or
- biotin-conjugated anti-goat IgG at 1:2000 (Dako)

diluted in blocking buffer according to the primary antibody used. Following incubation with the second antibody the membrane was washed again with TTBS as described above. The blot was then incubated for 1hr at room temperature with alkaline phosphatase-conjugated streptavidin (R&D kit) diluted in blocking buffer. The membrane was washed again.

For colour development 66μl of colour reagent A (50mg/ml p-nitro blue tetrazolium chloride in 70% N,N dimethylformamide) were added to 10ml alkaline phosphatase (AP) buffer (100mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$ pH 9.5, Appendix 1), the solution mixed and then 33μl of colour reagent B (50mg/ml 5-bromo-4-chloro-3-indolylphosphate

p-toluidine salt in N,N-dimethylformamide) were added and the solution mixed again. Once the colour had developed, the reaction was stopped by discarding the colour development solution and and rinsing the membrane with water.

The molecular weight of a band on a gel or Western blot was interpolated from a calibration curve constructed by plotting Log_{10} molecular weight of the molecular weight markers (Y axis) vs relative mobility (Rf) (X axis). A typical calibration curve is shown in Fig 2.5.

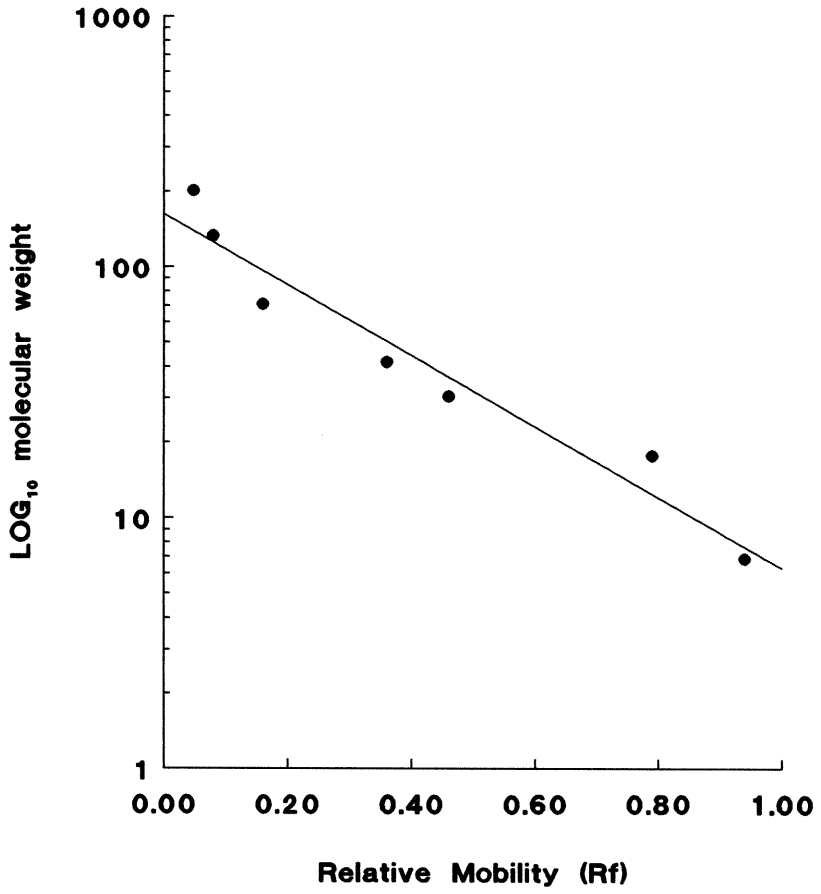


Fig 2.5 Typical calibration curve of SDS-PAGE molecular weight markers.

2.15 Measurement of cell proliferation

Mesangial cell proliferation was measured by ^{3}H thymidine incorporation using a modified method of Greenberg D *et al* [1977].

Confluent, quiescent mesangial cells were exposed to MPCM or medium alone for 3 days. After this time 1μCi/well of ^{3}H-thymidine (Amersham, TRK61) was added to each well directly or the culture supernatants were removed and the wells replaced with 1ml fresh RPMI + 0.5% FCS containing 1μCi/well of ^{3}H-thymidine. The cells were incubated for a further 24hr after which the culture media were discarded and the wells rinsed once with 1ml PBS. 1ml of 0.1mM non-radioctively labelled thymidine in RPMI + 0.5% FCS was then added to each well and incubated at 37°C for 20min. The wells were washed once with ice-cold PBS, twice with 10% TCA (trichloroacetic acid) and then once again with PBS. 250μl of 0.5M NaOH was added to each well and the plate incubated at 60-70°C for 30min to dissolve the cell monolayer. 200μl of cell lysate from each well was added to 4ml of Ecoscint A scintillation fluid (National Diagnostics) in a scintillation vial followed by 20μl of concentated HCl. The scintillation vials were thoroughly vortexed, placed into counting vials and counted on a LKB 1219 liquid scintillation counter with a ^{226}Ra external standard and quench correction.

2.16 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that catalyses the interconversion of lactate and pyruvate. Release of this enzyme into the culture supernatants can be used to assess cell viability/cytotoxicity [Liberek T *et al*, 1993]. LDH activity was determined from the rate of oxidation of reduced nicotinamide adenine dinucleotide (NADH),

measured as a change in optical density at 340nm, during the conversion of pyruvate to lactate using commercial assay reagents (Sigma).

LDH reagents A (0.194mM NADH in 54mM phosphate buffer pH 7.5) and B (16.2mM pyruvate containing nonreactive stabilisers and fillers) were reconstituted according to the manufacturer's instructions (Sigma). The spectrophotometer (Cecil CE 2040) wavelength was set at 340nm and zeroed against water. 700μl reagent A followed by 30μl sample was added to a cuvette, mixed and incubated for 60s. After this time 30μl of reagent B was added with thorough mixing. After a further 30s the absorbance reading at 340nm was measured (t=0). The decrease in absorbance at 340nm was subsequently measured at one minute intervals for 10 min. LDH activity was determined as follows:

$$\text{LDH activity (U/L)} = \frac{\Delta \text{ A per min x TV x 1000}}{\text{6.22 x LP x SV}}$$

where:

ΔA per min = change in absorbance per minute at 340nm.

TV = Total volume (ml)

SV = Sample volume (ml)

6.22 = Millimolar absorptivity of NADH at 340nm

LP = Light path (1cm)

1000 = Conversion of units per ml to units per l.

LDH release into culture supernatants was expressed as a percentage of LDH activity in cell monolayer lysates which had been prepared by scraping the monolayers into 400μl/well

RPMI 1640 + 0.5% FCS, sonicating for 15s and centrifuging for 2min at 11600g. The LDH content of neat MPCM was also assessed.

2.17 Identification of *de novo* synthesised fibronectin by mesangial cells

Newly synthesised fibronectin was identified by biosynthetically labelling mesangial cell secreted fibronectin with ^{35}S-methionine, immunoprecipitating out the fibronectin, running the immunoprecipitated protein out on SDS-PAGE and visualising the radioactive band by autoradiography.

2.17.1 Biosynthetic labelling

Confluent quiescent mesangial cells were exposed to MCPM or medium alone diluted 1:1 with methionine-free RPMI + 0.5% FCS (Sigma) for 72hr. 18hr prior to the end of the culture period each well was pulsed with 50μCi/well of L-^{35}S-methionine (Amersham, SJ 1515). The culture media were retained and the cell monolayers washed twice with PBS. Cell lysates were prepared as previously described (section 2.5).

2.17.2 Immunoprecipitation

20μl of 1mg/ml goat anti-rat fibronectin (Calbiochem; antibody recommended by manufacturers for immunoprecipitation) was added to 500μl of tissue culture supernatant or 200μl of cell lysate. Normal goat serum (Dako) was used as a control. The samples were incubated overnight at 4°C. 50μl of insoluble protein A cell suspension (10% wet w/v of non viable S. aureus (Cowan Strain) cells in 0.04M sodium phosphate buffer pH 7.2, 0.15M NaCl, containing 0.05% sodium azide, Sigma) was then added to precipitate out the antigen-antibody complexes and incubated for 4hr at 4°C. The samples were then

centrifuged for 10min at 4000rpm (3000g) and the pellets washed three times with ice-cold immunoprecipitation buffer (PBS containing 0.5M NaCl, 0.1% SDS, 1% Triton X 100, pH 7.4, Appendix 1) vortexing the pellet thoroughly between each wash. Finally the pellets were washed with ice-cold PBS and dissolved in 70μl of SDS-PAGE sample buffer containing 3% SDS and 10% 2-mercaptoethanol and heated for 7min in a boiling water bath. The dissolved pellets were centrifuged in a microcentrifuge for 30s.

The radiolabelled proteins were resolved by SDS-PAGE on 5% polyacylamide gels and dried as previously described (section 2.11).

2.17.3 Autoradiography

The dried gels were placed in a Kodak C-2 cassette containing two Kodak X-Omatic intensifier screens. A 13x18cm sheet of X-OMAT AR: XAR-S film was then aligned on the dried gel. The cassette was closed, placed in a plastic black bin liner and placed in a -70°C freezer for approximately 1 week. All autoradiographic procedures were carried out in the dark room using a 7.5W red safety light.

2.17.3.1 Development of autoradiographs

After an appropriate time, the cassette was taken out of the -70°C freezer and allowed to come to ambient temperature. The film was removed from the cassette and placed in approximately 500ml Kodak GBX developer and replenisher (Appendix 1). After about 1min in the developer solution (with gentle agitation) the film was placed briefly into 500ml water and then into 500ml of Kodak GBX fixer and replenisher (Appendix 1) for approximately 3min. The film was rinsed in water and allowed to dry. The bands were quantified by scanning densitometry on a LKB Ultroscan laser densitometer (LKB).

2.18 Biosynthetic labelling of MPCM

Macrophages were isolated and plated into 25cm^2 flasks as previously described (section 2.2). Adherent macrophages were washed and exposed to 1μg/ml LPS and 8μl (60μCi) of L-^{35}S-methionine in methionine-free, serum-free RPMI per flask. The flasks were incubated at 37°C for 16hr after which they were washed three times. 7ml of fresh methionine-free, serum-free RPMI was added and the flasks cultured for a further 48hr to generate the ^{35}Smet-MPCM.

2.19 Cytokine ELISAs

Cytokines measurements were carried out with commercially available ELISA assays for the following cytokines:

- Human TGFß$_1$ (Predicta, Genzyme Diagnostics., Quantikine, R&D)
- Human PDGF-AB (Biotrak, Amersham)
- Murine TNFα (Factor-Test-X, Genzyme Diagnostics)
- Murine IL-1ß (Intertest-1ßX, Genzyme Diagnostics)

All assays were carried out according to the manufacturer's instructions in twelve 8 well strips in a microtitre plate format. All assays were equilibrated at room temperature prior to use and all washes were carried out in wash buffer as described for the fibronectin assay. Samples and standards were assayed in duplicate.

2.19.1 TGFß$_1$ (Predicta)

a) Activation of standards and samples

TGFß$_1$ standards and samples were activated by acidification. 20μl of 1N HCl was added to 460μl of TGFß$_1$ standard (100ng/ml) or 400μl of sample. The tubes were vortexed and

incubated at 4°C for 1hr. The standards were neutralised by addition of 20μl 1N NaOH while the samples were neutralised with 15μl 1N NaOH.

A standard curve was prepared using 2.5 fold serial dilutions (4 - 0.1ng/ml)

b) Assay

100μl of standard or sample was added to the wells of a microtitre plate which had been pre-coated with a mouse monoclonal anti-human TGFß$_1$ antibody. The plate was incubated at 37°C for 1hr. After four washes, 100μl of anti-TGFß$_1$ HRP conjugate was added to each well and incubated for 1hr at 37°C. Following four more washes, 100μl of substrate reagent (tetramethylbenzidine and 0.03% H_2O_2 in buffered solution) was added to each well and incubated at room temperature for 20min. The reaction was stopped by addition of 100μl stop solution (mixture of acids (<2N)) and the absorbance read at 450nm. Sample values were interpolated from a standard curve and multiplied by a 2.17 dilution factor.

2.19.2 TGFß$_1$ (Quantikine)

When the R&D TGFß$_1$ ELISA became available it was found to be more sensitive than the Genzyme ELISA (31.2 vs 100 pg/ml respectively). Subsequently this became the TGFß assay of choice.

a) Activation of samples

80μl of 1N HCl was added to 400μl of sample, vortexed and left for 10 min at room temperature. The samples were then neutralised by addition of 80μl of 1.2N NaOH/0.5M HEPES free acid (Appendix 1) and vortexing.

The TGFß$_1$ standard did not need to be activated in this assay. Lyophilised TGFß standard

was reconstituted with calibrator diluent to give a 2000pg/ml stock. A standard curve was then prepared using two-fold serial dilutions (2000 - 31.2pg/ml).

b) Assay

200μl standard or sample was added to the wells of a microtitre plate which had been pre-coated with recombinant human TGFß soluble type II receptor. The plates were covered and incubated for 3 hr at room temperature. After 4 washes 200μl of polyclonal HRP-conjugated anti-TGFß antibody was added and incubated for 1.5hr at room temperature. After a further four washes 200μl of substrate solution (equal volumes of colour reagent A (stabilised H_2O_2) and colour reagent B (stabilised tetramethylbenzidine chromogen) were added and incubated at room temperature for 20min. 50μl of stop solution (2N H_2SO_4) was added and the aborbance at 450nm was determined. Sample values were interpolated from the standard curve and multiplied by a 1.4 dilution factor.

2.19.3 PDGF (Biotrak)

a) Standard curve

Lyophilised PDGF-AB standard was reconstituted using 5ml standard diluent (buffered protein base with preservative). The standard curve was prepared using two-fold serial dilutions (2000 - 31.3pg/ml).

b) *Assay*

50μl of assay diluent was added to the wells of a microtitre plate pre-coated with a murine antibody to PDGF-AA. 200μl of standard or sample was added to duplicate wells and incubated for 2hr at room temperature. After four washes 200μl of polyclonal HRP-

conjugated anti-PDGF-BB antibody was added to each well and incubated for 2hr at room temperature. Following four more washes 200μl of substrate solution (equal volumes of colour reagents A and B) was added and incubated for 20min at room temperature. The reaction was stopped by addition of 50μl stop solution (1M H_2SO_4). The absorbance at 450nm was measured and sample values interpolated from the standard curve.

2.19.4 TNFα (Factor-Test-X)(murine and rat)

a) Standard curve

Murine or rat TNFα standard (8.96ng/ml) was diluted using serial 4-fold dilutions (2240-35pg/ml).

a) Assay

100μl of standard or sample (1:2 dilution) were added to the wells of a microtitre plate pre-coated with hamster anti-murine TNFα monoclonal antibody. The plate was incubated for 2hr at 37°C. After four washes 100μl of polyclonal HRP-conjugated goat anti-mTNFα was added and incubated for 1hr at 37°C. After four more washes 100μl of substrate solution (equal volumes of colour reagents A and B) was added and incubated for 15min at room temperature. The reaction was stopped by addition of 100μl stop solution (1M H_2SO_4) and the absorbance was read at 450nm. Sample values were interpolated from the standard curve and multiplied by a dilution factor of 2.

2.19.5 IL-1ß (Intertest-1ßX)

a) Standard curve

The 9.6ng/ml murine IL-1ß standard was diluted 1:10 followed by serial four-fold dilutions

to give a standard curve in the 960-15pg/ml range.

b) Assay

100μl standard or sample were added to the wells of a microtitre plate which had been pre-coated with an anti-mIL-1ß monoclonal antibody and incubated for 1hr at 37°C. After four washes, 100μl biotinylated anti-mouse IL-1ß was added to each well and incubated for 1 hr at 37°C. The plate was washed again and 100μl of HRP-conjugated streptavidin was added to each well and incubated for 30min at 37°C. After a final wash, 100μl of substrate solution (equal volumes of colour reagents A and B) was added to each well and incubated for 10min. The reaction was stopped by adding 100μl stop solution (1M H_2SO_4) per well. The absorbance at 450nm was read and sample values interpolated from the standard curve.

2.20 PDGF and TGFß cell binding assays

In order to assess the physical effect of suramin on the binding of growth factors to mesangial cells a simple semi-quantitative assay on fixed mesangial cells was developed.

Confluent, quiescent mesangial cells in a 24 well plate were fixed with 200μl/well 0.25% gluteraldehyde for 10min at room temperature. The plate was washed twice with 2ml HBSS/well. The fixed mesangial cells were then treated with 200μl/well of either:

- 10ng/ml PDGF (or $TGF\beta_1$) $\pm$ 150μg/ml suramin or
- medium alone $\pm$ 150μg/ml suramin

for 1hr at room temperature. Following incubation the plate was washed three times with ELISA wash buffer and blocked with 2% BSA (in wash buffer) for 30min. 200μl of goat anti-human PDGF antibody (or rabbit anti-human TGFß) at 20μg/ml was added to each

well and incubated for 1hr at room temperature. The plate was then washed three times in wash buffer. 200μl/well of rabbit anti-goat Ig HRP conjugated antibody (1:5000 dilution, Dako) (or goat anti-rabbit Ig HRP conjugated antibody, 1:1000 dilution, Dako) was added to each well and incubated for a further hour at room temperature. Following another three washes 200μl/well OPD substrate solution was added (see ELISA method section 2.6). Once a suitable colour intensity had been reached the reaction was stopped with 200μl 1M H_2SO_4. Duplicate 200μl aliquots from each of the 24 wells were transfered to the wells of a 96 well plate and the optical density read on the titertek plate scanner.

MOLECULAR BIOLOGY

It became necessary to look at certain aspects of the study at the molecular level. All the techniques were set up by myself using protocols and probes furnished by Dr T Johnson of the Sheffield Kidney Institute.

2.21 Avoiding RNAse contamination

When working with RNA it is essential to avoid RNAse contamination. Although not essential for DNA work, all molecular biology techniques used were carried out in an RNAse free manner. All experimental and preparation work was carried out with gloved hands. Glassware was baked at 200°C for 4hr. Sterile plasticware was considered to be RNAse free. Non-sterile but fresh plasticware was autoclaved. All the water for making solutions was of nanopure grade and was rendered RNAse free by treatment with diethyl pyrocarbonate (DEPC) (Sigma, Appendix 1). All reagents and chemicals were of molecular biology grade and deemed to be RNAse free by the manufacturers. Chemicals were

weighed by pouring dried chemicals directly from the bottle - spatulas were never used. Any equipment which could not be autoclaved (electrophoresis cells, pH probes etc) was cleaned by vigorous rubbing with RNAse Away solution (Gibco).

2.22 Probes

All the probes used were generous gifts. Dr T Johnson (Sheffield Kidney Institute) carried out all the bacterial transformations of the probes into E.Coli (DH5α strain) with the exception of the MCP-1 cDNA. Small aliquots of Luria Broth (Millers LB broth base, 25g/L, Gibco) containing 50μg/ml ampicillin were innoculated with E Coli containing the various plasmids and were transfered to Leicester. The innocula were then processed as described in the next sections.

2.22.1 Fibronectin

A clone of rat fibronectin cDNA (p-SR270) from the exons encoding the 10th and 11th type 1 repeat near the C-terminal end of the molecule was a gift from Dr RO Hynes (Massachusetts Institute of Technology). This was a Stul-EcoR1 fragment, 270 bases in length, purified and inserted between the Hinc II and EcoR1 sites of pGEM2 plasmid. The sequence of this fragment is published in Schwarzbauer JE *et al* [1983].

2.22.2 Cyclophilin

A cDNA clone of human liver cyclophilin (pBHLCP11) was a gift from SmithKline Beecham Pharmaceuticals. Cyclophilins are a family of ubiqitous, highly conserved, abundant proteins believed to be the target proteins for Cyclosporin-A [Handschumacher R *et al,* 1984]. Cyclophilin was used as the 'house-keeping gene' in order to correct for

differences in RNA loading [Lopez JF *et al,* 1992] (Cyclophilin gene expression was unaffected by treatment with MPCM or cytokines in this system).

2.22.3 TGFß$_1$

The cDNA clone of murine TGFß$_1$ was a gift from Dr R Akhurst (Department Medical Genetics, Glasgow University). The cDNA is a 600bp fragment inserted into a pBluescribe plasmid. A description of the clone is published in Millan F *et al* [1991].

2.22.4 PDGF B chain

The cDNA clone of murine PDGF-B chain (CB8B.A1A2) was a gift from Dr CD Stiles (Dana Faber Cancer Institute, Harvard Medical School, Boston). The cDNA is a 325 base AvaI-AvaII fragment of a genomic clone blunt-end cloned into the Sma I site of pGEM-1. A description of the clone is published in Mercola M *et al* [1990].

2.22.5 α1 chain (IV) collagen

The cDNA clone of murine α1 (IV) collagen (pPE123) was a gift from Dr M Kurkinen (Department of Medicine, University of Medicine and Dentistry of New Jersey-Rutgers Medical School, Piscataway, New Jersey). The 1.8kb insert codes for the 204 amino acids at the C-terminus of the NCI domain of the α1 (IV) collagen chain. A description of the clone is published in Kurkinen M *et al* [1987].

2.22.6 Laminin B1 chain

The cDNA clone of murine laminin B1 chain (pPE386) was also a gift from Dr M Kurkinen. The 1.1kb insert codes for approximately 300 amino acids at the C terminus of

the laminin B1 chain. A description of the clone is published in Barlow DP *et al* [1984].

2.22.7 TIMP1

The cDNA clone of murine TIMP1 was a gift from DT Denhardt (Rutgers University, Piscataway, New Jersey, USA). The cDNA is a 720bp fragment inserted in to a pSP64 plasmid. A description of the clone is published in Waterhouse P *et al* [1990].

2.22.8 MCP-1

The cDNA clone of rat MCP-1 was a gift from Dr T Yoshimura (Laboratory of Immunobiology, Frederick Cancer Institute, Bethesda Maryland, USA) and was provided in the plasmid form by Dr F Tamm (Renal Unit, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London). The cDNA is a 665bp fragment inserted into a pBLUEScribe plasmid. A description of the clone is published in Yoshimura T *et al* [1991].

2.23 Preparation of cDNAs

2.23.1 Bacterial cultures

An E.Coli innoculum was cultured in 30ml of Luria broth (25g/L containing 50μg/ml ampicillin) (Sigma) overnight at 37°C after which it was transferred into 400ml LB Broth containing ampicillin and cultured for a further 24hr.

2.23.2 Plasmid preparation

Plasmid preparation and purification was carried out at room temperature using a Wizard Maxipreps DNA Purification System (Promega) according to manufacturer's instructions.

The 400ml culture was divided equally between two 250ml centrifuge bottles and centrifuged at 5000g for 10min in a room temperature SLA-1500 fixed angle rotor (Sorvall) in a RC 5B Plus high speed centrifuge (Sorvall). The supernatants were decanted and each pellet was resuspended in 7.5ml Cell Resuspension Solution (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100μg/ml RNAse A). To aid resuspension the pellets were disrupted by pipetting up and down until no clumps were visible. 7.5ml Cell Lysis Solution (1% SDS in 0.2M NaOH) was added to each pellet and the solutions mixed gently, but thoroughly, in a shaker incubator set at ambient temperature for about 20min, until the solutions became clear and viscous. 15ml of Neutralisation Solution (1.32M potassium acetate, pH 4.8) was then added and immediately mixed by inversion in the centifuge bottles. The bottles were centrifuged at 14,000g for 15min in a room temperature rotor. The cleared supernatants were then filtered through Whatman no.1 filter paper into clean graduated measuring cylinders and the volumes measured. The supernatants were then transferred into 50ml centrifuge tubes. Half the supernatant volume of room temperature isopropanol (Sigma) was added and mixed by inversion. The solutions were then centrifuged at 14,000g for 15min in a SS-34 fixed angle rotor (Sorvall). The supernatants were carefully discarded and each DNA pellet was resuspended in 1ml Tris-EDTA (TE) buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA, diluted from a 100X concentrate, Sigma) and frozen at -70°C.

2.23.3 Plasmid purification

5ml of Wizard Maxipreps DNA Resin was added to each of the thawed pellets of plasmid preparation and swirled to mix thoroughly. The Resin/DNA mix from the two pellets was applied to a Maxicolumn. The Maxicolumn was then connected to a vacuum source in order to pull the Resin/DNA mix into it. 13ml of Column Wash Solution (200mM NaCl, 20mM Tris-HCl, pH 7.5, 5mM EDTA - diluted with 95% ethanol to give a final ethanol concentration of 55%) was added to the tubes which had contained the pellets. The washings were swirled and applied to the column (to ensure thorough transfer of all the Resin/DNA). A vacuum was applied to draw the Wash Solution through the Maxicolumn. A further 12ml of Column Wash Solution was added to the column and the vacuum applied again. The column Resin was then rinsed with 5ml 80% ethanol after which a vacuum was applied for 1min. The column was then inserted into a 50ml screw cap tube and centrifuged using a swing-out bucket rotor in a bench centrifuge at 2500 rpm for 5min. The Maxicolumn (containing the Resin/DNA) was removed and both the centrifuge tube and liquid were discarded. The Resin was then thoroughly dried by placing it back on the vacuum source. The Maxicolumn was placed into another 50ml screw cap tube and 1.5ml of pre-heated (65-70°C) TE buffer was added to the column and left for 1min. The DNA was eluted from the column by centrifugation for 5min at 2500 rpm in a bench centrifuge with a swing-out rotor as before. The column was discarded and the eluted DNA in the bottom of the 50ml tube was frozen at -70°C after removing a small aliquot (4μl) to measure the DNA concentration.

2.23.4 Determination of DNA concentration

4μl of purified plasmid was diluted in 400μl water (1:100 dilution) and the absorbances at 260nm and 280nm were measured on a Cecil spectrophotometer.

For DNA A_{260} 1 = 50μg/ml

Therefore: A_{260} x 100 (dilution) x 50 x volume (ml) = total DNA (μg)

The ratio A_{260}/A_{280} gives an index of the purity of the sample and should typically be about 1.8.

2.23.5 Excision of cDNA from plasmid

For the excision of cDNA 30μg plasmid was mixed together with the appropriate restriction endonucleases and buffers and incubated at 37°C for 1-2 hr with continuous agitation. Combinations of the following were used:

- Black pallette buffer (10mM Tris-HCl, pH 7.2, 300mM NaCl, 5mg/ml BSA, 0.5mM EDTA, 5.0mM EGTA, 5.0mM 2-mercaptoethanol 0.2%(w/v) Triton X-100, (Sigma)
- EcoR1 restriction endonuclease (125 units/μl, Sigma)
- Hind III restriction endonuclease (20 units/μl, Sigma)
- Bam H1 restriction endonuclease (20 units/μl, Sigma)
- BSA (20mg/ml)
- DEPC-water

The components and volumes appropriate to each cDNA digestion are summarised in table 2.1.

Table 2.1 Summary of cDNA digestion components.

cDNA	Plasmid	Buffer	EcoR1	HindIII	BamH1	BSA	Water
Fibronectin	39μl	10μl	2μl	8μl	-	-	41μl
Cyclophilin	29.5μl	10μl	4μl	10μl	-	-	46.5μl
TGFß	95μl	10μl	4μl	10μl	-	-	26μl
PDGF	58μl	10μl	5μl	-	10μl	2μl	15μl
Coll IV	44μl	10μl	5μl	10μl	-	-	31μl
Laminin	44.5μl	10μl	5μl	10μl	-	-	30.5μl
TIMP-1	130μl	20μl	5μl	12μl	-	-	33μl
MCP-1	10μl	5μl	2μl	-	-	-	33μl

2.23.6 Isolation of cDNA from plasmid digest

2.23.6.1 Gel preparation

A 1% agarose gel was made by adding 150ml of 1X Tris-Acetate-EDTA (TAE) buffer (40mM Tris, 1mM EDTA pH 8.0, 0.2M acetic acid, pH 7.6, (diluted from a 10X concentrate, Gibco)) to 1.5g agarose (Sigma) and heating in a boiling water bath until the agarose had completely dissolved. The agarose solution was then allowed to cool to about 50°C. 0.5μg/ml ethidium bromide (Sigma) was added to the gel solution which was mixed by swirling. The agarose solution was poured into a gel tray (which had had its open ends blocked off with masking tape). An 8 well comb (11mm x 2mm x 10mm, Flowgen) was slotted into the gel tray and the gel was left to set for about 30min.

2.23.6.2 Sample preparation

Equal volumes of plasmid digest and 10X DNA sample loading buffer (20% Ficol 400K, 0.1M EDTA, pH 8.0, 1% SDS, 0.25% bromophenol blue, (Sigma, Appendix 1)) were added together, vortexed and briefly pulse centrifuged in a microfuge to pool the solution at the bottom of the tube. 4μl of DNA standard ladder (λ DNA/Hind III fragments, 0.5μg/μl, Gibco) was mixed with 5μl 2X saline-sodium citrate (SSC) (0.3M NaCl, 0.03M Na citrate, pH 7.0) and heated to 65°C for 10min. 10μl of DNA sample loading buffer was then added to the standards.

2.23.6.3 Loading and electrophoresis

Once the agarose had set, the masking tape and comb were removed and the gel tray replaced into the horizontal electrophoresis cell (model MH1510, Flowgen). TAE buffer was then poured into the electrophoresis chamber until the gel was completely submerged.

The sample (approximately 50μl/well) and the standard (0.1μg/mm lane width) were loaded onto the gel. The gel was electrophoresed at 100V for about 2hr. A typical example of a gel showing the excised cDNA distal to the plasmid is shown in Fig 2.6.

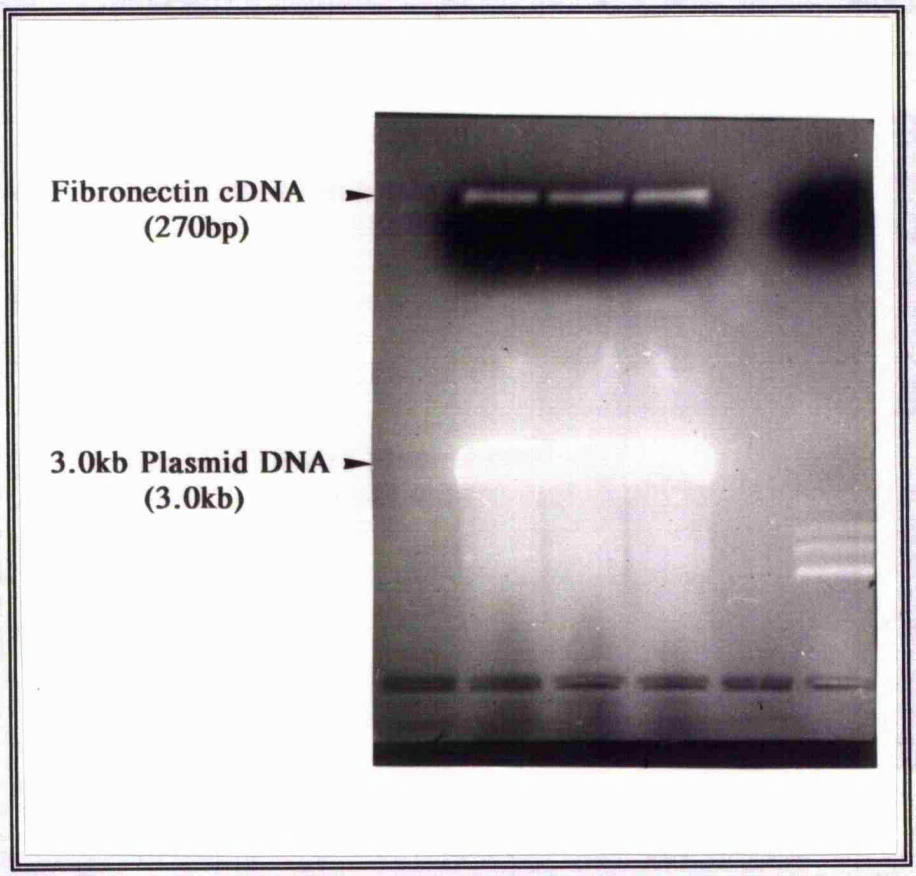


Fig 2.6 Isolation of fibronectin cDNA from plasmid digest

Example of a 1% TAE agarose gel stained with ethidium bromide under UV light showing excised 270bp fibronectin cDNA distal to the plasmid.

2.23.6.4 Extraction of cDNA from agarose gel

A Sephaglas BandPrep Kit (Pharmacia) was used to extract the cDNA from the agarose gel according to the manufacturer's instructions.

Following electrophoresis, the gel was viewed on a UV transilluminator (Flowgen) to locate the cDNA band. Using a clean scalpel, the slices of agarose containing the cDNA bands were excised as close to the bands as possible and placed into pre-weighed 1.5ml microcentrifuge tubes. The tubes were weighed again and the weight of agarose calculated by subtraction. 1μl of Gel Solubiliser (buffered NaI solution) per mg of agarose was added to the tubes. These were vortexed vigorously and incubated at 60°C for 10min or until the agarose slice was completely dissolved. 5μl of previously vortexed Sephaglas BP suspension (20% w/v Sephaglas BP in aqueous solution) was added for each μg DNA and vortexed gently. The mixture was incubated for 5min at room temperature, vortexing every minute to resuspend the Sephaglas. The tubes were pulse spun for 30s in a microfuge and the supernatants carefully discarded without disturbing the Sephaglas pellet. 40μl of Wash Buffer (20mM Tris-HCl, pH 8.0, 1mM EDTA, 0.1 mM NaCl solution to which 18ml absolute ethanol had been added before use)(ie 8X the volume of Sephaglas) was added, vortexed and then pulse spun for 30s. Again the supernatant was carefully discarded. This wash step was performed three times. After the third wash the tubes were inverted and the Sephaglas allowed to dry for at least 10min. 20μl of Elution buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) was added and the tubes were vortexed to resuspend the pellets and incubated for 5min at room temperature with periodic agitation. The tubes were again spun for 1min in a microfuge. The supernatant was carefully removed and placed into a clean tube. A 5μl aliquot was taken for the estimation of DNA concentration while the rest was

frozen at -70°C until required.

2.23.6.5 Quantitation of cDNA

A 1% TAE gel was prepared as previously described (section 2.22.6.1). A 16 well comb (5mm x 1mm x 10mm) was used. 8μl, 4μl, 2μl, 1μl and 0.5μl aliquots of standard λDNA/Hind III (0.5μg/μl) fragments were premixed with 5μl of 2x SSC and heated in a 65°C water bath for 10 min. 10μl of DNA sample loading buffer was added to the DNA standards and to 5μl of purified cDNA. The standards and samples were loaded on the gel and electrophoresed in TAE buffer for 2hr at 100V. The concentration of cDNA was determined by scanning the fluorescent gel using the Gel Doc programme on the Bio-Rad Scanning Laser Densitomer and comparing the fluorescence of the cDNA with that of the DNA standards. Fig 2.7 shows an example of a gel showing the purified cDNA against DNA ladder standards.

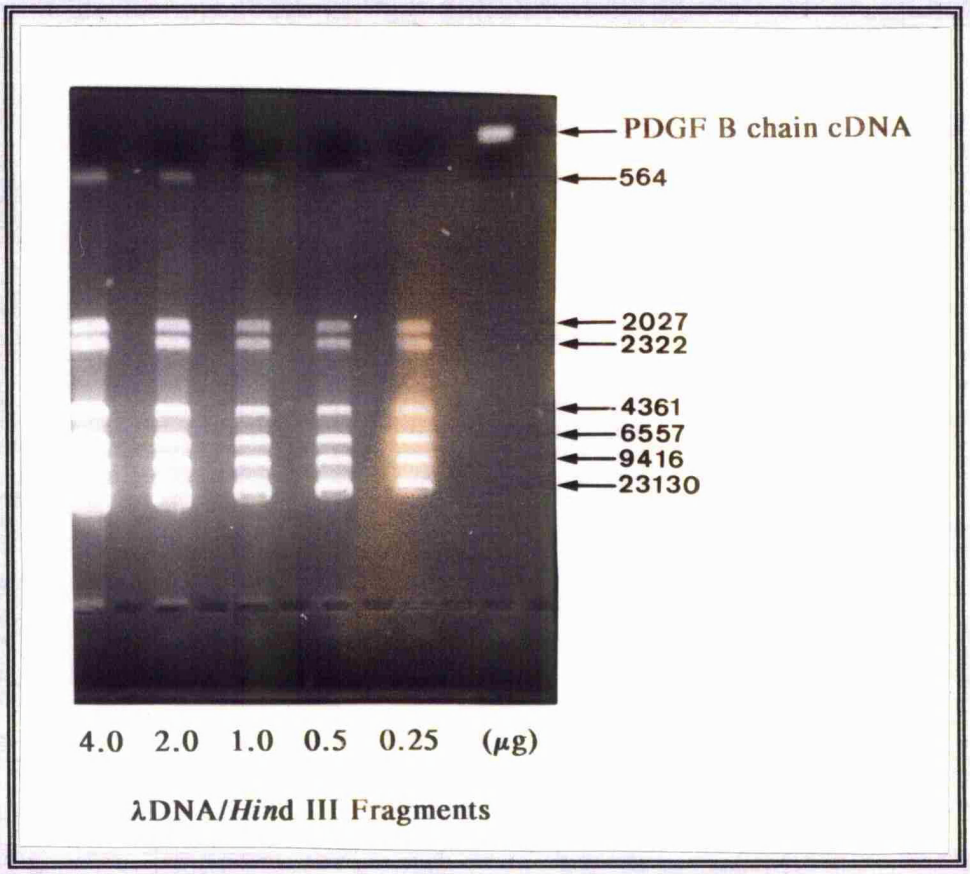


Fig 2.7 Quantitation of cDNA

Example of 1% TAE agarose gel stained with ethidium bromide under UV light showing the 325 bp PDGF B chain cDNA against a series of DNA ladders (λDNA/Hind III fragments) at 4, 2, 1, 0.5, 0.25 μg DNA/lane.

2.24 ^{32}P-dCTP labelling of cDNA probes

Labelling of probes was carried out using the Prime-a-Gene Labelling System (Promega) which is based on the method of Feinberg and Vogelstein [1983]. Labelling was carried out according to manufacturer's instructions.

All labellings were carried out in a designated radioactive area using perspex screens for protection.

25ng of cDNA was mixed with 5μl of dilution buffer (10mM Tris-HCl, pH 7.4, 5mM NaCl, 0.1mM Na-EDTA, Appendix 1) in a microfuge tube and denatured by heating at 95-100°C for 5min. The tube was then cooled on ice and the following additions made:

- nuclease-free water (to give a final sample volume of 50μl)
- 10μl 5X labelling buffer (250mM Tris-HCl, pH 8.0, 25mM $MgCl_2$, 10mM DTT, 1M HEPES, pH 6.6, 26 A_{260} u/ml Random Hexadeoxyribnucleotides)
- 2μl of unlabelled dNTPs (dATP, dGTP and dTTP mixed in equal volumes, each at 20μM final concentration).
- 2μl nuclease-free BSA (400μg/ml final concentration).
- 5μl [α-^{32}P]dCTP (50μCi, 3000Ci/mmol)
- 1μl of DNA Polymerase 1, Large (Klenow) Fragment (100 u/ml final concentration).

The mixture was vortexed, pulse centrifuged to bring down all the reaction mixture from the sides of the tube and incubated at room temperature for at least 1hr.

2.24.1 Removal of unincorporated nucleotides

Unincorporated nucleotides were removed by size exclusion chromatography using Sephadex G-50 Nick spin columns (Pharmacia). The columns were used according to manufacturer's instructions.

The top cap of the column was removed, the excess liquid poured off and the column rinsed with approximately 3ml TE buffer. The bottom cap was then removed and the column supported over a 30ml tube. It was equilibrated with approximately 3ml TE buffer. 50μl of ^{32}P-labelled probe solution was applied and allowed to enter the gel bed. 400μl of TE buffer was then added and allowed to enter the gel bed. The column was placed over a microfuge tube inside a 30ml tube for sample collection. The purified probe was eluted from the column with 400μl of TE buffer.

2.25 Northern blotting

2.25.1 RNA extraction

RNA extraction was carried out using TRIzol reagent (Gibco), a mono-phasic solution of phenol and guanidinium isothiocyanate, using a modified method of Chomczynski P and Sacchi N [1987]. The TRIzol reagent was used according to the manufacturer's instructions.

2.25.1.1 Cell lysis

Following stimulation of mesangial cells with MPCM, cytokines or medium alone the tissue culture supernatants were removed and the cells were lysed by addition of 1ml TRIzol reagent (Gibco) with trituration. The flasks were incubated for 5min at room

temperature to allow complete dissociation of nucleoprotein complexes.

2.25.1.2 Phase separation

The cell lysates were transferred into microfuge tubes and 0.2ml chloroform were added per 1ml of TRIzol reagent. The tubes were capped and shaken vigorously for 15s and incubated at room temperature for a further 2-3min. The samples were then centrifuged at 12000rpm in a microfuge for 15min at 4°C.

2.25.1.3 RNA precipitation

Following centrifugation the colourless, aqueous, upper phases were transfered into fresh tubes. The RNA was precipitated from the aqueous phase by addition of 0.5ml isopropanol/ml TRIzol reagent. The samples were vortexed, incubated at room temperature for 10min and centrifuged at 12000rpm for 10min at 4°C.

2.25.1.4 RNA wash

Following centrifugation the supernatants were removed and the pellets were washed once with 1ml 75% aqueous ethanol. The samples were vortexed and centrifuged at 8000 rpm for 8min at 4°C.

2.25.1.5 Redissolving the RNA

At the end of the procedure the RNA pellet was air dried for about 10min. (It is important not to let the RNA pellet completely dry as this will impede solubility). 20μl of DEPC-water was then added to each pellet and incubated for about 10min at 37°C to ensure complete dissolution.

2.25.1.6 Quantitation of RNA

2μl of RNA solution was diluted in 400μl DEPC-water and the absorbance at A_{260} and A_{280} were measured. The RNA concentration was then calculated according to the following formula:

for [RNA], A_{260}= 1 = 40μg/ml

therefore, RNA in sample (μg) = A_{260} x 200 (dilution) x 40 x 0.02 (volume (ml))

The ratio of A_{260}/A_{280} was determined to give an indication of the quality of the RNA preparation. The ratio was typically about 1.6.

The samples were frozen at -70°C until ready for use.

2.25.2 Electrophoresis of RNA samples.

2.25.2.1 Gel Preparation

150ml MOPS buffer (20mM 3-[N-Morpholino]propanesulfonic acid (MOPS), 5mM sodium acetate, 1mM EDTA, Appendix 1) was added to 1.5g agarose and heated in a water bath to dissolve the agarose. The agarose was allowed to cool to 50°C after which 7.8ml 37% formaldehyde was added with swirling. The agarose was poured into the gel tray as described before and allowed to set.

2.25.2.2 Sample preparation

30μg of RNA solution was added per microfuge tube followed by 25μl electrophoresis sample buffer (Appendix 1) containing 40μg/ml ethidium bromide. The samples were then heated at 65°C in a water bath for 15min. 3μl/well of 0.24-9.5 Kb RNA ladder (Sigma)

(made up and heated in the same way as the samples) was loaded on the same gel as a size standard.

2.25.2.3 Electrophoresis

Prior to sample loading the electrophoresis tank was filled with MOPS electrophoresis buffer. The samples were then loaded into the wells and electrophoresed at 80V until the dye front has gone 3/4 of the distance of the gel.

2.25.2.4 Integrity of RNA

Following elecrophoresis the gel was placed on a TFX 20M UV transilluminator (Flowgen) in order to visualise the RNA. The 28S (5kb) and the 18S (2kb) ribosomal subunits should be clearly visible at a ratio of 2:1 indicating no significant degradation of RNA.

The gel was photographed using a Polaroid DS 34 direct screen instant camera fitted with a 0.85X electrophoresis hood and a tiffen 40.5mm 15 orange filter. The camera was loaded with Polaroid Black-and-White print film type 667. Camera settings were F number 5.6 and 1/30s or 1/15s exposure time (depending on the brightness of the bands).

Fig 2.8 shows the typical staining pattern of the ribosomal subunits.

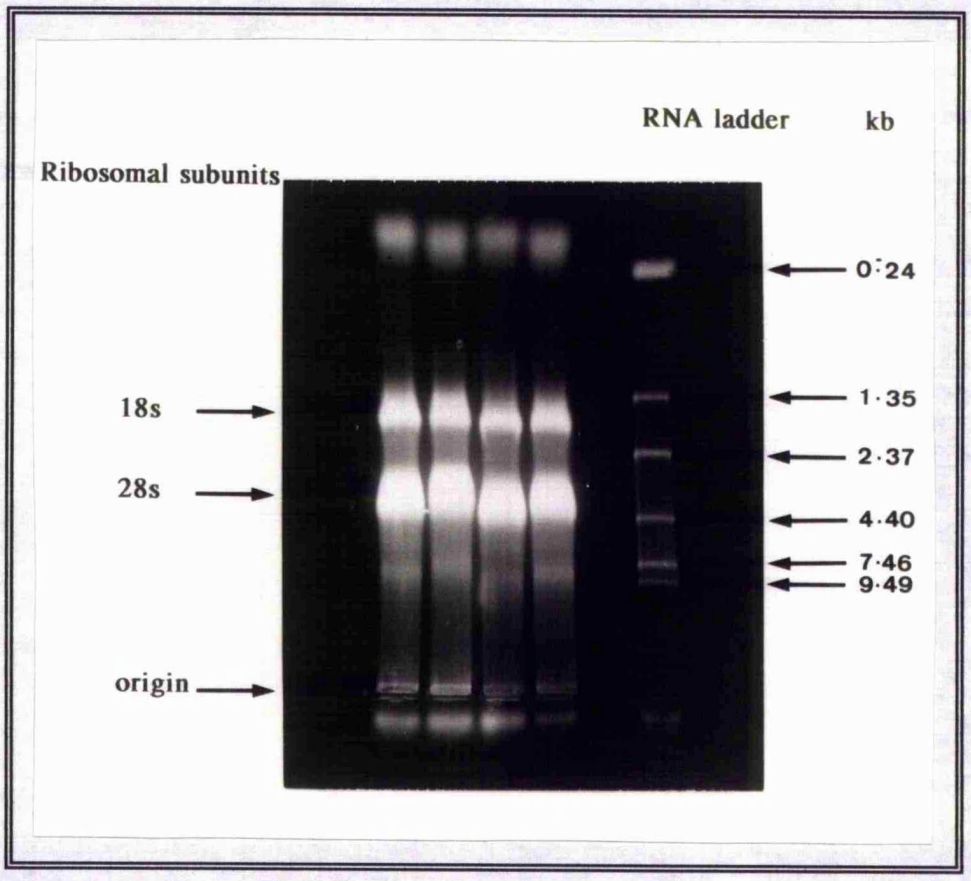


Fig 2.8 Integrity of RNA

Example of a 1% MOPS agarose gel with ethidium bromide stained samples under U.V light showing the 28s and 18s ribosomal subunits.

2.25.3 Northern Transfer

After electrophoresis and photography the gel was inverted and soaked in DEPC-water for 30min followed by 20X SSC for 5min to get rid of the formaldehyde. After soaking the gel was placed on to the transilluminator and exposed to UV light for approximately 5min to aid in subsequent transfer. During gel soaking the capillary transfer apparatus was set up:-

0.5L of 20X SSC was poured into a plastic tray. A glass plate which was longer but narrower than the plastic tray was placed on top. A piece of double-folded Whatman 3MM paper was placed around the glass plate such that the ends of the paper dipped into the buffer forming a wick. When the filter paper on the glass plate was thoroughly wetted it was smoothed out with a sterile pipette to get rid of air bubbles. The agarose gel was then placed on the wetted filter paper, well side down. A piece of Hybond-N nylon membrane (Amersham) was cut to the size of the gel, wetted in 20X SSC and placed on top of the gel. Cling-film was then placed around the perimeter of the gel to ensure that capillary transfer of buffer occured only through the gel. Two sheets of Whatman 3MM paper soaked in 20X SSC were placed on top of the nylon membrane followed by 10 sheets of Quick-Draw paper (Sigma) and a stack of paper towels about an inch thick. Lastly a glass plate was placed on top of the towels which was weighed down by a 500g weight. Transfer was allowed to proceed for approximately 18hr during which time wet towels were replaced.

This set up allows a flow of liquid from the reservoir through the gel and the nylon membrane eluting the RNA from the gel and depositing it on the nylon membrane.

Following transfer the towels were removed and gel and nylon membrane were placed on the UV transilluminator membrane-side down to check that transfer had occurred efficiently. The positions of the wells were marked onto the membrane as well as the positions of the 28S and 18S ribosomal subunits. The gel was discarded and the membrane rinsed in DEPC-water to remove any pieces of agarose. The membrane was then dried at 80°C for 10min.

2.25.4 UV cross-linking

In order to fix the RNA to the nylon membrane it was placed inside a UV cross-linker (Amersham) which had been pre-calibrated for use with Hybond-N nylon membranes. The membrane was then wrapped in cling film, sealed in a plastic bag and stored at 4°C ready for hybridisation.

2.25.5 Pre-hybridisation

The blotted membrane was placed into a 35 x 300 mm hybridisation bottle. The following were then added together (10ml/bottle) to give the stated final concentrations:

- 1.0ml DEPC-water
- 5.0ml De-ionised formamide (50%)
- 2.5ml 20X saline-sodium phosphate-EDTA (SSPE) (0.2M phosphate buffer, pH 7.4, 2.98M NaCl, 0.02M EDTA, Gibco) (5X)
- 1.0ml 50X Dendardt's (1% Ficoll 400kD, 1% polyvinylpyrolidone, 1% BSA, Sigma) (5X)
- 0.5ml 20% SDS (1%)

The solution was pre-warmed to the hybridisation temperature (37°C for fibronectin cDNA and 42°C for the other probes). 0.5ml of salmon sperm DNA (10mg/ml, Gibco) was boiled for 5min and then immediately quenched on ice. 200μl of the denatured DNA was added per 10ml of pre-hybridisation solution. This was then poured into the hybridisation bottle containing the membrane. The bottle was placed into a hybridisation oven (Hybaid) and incubated for 4hr at the appropriate temperature.

2.25.6 Hybridisation

The hybridisation solution was made up and warmed to the hybridisation temperature. 200μl salmon sperm DNA was boiled for 2min then added to freshly ^{32}P-labelled probe. The probe and salmon sperm DNA were denatured at 95°C for 5min, quenched on ice and added to the hybridisation solution. The pre-hybridisation solution was removed from the hybridisation bottle and replaced with hybridisation solution containing the probe. Hybridisation was carried out overnight at 37°C (fibronectin) or 42°C (other probes).

2.25.7 Stringency washes

Following hybridisation the hybridisation solution was poured off and the membrane was washed with 50ml aliquots of the following solutions at the given temperatures:

- 2X SSPE, 0.2% SDS at room temperature for 15min
- 2X SSPE, 0.2% SDS at room temperature for 15min
- 0.2X SSPE, 0.2% SDS at 65°C for 30min
- 0.2X SSPE, 0.2% SDS at 65°C for 30min

All washes were carried out in the hybridisation bottles in the hybridisation oven.

2.25.8 Autoradiography

Following the stringency washes the membrane was wrapped in cling-film, placed into an exposure cassette, exposed to Kodak Xomat LS film and placed at -70°C. The exposure time was adjusted according to the activity of the probe on the membrane (as assessed using a Geiger counter) in order to give an image which was readily visualised. The film was developed as described earlier. The bands were scanned using an imaging laser densitometer (model GS 700, BioRad).

2.25.9 Stripping membrane of probe

1L of boiling stripping solution (0.1X SSPE, 1% SDS, Appendix 1) was poured over the membrane in a tray and gently agitated until the stripping solution had completly cooled. The membrane was then allowed to dry on Whatman 3MM filter paper. The membrane was not allowed to dry out if it was to be stripped for reuse.

2.26 Methylene blue staining of RNA

In order to have a set of size markers which compared directly in scale with the bands on the autoradiograph, the RNA ladder on the membrane was stained with methylene blue.

The lane containing the RNA ladder was carefully cut from the membrane and soaked in 5% acetic acid for 15min at room temperature. The membrane was then transferred to a solution of 0.5M sodium acetate pH 5.2 and 0.04% methylene blue (Appendix 1) for 10min at room temperture. The membrane was rinsed in water for approximately 10min after which it was dried so that sharp RNA bands could be visualised. The typical staining pattern of the RNA ladder is shown in Fig 2.9.

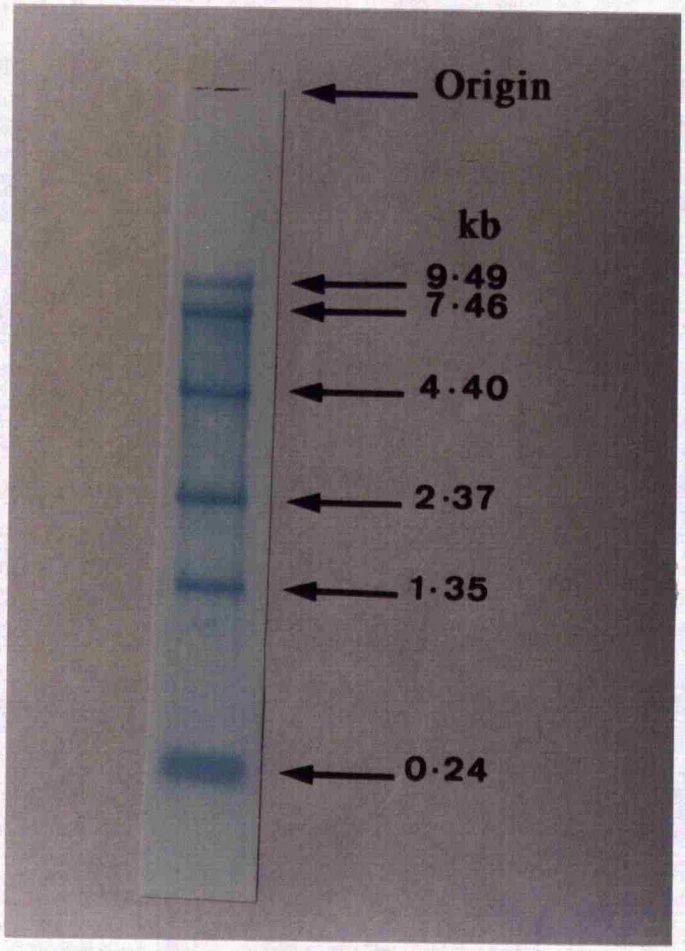


Fig 2.9 Methylene blue staining

Example of an RNA ladder stained with methylene blue.

2.27 Data processing and statistics

2.27.1 Data presentation

Throughout the study it was noticed that quantitatively the response of mesangial cells with respect to fibronectin secretion varied between experiments, depending on a number of parameters including mesangial cell line and passage number, batch of MPCM, batch of FCS etc. Qualitatively however, within assay results were consistent. In order to allow comparison of multiple experiments results were expressed as percentages or fold increases compared to control. However, where it was appropriate to present absolute values (and replicate experiments differed by more than 20%), then representative experiments are presented.

2.27.2 Statisticical analysis

Data were expressed as means $\pm$ SEM. For comparison of means between two groups an unpaired *t*-test was used. To compare values between multiple groups an analysis of variance (ANOVA) with a Bonferroni correction was applied. Statistical significance was defined as $p<0.05$. All computations were carried out using 'in house' (c-stat and ANOVA) statistical computer packages.

CHAPTER THREE

Effect of macrophage secretory products on mesangial cell matrix production.

3.1 Introduction

As early as 1953 studies by Jones demonstrated an increased number of macrophages in the mesangium following renal damage. These studies were later corroborated and elaborated on by other investigators [Schreiner GF *et al*, 1978., Floege J *et al,* 1992a]. The precise mechanism whereby an influx of macrophages results in the initiation of the sclerotic process are yet to be fully understood. Macrophages are known to secrete a number of biologically active substances into their local milieu [Nathan CF, 1987], it is conceivable that these secretory products could modulate the proliferation and production of extracellular matrix proteins by resident glomerular cells. A number of investigators have shown that macrophage secretory products can directly modulate mesangial cell proliferation [Lovett DH *et al*, 1983a., Matana J and Singhal PC, 1993., Ooi YM *et al*, 1983., Weissgarten J *et al*, 1993]. Lovett and co-workers [1983a] tentatively attributed this activity, which could only be seen in the presence of 20% FCS, to IL-1ß. To date the effects of long term, *in vitro* exposure of mesangial cells to macrophage secretory products or conditioned medium (MPCM) have been largely unexplored. Mosquero JA [1993] demonstrated that MPCM derived from macrophages elicited by various agents enhanced proliferating mesangial cell fibronectin production. Similarly Narita I *et al,* [1991] demonstrated that factors derived from LPS stimulated human monocytes caused non quiescent rat mesangial cells to upregulate their production of glycosaminoglycans. Matana and Singhal [1994] reported enhanced proliferation and matrix synthesis in response to secretory products derived from macrophages activated by Fc-receptor-mediated endocytosis.

Since macrophages are of pathogenetic importance to the development of glomerulosclerosis, the present study was undertaken to establish the role played by macrophage secretory products on the initiation of matrix deposition by mesangial cells - functionally the most important cells within the glomerulus.

3.2 The experimental model

The test system used in the majority of the experiments in this thesis involved looking at the effects of thioglycollate elicited, LPS stimulated, adherent rat peritoneal macrophages or conditioned medium generated from the culture of these cells, on the fibronectin production of cultured rat mesangial cells. Fibronectin was the matrix protein of choice since it has been shown to accumulate in the glomerulus in many of the conditions leading to glomerulosclerosis [Dixon AJ *et al*, 1980]. The experiments were carried out on quiescent cells grown in 0.5% FCS to both reflect the state of activation of the *in vivo* mesangial cell and to dissociate any mitogenic effects from those related to stimulation of matrix production.

3.3 Effect of macrophage / mesangial cell co-culture on fibronectin production.

3.3.1 Materials and Methods

Thioglycollate elicited peritoneal macrophages (isolated as described in chapter 2 section 2.2) were added to confluent, quiescent mesangial cells in 24-well plates at final (macrophage) cell densities of 2.5×10^5, 1.25×10^5, 6.25×10^4, 3.13×10^4, 1.56×10^4, and 0 cells/ml/well. In parallel, macrophages at the same cell densities were cultured directly on the 24-well plates as controls for any macrophage fibronectin production. After 3 days of incubation the supernatants and cell lysates were assayed for fibronectin. (methods described in chapter 2 sections 2.4-2.6).

3.3.2 Results

Macrophages cultured in the presence of 0.5% FCS for 3 days generated small amounts of fibronectin. The amount of fibronectin produced (secreted and cell associated) depended on the number of macrophages cultured (Fig 3.1a). Direct co-culture of macrophages with mesangial cells resulted in a significantly greater fibronectin production than was seen with macrophages or mesangial cells alone in both the secreted and cell associated forms (Fig 3.1a and b note the difference in scales).

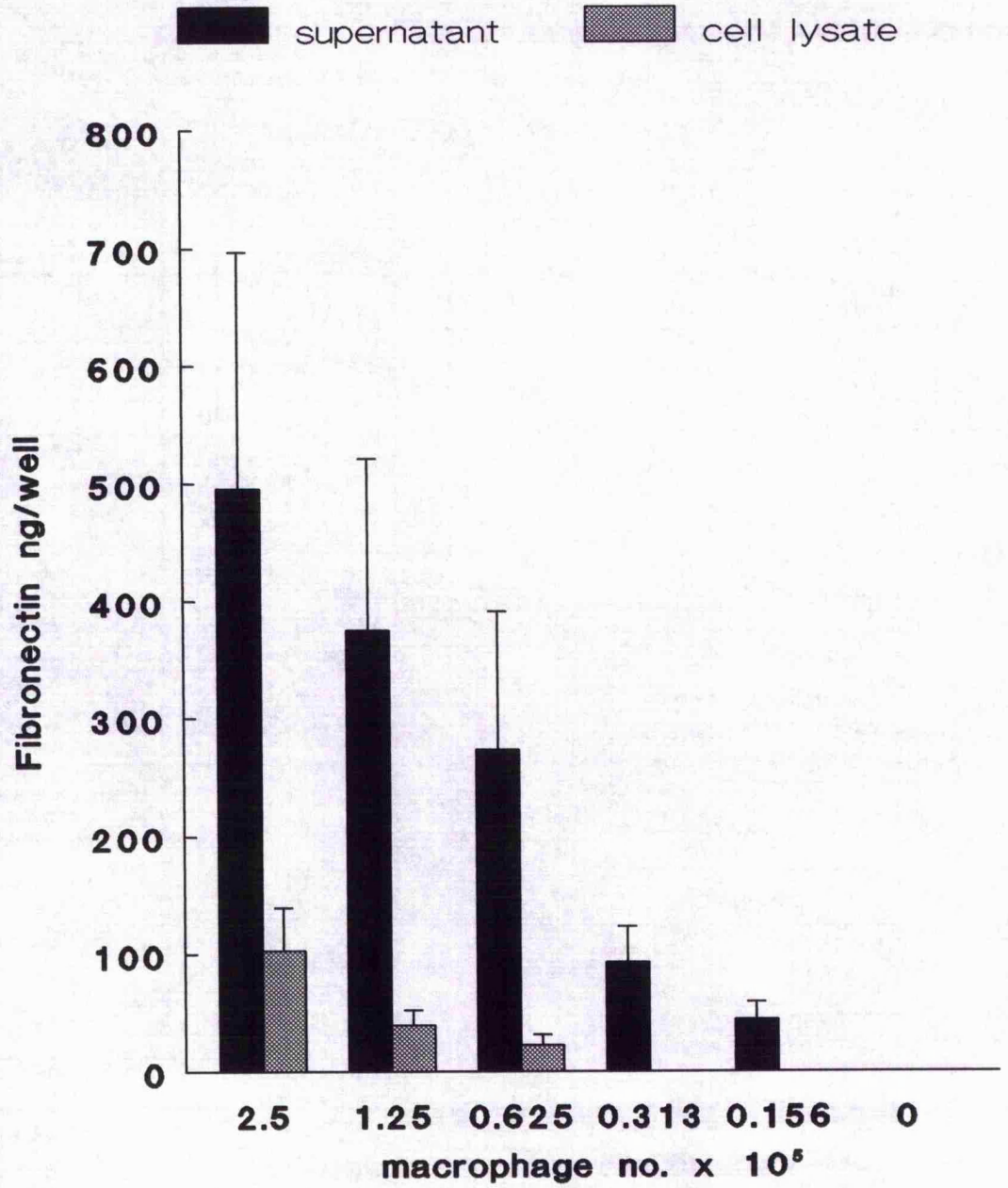


Fig 3.1a Effect of macrophage culture on fibronectin production.

Macrophages were cultured for 3 days in RPMI containing 0.5% FCS. Tissue culture supernatants and cell lysates were assayed for fibronectin. Values are mean ± SEM of 6 wells.

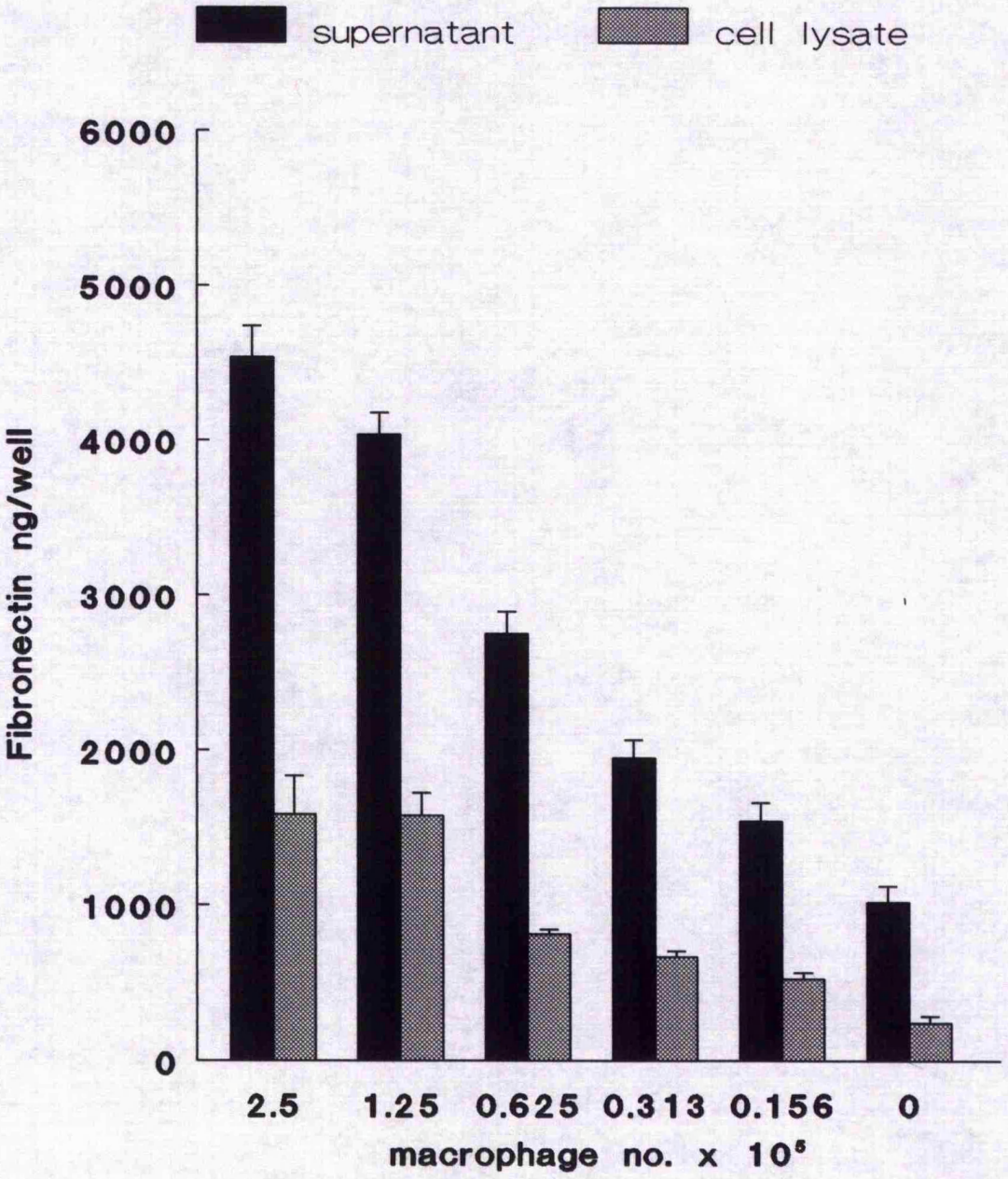


Fig 3.1b Effect of mesangial cell/macrophage co-culture on fibronectin production. Mesangial cells were co-cultured in the presence of decreasing numbers of macrophages (2.5, 1.25, 0.625, 0.313, 0.156 x 10^5 cells/ml/well) for 3 days. Supernatants and cell lysates were assayed for fibronectin. Values represent means ± SEM of 8 wells.

3.4 Effect of MPCM on fibronectin production by mesangial cells.

In order to confirm that the macrophage effect on mesangial cell fibronectin production was due to secreted factors and could occur independently of cell:cell contact, mesangial cells were exposed to macrophage conditioned medium (MPCM).

3.4.1 Materials and Methods

Confluent, quiescent mesangial cells were exposed to 100, 50, 20, 10 and 1% solutions of MPCM or to medium alone. The culture supernatants were sampled at days 0, 3, 5 and 7 and assayed for fibronectin. The cell monolayers were lysed on day 7 and assayed for cell associated fibronectin or for protein and DNA (chapter 2 sections 2.4-2.8)

3.4.2 Results

Although macrophages cultured for 3 days in medium containing 0.5% FCS generated small amounts of fibronectin, the serum free conditions of macrophage culture employed to produce MPCM did not result in measurable levels of fibronectin in MPCM.

Cultured rat mesangial cells constitutively secreted low levels of fibronectin into the culture medium, addition of MPCM significantly enhanced this production in a time dependent manner (Fig 3.2). Fibronectin accumulation was also MPCM dose dependent (Fig 3.3a). When fibronectin production was expressed as a function of total cell DNA or total cell protein the dose response was still observed (Figs 3.3b and c) indicating that the effect was not secondary to a stimulation of cell proliferation ie more cells giving rise to more fibronectin.

When the total DNA content per well was calculated for each concentration of MPCM, it was found to increase slightly, though significantly, in the presence of 10% and 20%

MPCM (Table 3.1). However, the increase in DNA could not account for the observed increases in fibronectin levels.

Table 3.1 Effect of MPCM dose on the DNA content of mesangial cell cultures.

%MPCM	DNA μg/well	p value (vs 0%MPCM)
0	3.35±0.25	
100	3.2±0.11	NS
50	4.15±0.15	NS
20	4.8±0.53	0.006
10	4.6±0.32	0.02
1	3.45±0.38	NS

MPCM also caused an increase in cell associated fibronectin. Of the total amount of fibronectin produced by mesangial cells approximately 80% was secreted into the supernatant, whilst 20% was cell associated (427.5 ± 59 ng/μg cell protein in supernatant vs 80.1 ± 16.1 ng/μg cell protein in cell lysate - values are means ± SEM (n=4)).

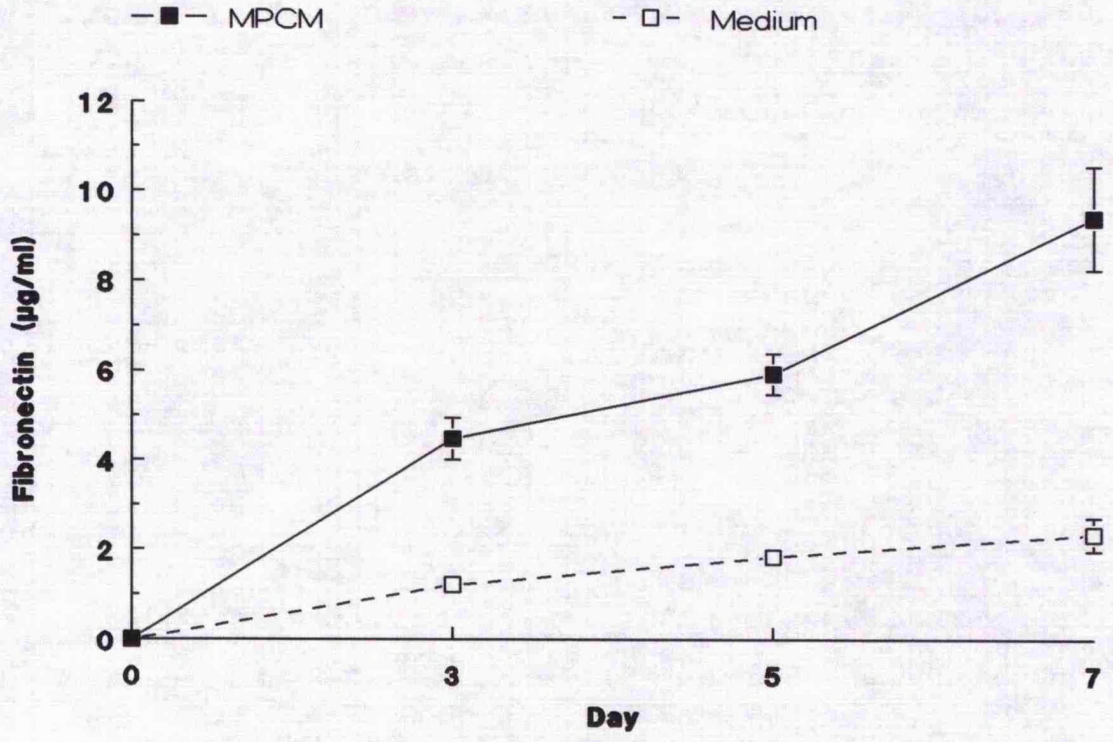


Fig 3.2 Time course of fibronectin production.

Mesangial cells were exposed to a 50% solution of MPCM or medium alone. The culture supernatants were sampled at days 0, 3, 5 and 7 and assayed for fibronectin. Values are means ± SEM (n=3) each carried out in quadruplicate.

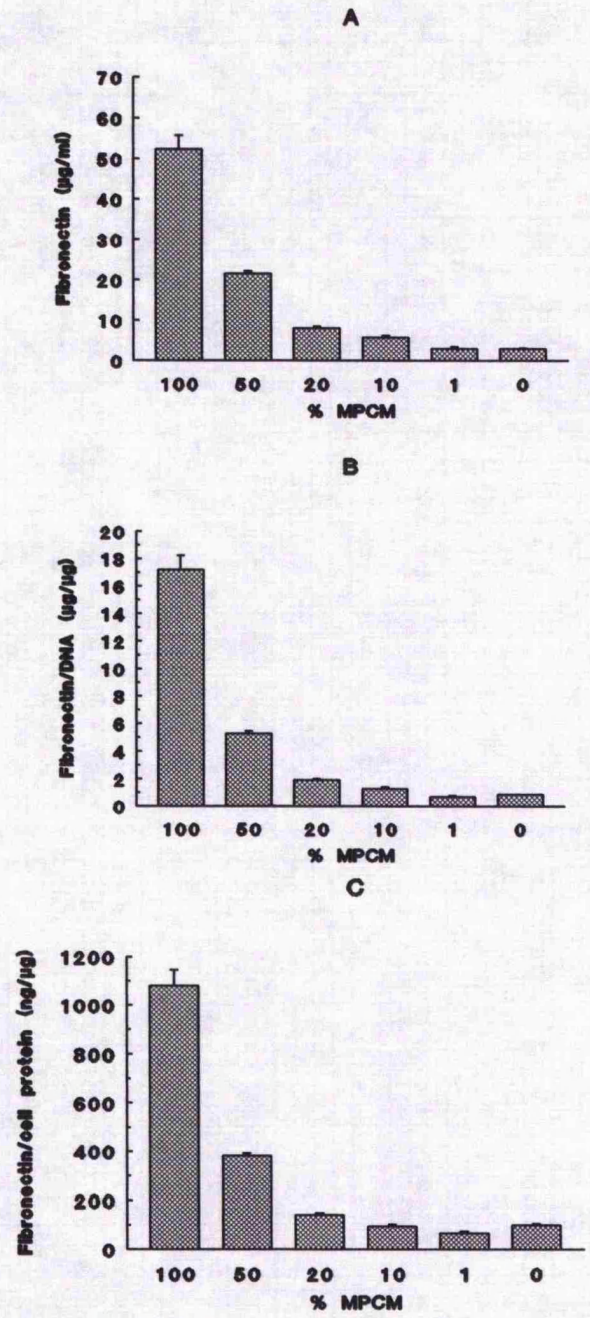


Fig 3.3 Dose response of fibronectin production.

Mesangial cells were exposed to 100, 50, 20, 10, 1 and 0% MPCM. Supernatants were sampled at day 7 and assayed for fibronectin. Fibronectin production is expressed as a) µg/ml, b) µg/µg cell DNA, c)ng/µg cell protein. Representative experiment of three carried out in quadruplicate is presented.

3.5 Effect of MPCM on mesangial cell proliferation

^{3}H-thymidine incorporation is a conventional way of measuring cell proliferation. Strictly speaking it reflects the number of cells in S phase during the labelling period. In order to confirm that the observed fibronectin accumulation was not secondary to a stimulation of cell proliferation ^{3}H-thymidine incorporation assays were carried out.

3.5.1 Materials and Methods

After 3 days of mesangial cell culture in the presence of MPCM or medium alone, 1μCi ^{3}H-thymidine was added directly to each well and ^{3}H-thymidine incorporation was measured.

In parallel experiments (using the same batch of MPCM and mesangial cell line) the existing tissue culture medium containing MPCM or medium alone was replaced with fresh RPMI + 0.5% FCS prior to addition of ^{3}H-thymidine (chapter 2, section 2.15).

Cell cytotoxicity/viability was assessed using the LDH release assay (chapter 2, section 2.16). LDH activity in mesangial cell culture supernatants was expressed as a percentage of the activity in the corresponding cell lysates (results were corrected for any LDH activity inherent in MPCM *per se*).

3.5.2 Results

^{3}H-thymidine incorporation assays demonstrated that MPCM caused a dose dependent suppression of proliferation when compared to medium alone (Fig 3.4).

Assessment of LDH activity revealed that this decrease in ^{3}H-thymidine uptake could not be accounted for by an increase in cytotoxicity. On the contrary, the activity of LDH in mesangial cell culture supernatants from cells exposed to 100-20% MPCM was

significantly lower than the basal activity of control solutions ($p<0.02$). There was no significant difference in LDH activity between the supernatants of cells exposed to medium alone and 1% MPCM (Fig 3.5).

When MPCM and medium were replaced with fresh medium prior to addition of ^{3}H-thymidine, there was no significant difference in ^{3}H-thymidine uptake between cells previously treated with MPCM or medium alone (Fig 3.6) suggesting that MPCM contains an agent capable of reversibly suppressing proliferation. Alternatively, the uptake of thymidine by mesangial cells may somehow be affected by factors present in MPCM.

The ^{3}H-thymidine incorporation assays are at variance with the results of the total cell DNA/well data which demonstrated an increase in DNA with low concentrations of MPCM. Taken together however, the data suggest that the state of mesangial cell proliferation in response to MPCM is dependent on the MPCM dose and by implication on the concentration of a factor(s) in MPCM. The data conclusively show that the observed increase in fibronectin levels does not occur as a direct result of an increased number of cells.

Support to the concept that MPCM might inhibit mesangial cell proliferation at high doses is provided by the observed effects of MPCM on cell protein content. The protein content of mesangial cells exposed to MPCM was compared to that of cells exposed to medium alone. The 'MPCM:medium' protein ratio from 50 assays was 0.89 ± 0.03 ($p<0.001$).

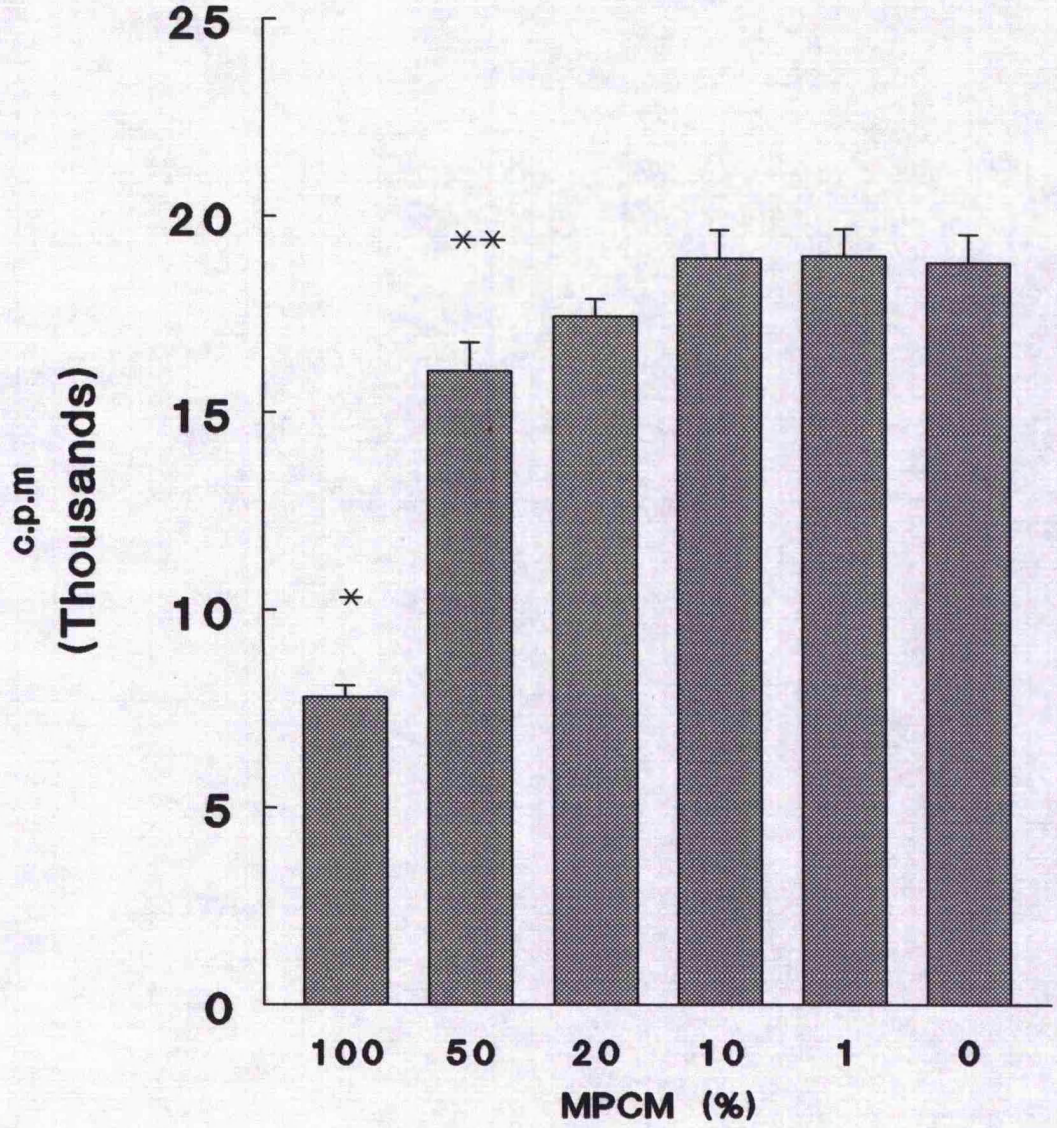


Fig 3.4 Effect of dose of MPCM on mesangial cell proliferation.

1μCi/ml ^{3}H-thymidine was added to mesangial cells exposed to 100, 50, 20, 10, 1 and 0 MPCM. Each value represents the mean $\pm$ SEM of 8 wells. *$p < 0.001$, **$p < 0.003$ vs medium alone (0% MPCM).

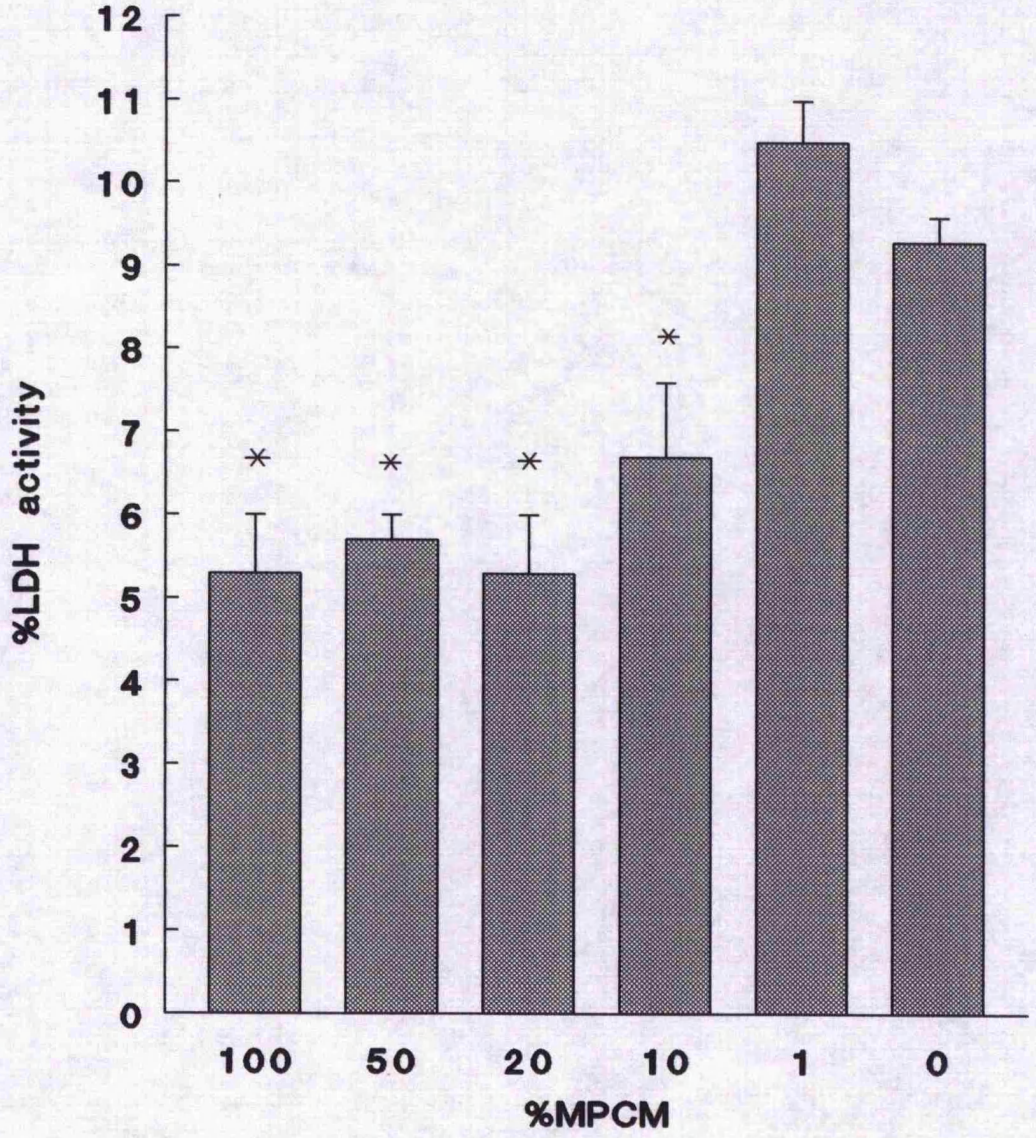


Fig 3.5 Effect of dose of MPCM on mesangial cell viability.

LDH activity was determined in the tissue culture supernatants of cells exposed to MPCM or medium alone and expressed as a percentage of the activity in sonicated cell lysates. Each value represents the mean ± SEM from three experiments each carried out in quadruplicate. *$p < 0.02$ vs medium alone.

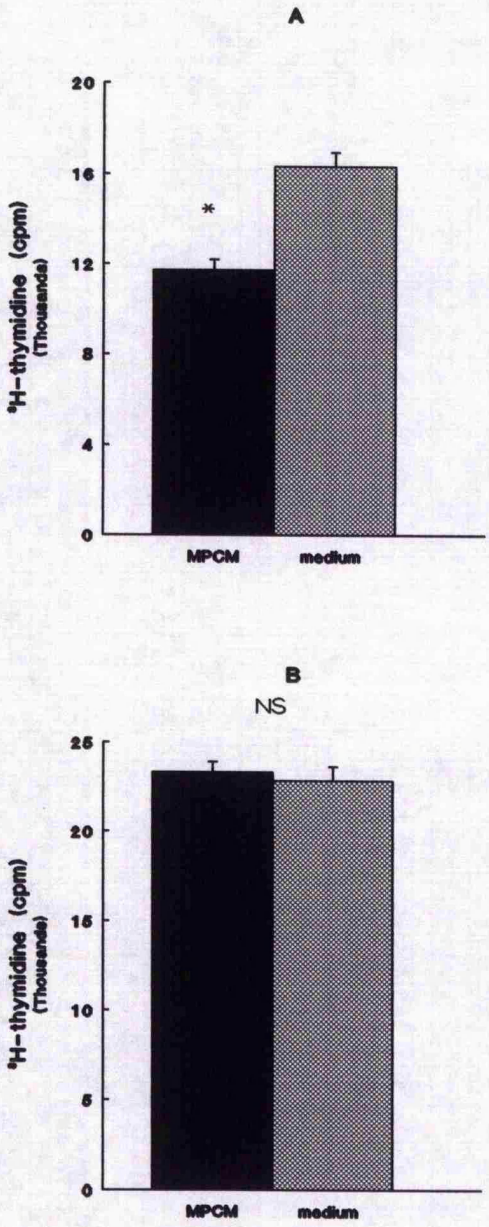


Fig 3.6 Effect of replacing MPCM with fresh medium on mesangial cell proliferation. 1μCi/ml ^{3}H-thymidine was added directly to mesangial cells or after the tissue culture supernatants containing MPCM or medium alone had been replaced with fresh RPMI + 0.5% FCS. Each value represents the mean $\pm$ SEM of 8 wells. *$p < 0.001$ vs medium alone.

3.6 Effect of LPS stimulation of macrophages on activity of MPCM

LPS can induce the expression of lots of cytokines including TNFα, IL-1α, IL-1ß, IL-6, M-CSF, and IFN α and ß. Each of these can in turn increase the magnitude of their own expression or expand the character of the response to include greater functional diversity or both [Nathan CF, 1987]. In order to observe the effects of LPS stimulation of macrophages on their ability to induce fibronectin by mesangial cells MPCM was generated using both LPS stimulated and unstimulated macrophages.

3.6.1 Materials and Methods

Thioglycollate elicited, adherent rat peritoneal macrophages were cultured in the presence or absence of 1μg/ml LPS for 16hrs. The "LPS supernatants" were removed and the cells washed and cultured for a further 48hr in serum-free RPMI to generate the conditioned medium. Confluent, quiescent mesangial cells were then exposed to the conditioned media for 7 days following which the supernatants were assayed for fibronectin.

3.6.2 Results

Conditioned medium from thioglycolate elicited macrophages that had not been stimulated with LPS had a markedly reduced ability to stimulate mesangial cell fibronectin production than conditioned medium from standard LPS stimulated macrophages (Fig 3.6).

These data indicate that although conditioned medium from elicited macrophages has the ability to induce fibronectin production, LPS stimulation serves to potentiate this effect.

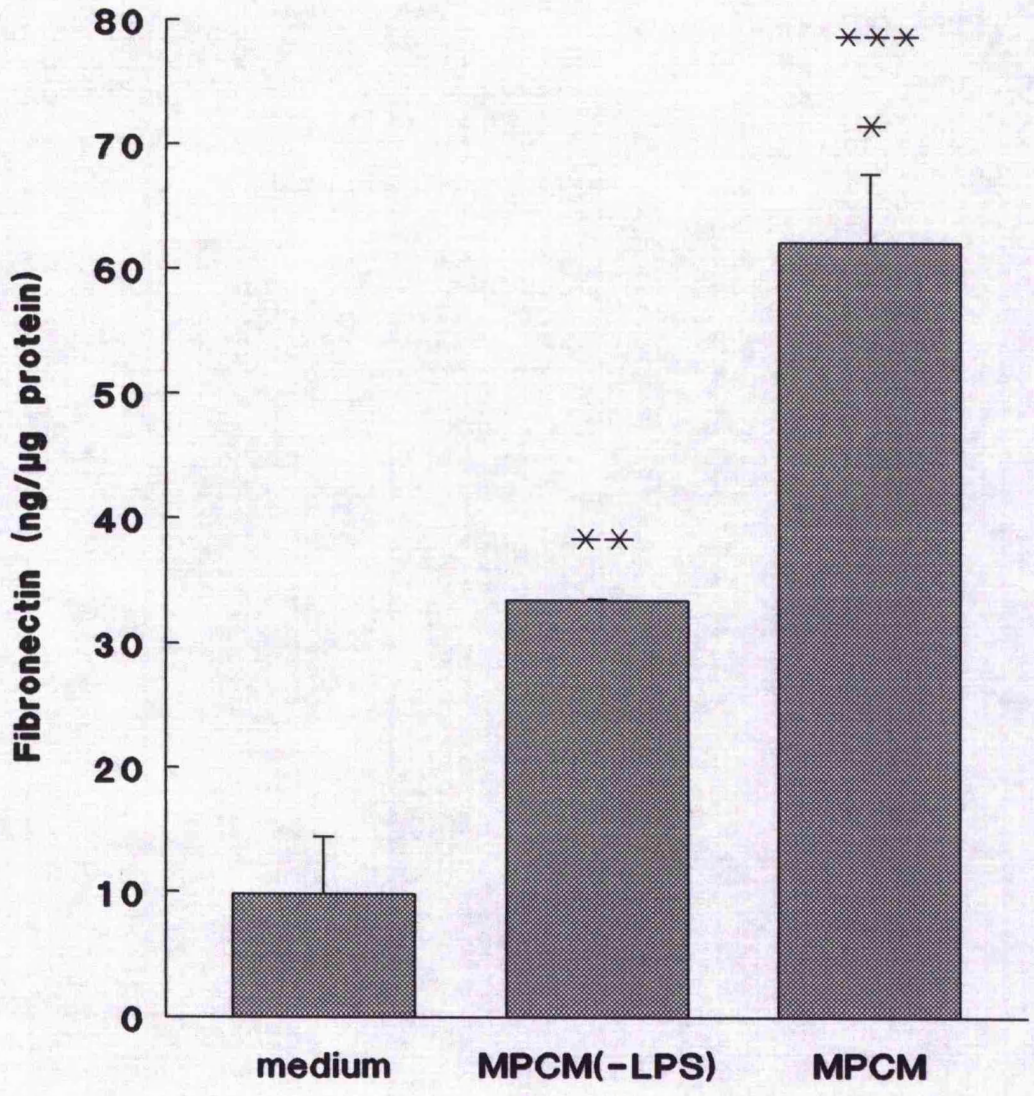


Fig 3.7 Role of LPS in MPCM mediated fibronectin production.

Mesangial cells were incubated in the presence of MPCM generated in the presence or absence of 1μg/ml LPS for 7 days. Supernatants were assayed for fibronectin. Values are means $\pm$ SEM (n=4) each carried out in quadruplicate. *p=0.006 vs medium, **p=0.001 vs medium, ***p=0.012 vs MPCM(-LPS).

3.7 Residual effects of LPS on mesangial cells

It has been shown that rat mesangial cells release the monokine TNFα in response to stimulation by LPS [Baud L *et al*, 1989]. Although the LPS used to stimulate the macrophages in the preparation of MPCM was washed away, it was necessary to ascertain the effects, if any, of residual LPS on mesangial cell fibronectin production.

3.7.1 Materials and Methods

Confluent, quiescent mesangial cells were exposed to 1μg/ml LPS in medium alone or in combination with MPCM. After 7 days incubation, supernatants and cell lysates were assayed for fibronectin.

3.7.2 Results

Addition of 1μg/ml LPS to mesangial cells in medium alone did not result in any upregulation of fibronectin production over basal levels (1210 $\pm$125 vs 1100 $\pm$97 ng/ml p=NS, for medium+LPS vs medium-LPS respectively). Addition of 1μg/ml LPS in combination with MPCM had no additional effect over MPCM alone on mesangial cell fibronectin production (2349 $\pm$124 vs 2426 $\pm$99 ng/ml p=NS, for MPCM+LPS vs MPCM-LPS respectively), suggesting that LPS activation of mesangial cells cannot account for the observed effects of MPCM.

3.8 Effect of MPCM on glomerular fibronectin production

As well as mesangial cells, glomeruli contain the other cellular components of the glomerulus such as the epithelial cells, resident macrophages and endothelial cells all of which may contribute to the fibrotic process *in vivo*. In order to assess the general effect of macrophage secretory products on glomerular fibronectin production, isolated glomerular cores (containing all the cells in the *in vivo* conformation) were exposed to MPCM.

3.8.1 Materials and Methods

Glomeruli were prepared by serial sieving of kidney homogenates as described in chapter 2 section 2.1.1). Following collagenase digestion the glomeruli were plated out into 24 well plates at a density of approximately 2000 glomeruli per ml of RPMI + 0.5% FCS. After 7 days incubation the supernatants were removed and set aside for assay of fibronectin. The remaining glomeruli were pooled washed and treated with 1% Nonidet P40 with sonication to generate a solution of glomerular cell associated fibronectin which was subsequently assayed for fibronectin.

3.8.2 Results

MPCM induced a 2.5 $\pm$ 0.26 fold increase in glomerular supernatant fibronectin levels and a 3.7 $\pm$ 0.82 fold increase in cell associated fibronectin levels (Fig 3.8).

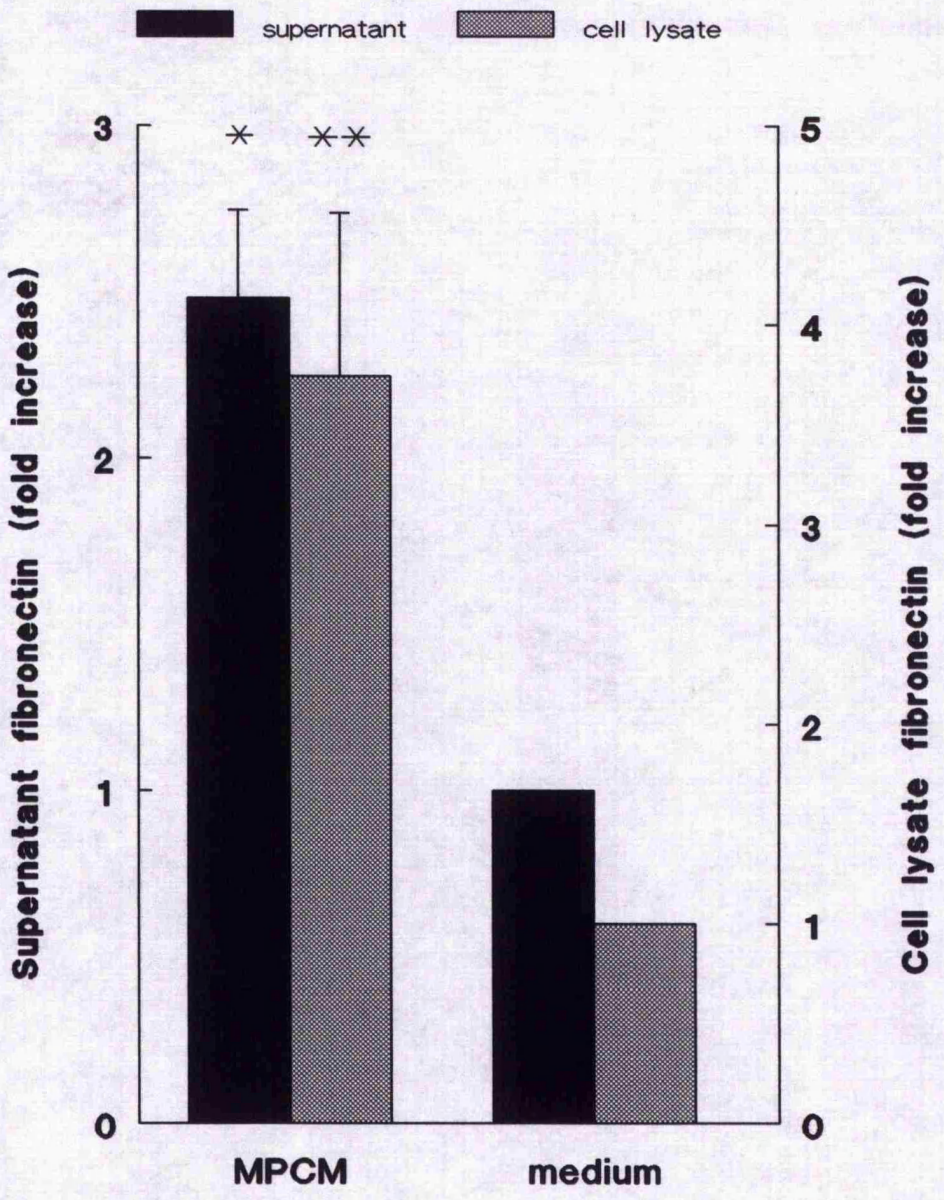


Fig 3.8 Effect of MPCM on glomerular fibronectin production.

2000 glomeruli exposed to 50% MPCM (0.5% FCS) or medium. Supernatants and glomerular lysates assayed for fibronectin. Values are means ± SEM (fold increase over medium) carried out in quadruplicate. (n=4) *p=0.001 and **p=0.015 vs medium.

3.9 Effect of mRNA and protein synthesis inhibitors on MPCM activity.

In order to assess whether *de novo* protein synthesis and mRNA transcription were involved in the observed increase in fibronectin production, mesangial cells were exposed to MPCM in the presence or absence of the metabolic inhibitors actinomycin D and cycloheximide.

3.9.1 Materials and Methods

Confluent quiescent mesangial cells were incubated for 24hr in the presence of MPCM or medium alone with or without the addition of 250ng/ml actinomycin D (Sigma) or 10μg/ml cycloheximide (Sigma). The concentrations chosen had been reported in the literature to inhibit mRNA transcription and protein synthesis respectively [Ignotz RA and Massague J, 1986., Penttinen RP *et al*, 1988]. Supernatants and cell lysates were then assayed for fibronectin.

3.9.2 Results

Secreted and cell associated fibronectin production was significantly decreased in the presence of both inhibitors (Table 3.2) although basal levels of fibronectin were unaffected. These data indicate that *de novo* mRNA transcription and protein synthesis are involved in the MPCM-mediated response.

Table 3.2 Effect of metabolic inhibitors on *de novo* mRNA transcription and protein synthesis. Fibronectin production is expressed as a percentage of that with MPCM alone. Values are means ± SEM (n=3) *p < 0.005 vs control.

	Fibronectin (% MPCM alone)		
Supernatant	Control	Actinomycin	Cycloheximide
MPCM	100	51.9±4.6*	51.4±7.8*
Medium	29.5±6.5	28.7±3.1	23.8±2.3
Cell lysate			
MPCM	100	51.8±1.1*	56.4±3.3*
Medium	55.3±4.5	53.1±3.4	51.6±4.4

3.10 Effect of MPCM on *de novo* fibronectin protein synthesis.

Fibronectin accumulation could result from increased fibronectin synthesis and/or an inhibition of degradation. In order to assess whether the time and dose dependent accumulation of fibronectin protein was due to increased fibronectin synthesis, mesangial cells were exposed to MPCM in the presence of ^{35}S-methionine. This way any new fibronectin synthesised would be biosynthetically labelled with the radioactive amino acid.

3.10.1 Materials and Methods

Confluent quiescent mesangial cells were exposed to 50% MPCM or medium alone and pulse labelled with ^{35}S-methionine. The supernatants and cell lysates were immunoprecipitated and electrophoresed on 5% polyacrylamide gels. The gels were autoradiographed and the 220kD fibronectin band was scanned (described in chapter 2 section 2.17).

3.10.2 Results

Autoradiographs of the immunoprecipitated, biosynthetically labelled fibronectin showed that exposure of mesangial cells to MPCM resulted in an increase in fibronectin synthesis over that of medium alone (Fig 3.9a and b). Semi-quantitative analysis of the 220kD fibronectin band by scanning densitometry showed 5.9 and 4.3 fold increases over control in the secreted and cell associated forms of fibronectin respectively (0.31 ± 0.06 vs 0.053 ± 0.007 $p < 0.001$, and 0.31 ± 0.07 vs 0.073 ± 0.016 $p < 0.005$, arbitrary densitometric units). The presence of additional bands below 200kD and particularly around 70kD could suggest an element of fibronectin degradation.

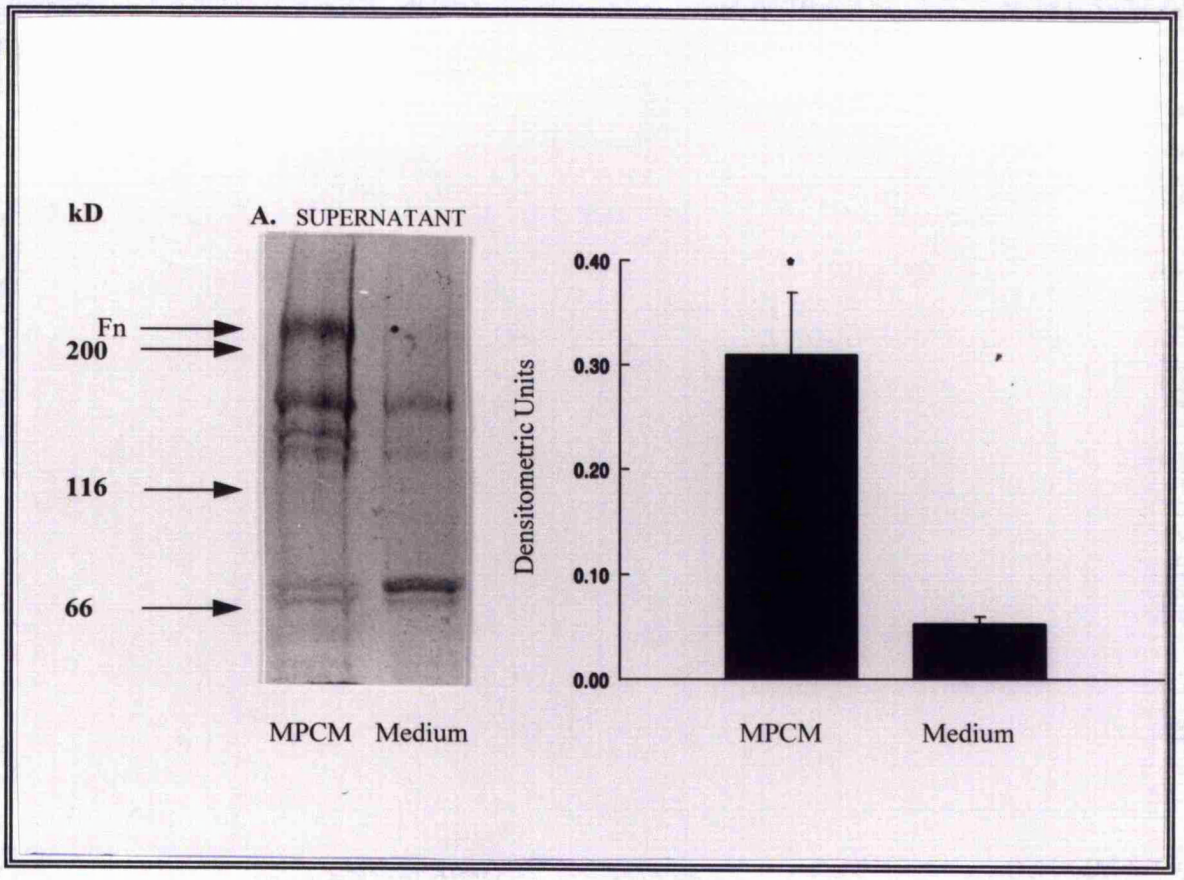


Fig 3.9a Effect of MPCM on *de novo* fibronectin synthesis.

Autoradiography and densitometric analysis of immunoprecipitated supernatant ^{35}S-fibronectin. A representative autoradiograph from three independent experiments is shown. Densitometric analysis of the 220kD fibronectin band incorporates the data from the three experiments.

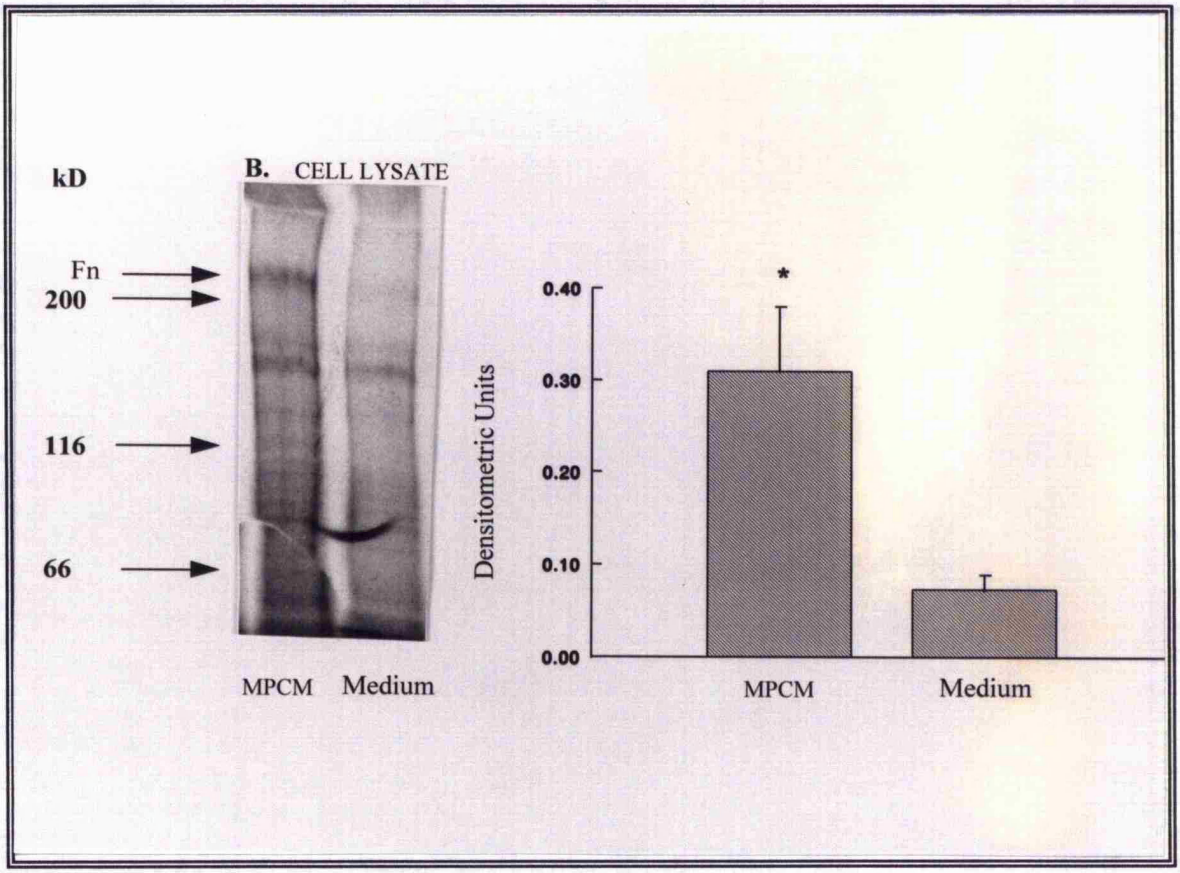


Fig 3.9b Effect of MPCM on *de novo* fibronectin synthesis.

Autoradiography and densitometric analysis of immunoprecipitated cell associated ^{35}S-fibronectin. A representative autoradiograph from three independent experiments is shown. Densitometric analysis of the 220kD fibronectin band incorporates the data from the three experiments.

3.11 Effect of MPCM on fibronectin gene expression

In order to confirm that the observed increases in supernatant and cell associated fibronectin levels were indeed due to an upreguation of fibronectin gene expression Northern blotting analysis was performed.

3.11.1 Materials and Methods

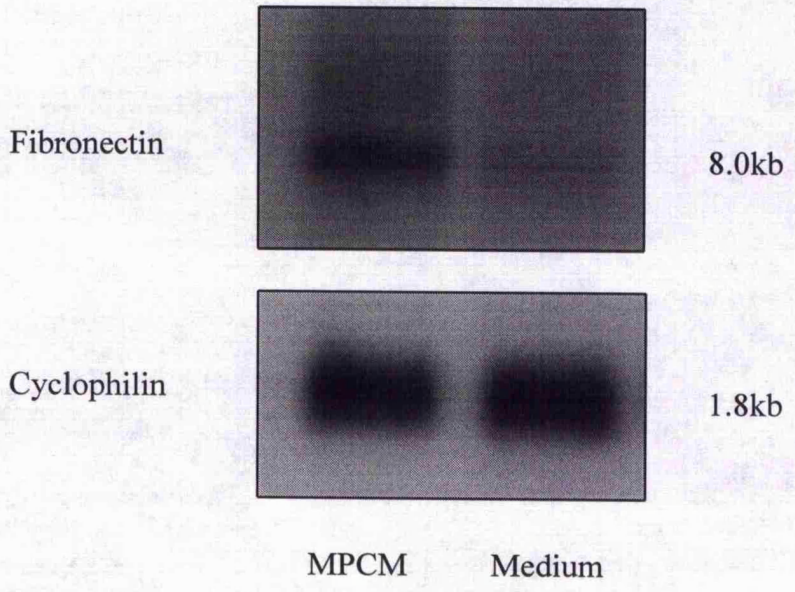
Total cellular RNA was isolated from confluent quiescent mesangial cells having been exposed to MPCM or medium alone for 24hr. Approximately 30μg RNA were fractionated on a 1% agarose gel. Following blotting and hybridisation with a ^{32}P-dCTP labelled fibronectin cDNA probe the membrane was autoradiographed and the resulting film was scanned on a laser densitometer. The membrane was subsequently stripped and reprobed with cyclophilin cDNA probe to normalise for RNA loading (chapter 2 section 2.25)

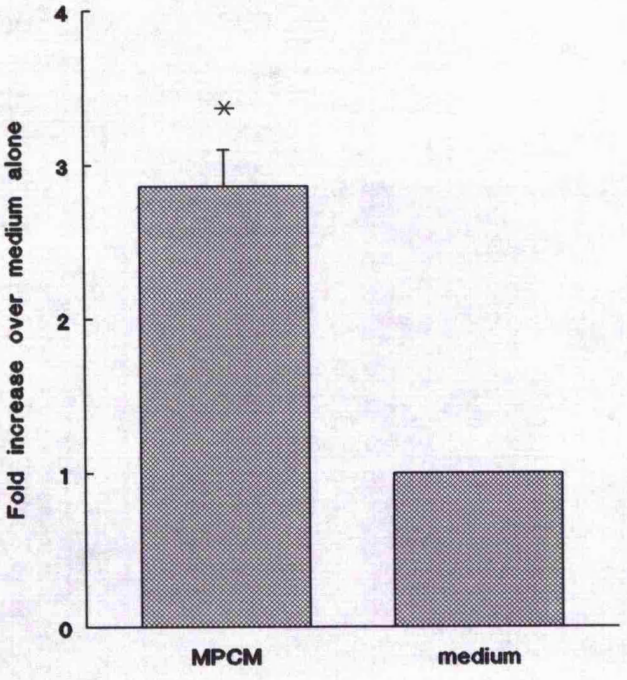
3.11.2 Results

Fig 3.10 shows a representative Northern blot illustrating an increase in mesangial fibronectin mRNA after 24hr treatment with MPCM. Analysis of the 8kb fibronectin band by scanning laser densitometry showed that fibronectin mRNA levels increased by 2.9 $\pm$ 0.24 fold over those of control cells exposed to medium alone (normalising for RNA loading with cyclophilin). A second larger fibronectin transcript ($>$9.49kb) was also seen but not included for analysis in this study.

Fig 3.10 Fibronectin gene expression in response to MPCM.

Northern blot of mesangial cell RNA following exposure to MPCM or medium alone for 24hr. One of five representative experiments is shown. Densitometric analysis incorporates the data from five experiments expressed as fold increase over medium alone normalised for RNA loading, *$p < 0.001$.





3.12 Effect of MPCM on the production of other matrix proteins

Laminin and collagen IV are matrix proteins more commonly associated with the basement membrane, although they are present in small amounts in the mesangium. However, under pathophysiological conditions mesangial levels of these matrix proteins are also increased.

Due to problems of sensitivity and specificity encountered in developing inhibition ELISAs for laminin and collagen IV, Northern blotting was carried out to detect the message for these matrix proteins in mesangial cells in response to MPCM.

3.12.1 Materials and Methods

Membranes which had already been probed with the fibronectin cDNA and cyclophillin were stripped and reprobed with α1 (IV) collagen cDNA and laminin B1 chain cDNA as described in chapter 2 section 2.25.

3.12.2 Results

MPCM induced a 3.1 $\pm$ 0.3 fold increase in α1 (IV) collagen mRNA (Fig 3.11) and a 4.9 $\pm$ 0.2 fold increase in laminin B1 chain mRNA (Fig 3.12) over control cells (normalised for RNA loading).

Fig 3.11 α1 (IV) collagen gene expression in respose to MPCM.

Northern blot analysis of mesangial cell RNA following exposure to MPCM or medium alone for 24hr. One of four representative experiments is shown. Densitometric analysis incorporates the data from all four experiments as fold increase over medium alone normalised for RNA loading, *$p < 0.001$.

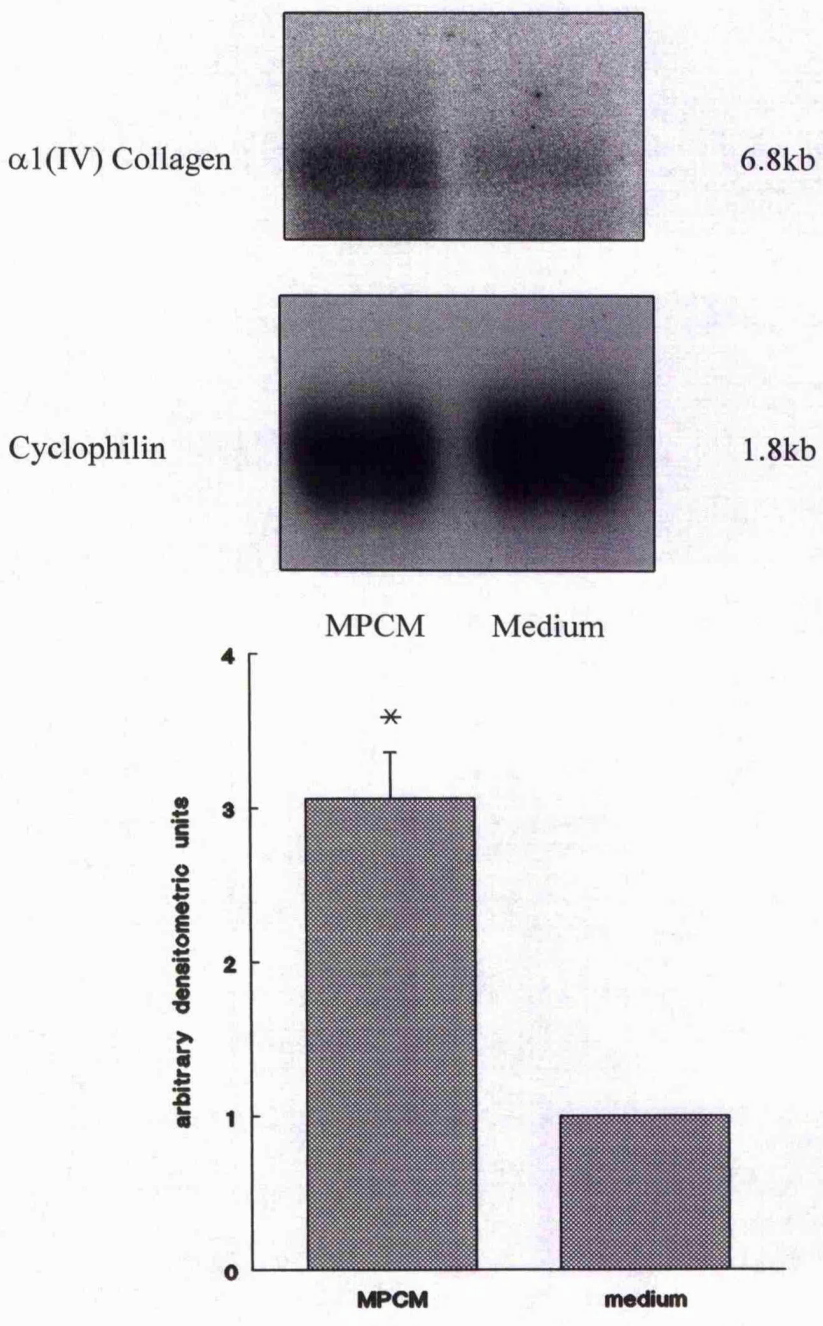
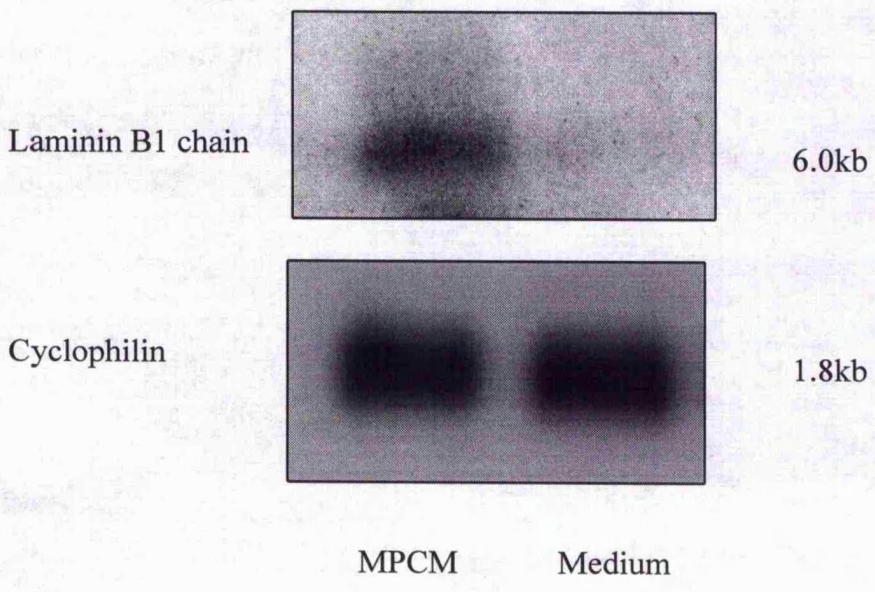
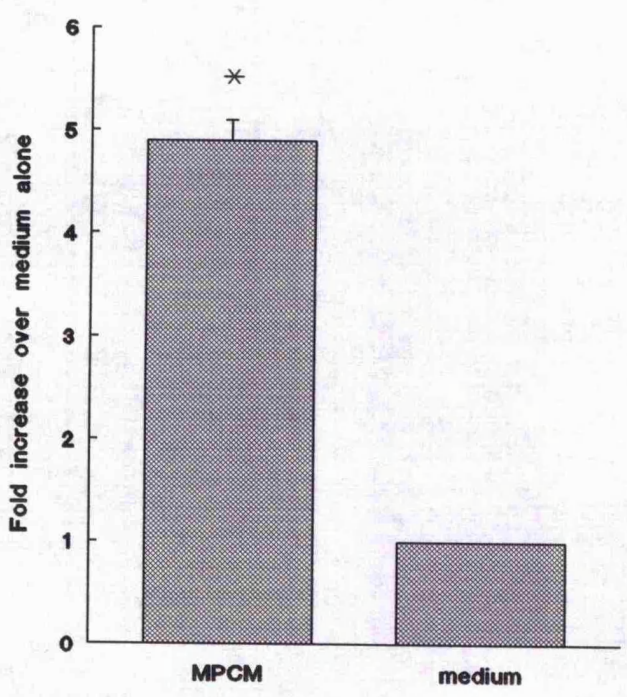


Fig 3.12 Laminin B1 chain gene transcrition in reponse to MPCM.

Nortern blot analysis of mesangial cell RNA following exposure to MPCM or medium alone for 24hr. One of four representative experiments is shown. Densitometric analysis incorporates the data from all four experiments expressed as a fold increase over medium alone normalised for RNA loading, *$p < 0.001$.





3.13 Effect of MPCM on TIMP gene expression

The data presented so far has shown that fibronectin accumulation in response to MPCM occurs, at least in part, as a result of increased fibronectin gene transcription and protein synthesis. Stromelysin-1 or the rat equivalent transin is a matrix metalloproteinase that is able to degrade various matrix components including fibronectin [Matrisian LM, 1990]. Inhibition of this enzyme would be expected to result in fibronectin accumulation. In order to assess whether an inhibition of matrix degradation may also play a role in MPCM-mediated fibronectin accumulation, Northern blot analysis was carried out to look for the message of the inhibitor of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1).

3.13.1 Materials and Methods

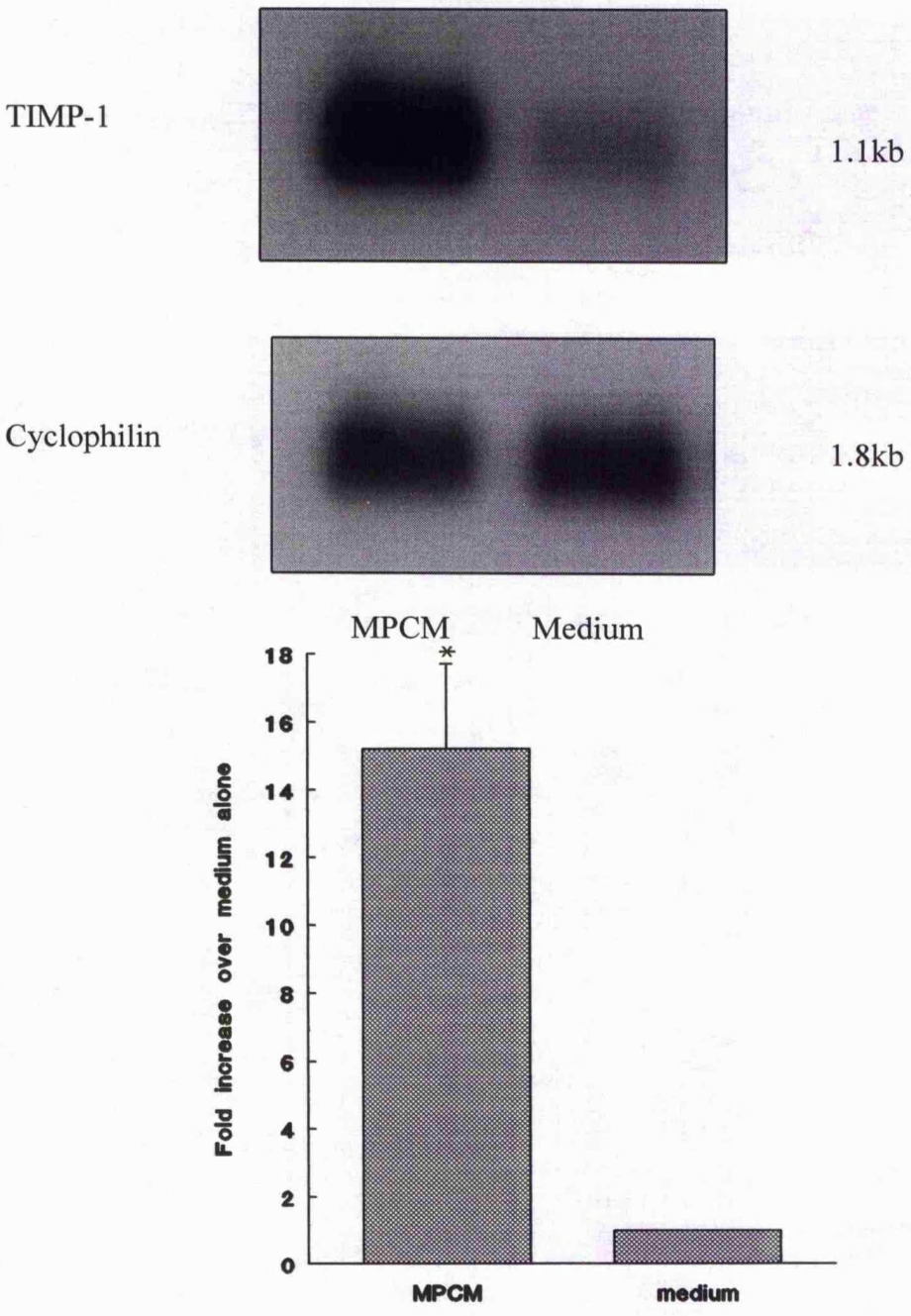
Membranes that had been previously probed for fibronectin and cyclophilin were stripped and reprobed with a ^{32}P-dCTP labelled TIMP cDNA.

3.13.2 Results

Exposure of mesangial cells to MPCM resulted in a 15.2 $\pm$ 2.5 fold upregulation of TIMP-1 gene transcription over control levels (Fig 5.13). Assuming that this increase in message results in increased translation of protein, which equals or exceeds the magnitude of any stromelysin production (enzyme and inhibitor interact in a 1:1 molar stoichiometry), then a reduction of matrix degradation may also be a contributary factor to the fibronectin accumulation observed in this system.

Fig 3.13 TIMP gene transcription in response to MPCM.

Northern blot of mesangial cell RNA following treatment with MPCM or medium alone for 24hr. One of six representative experiments is shown. Densitometric analysis incorporates the data from all six experiments expressed as fold increase over medium alone normalised for RNA loading, *$p < 0.001$.



3.14 Effect of MPCM on MCP-1 expression

The mesangial cell response to macrophages in the progression to glomerulosclerosis is almost certainly not limited to the direct modulation of matrix production and cell proliferation.

The chemokine MCP-1 is a potent chemoattractant for cells of the monocyte/macrophage lineage. MCP-1 has been shown to be expressed in the glomeruli of rats with anti-Thy-1 nephritis [Stahl RAK *et al*, 1993] and nephrotoxic nephritis [Rovin BH *et al*, 1994]. Moreover, Tam FWK *et al,* [1996] have demonstrated that glomerular MCP-1 expression correlates with monocyte influx in rats with nephrotoxic nephritis. Taking these observations further it has been reported that macrophage associated cytokines TNFα and/or IL-1ß upregulate MCP-1 gene transcription and protein synthesis in both human and rat mesangial cells [Zoja C *et al*, 1991., Rovin BH *et al*, 1992., Largen PJ *et al,* 1995] thus arguing a role for mesangial cell mediated macrophage recruitment and activation.

In order to ascertain whether MPCM could induce mesangial cell MCP-1 expression Northern and Western blotting were carried out to look for MCP-1 gene transcription and protein secretion respectively.

3.14.1 Materials and Methods

a) Northern blotting

Membranes which had been previously blotted with RNA extracted from mesangial cells exposed to MPCM and had been probed for fibronectin and cyclophilin were stripped and reprobed with a ^{32}P-dCTP-MCP-1 cDNA.

b) Western blotting

Concentrated MPCM, tissue culture supernatants from mesangial cells exposed to MPCM and recombinant rat MCP-1 (Serotec) at 10 and 1.0μg/ml (controls) were fractionated by SDS-PAGE on a 1.5mm, 15% gel and blotted on to nitrocellulose for 2hr at 90v. The membranes were blocked overnight with TBS + 1% BSA + 0.05% Tween 20. Immunostaining was carried out as described in chapter 2, section 2.14.2 using rabbit anti-rat MCP-1 (Serotec) at 1:1000 dilution and either HRP conjugated anti-rabbit Ig (Dako) at 1:750 dilution or ^{125}I-labelled anti-rabbit Ig (Amersham) at 4μCi/ml as second antibodies. MCP-1 was visualised using DAB or following autoradiography of the membranes.

3.14.2 Results

Exposure of mesangial cells to MPCM resulted in a 5.75 $\pm$ 0.75 fold increase in MCP-1 gene transcription over that of control (Fig 3.14).

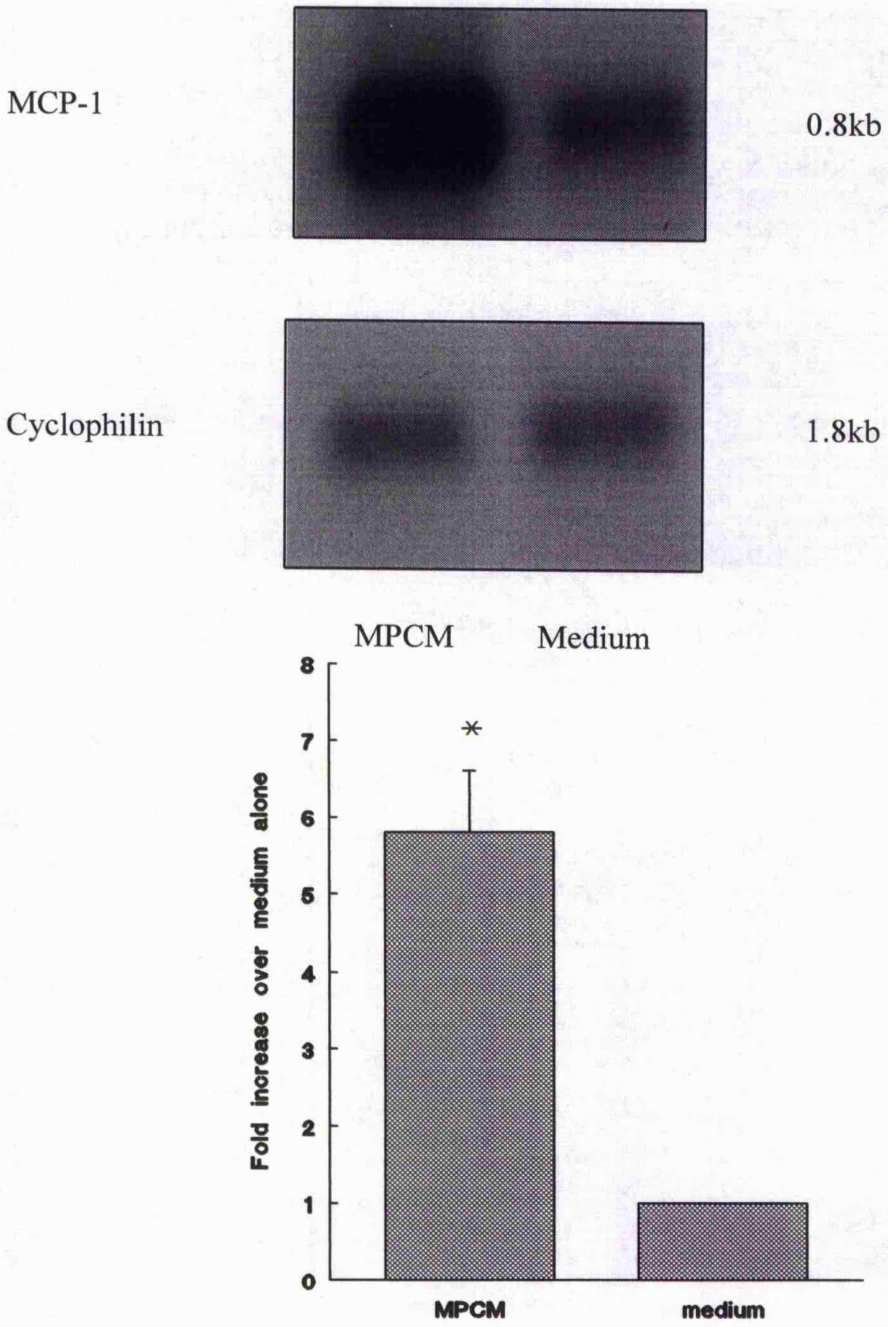
MCP-1 protein could not be detected in MPCM or in tissue culture supernatants by Western blotting. Of the two rat MCP-1 controls only the one at 10μg/ml could be detected indicating that the technique is not sensitive enough to be able to detect MCP-1 at physiologically or biologically relevant concentrations.

If translation of the MCP-1 transcript occurs then these data would suggest that macrophages themselves can induce a further influx of mononuclear cells into the glomerulus by stimulating mesangial cells to secrete MCP-1.

A positive feed back loop such as this would have to be counter-regulated otherwise macrophage accumulation with concomitant secretion of factors would continue. Since this does not happen clinically or in experimental models such a counter-regulatory mechanism must obviously exist.

Fig 3.12 Effect of MPCM on MCP-1 gene transcription.

Northern blot of mesangial cell RNA following exposure to MPCM. One of three representative experiments is shown. Densitometric analysis includes data from all three experiments expressed as fold increase over medium alone normalised for RNA loading, *$p < 0.001$.



3.15 Effect of TGFß on MPCM-mediated fibronectin production.

Certain cytokines have been shown to be potent modulators of monocyte/macrophage function. For example IL-10 can suppress the production of PGE_2 and numerous proinflammatory cytokines such as TNFα, IL-1, IL-6, IL-8 by activated monocytes [Fiorentino DF *et al*, 1991., De Waal MR *et al*, 1991., Niiro H *et al*, 1994]. Kitamura *M et al* [1995] have recently demonstrated that mesangial cells secrete a factor, which they identified as TGFß, that also suppresses cytokine expression by activated macrophages. Moreover, it has also been reported that TGFß can stimulate the release of IL-10 by mesangial cells [Fouqueray B *et al*, 1995].

With this in mind the aim of the next experiment was to observe whether TGFß could affect the production of the fibronectin stimulating factor(s) in MPCM thus providing a counter-regulatory mechanism modulating the pro-sclerotic effects of macrophages.

3.15.1 Materials and Methods

Confluent quiescent mesangial cells were exposed to MPCM (prepared as described in chapter 2, section 2) or to TGFß treated MPCM ($MPCM_{TGFß}$). $MPCM_{TGFß}$ was prepared by treating macrophages with 25ng/ml $TGFß_1$ during the LPS stimulation stage of MPCM generation. The LPS/TGFß medium was subsequently washed away after 16hr and the macrophages cultured in serum free RPMI as before. Mesangial cell supernatants and cell lysates were subsequently assayed for fibronectin (chapter 2, section 2.6.).

3.15.2 Results

$MPCM_{TGFß}$ had a significantly reduced ability to stimulate both secreted and cell associated fibronectin production in mesangial cells ($p < 0.001$ and $p < 0.02$ respectively) (Fig 3.15),

suggesting that TGFß suppressed the production of the fibronecting stimulating factor(s) elaborated by macrophages in response to thioglycollate and LPS stimulation. These data would support the observations of Kitamura M *et al* [1995], who found that TGFß was able to suppress the production of a number of cytokines by macrophages. The current data further demonstrate that macrophage production of fibronectin stimulating factors can also be suppressed following exposure to TGFß, which may be of relevance to the control of the sclerotic process. TGFß may thus contribute towards a counter-regulatory mechanism involved in macrophage:mesangial cell interactions.

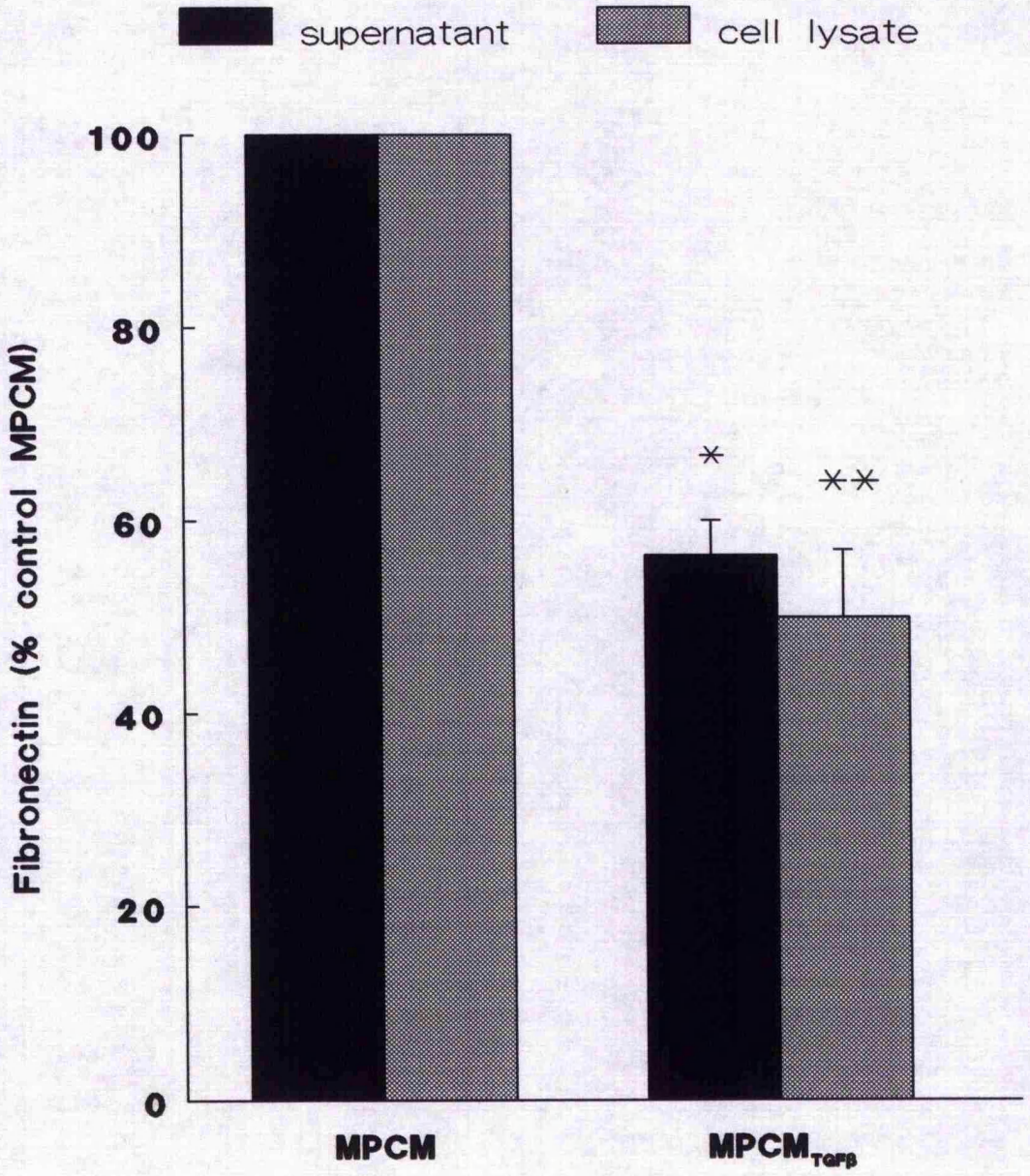


Fig 3.15 Effect of TGFß on MPCM mediated fibronectin production.

Mesangial cells were treated with MPCM and TGFß treated MPCM for 3 days. Supernatants and cell lysates were assayed for fibronectin. Values represent mean ± SEM, n=3 each carried out in quadruplicate. *$p < 0.001$, **$p < 0.02$ vs MPCM.

3.16 Discussion

The present study demonstrates that macrophage derived factors cause cultured mesangial cells to secrete fibronectin in a time and dose dependent manner. The fibronectin was derived from the mesangial cells since the matrix protein was not detected in the MPCM *per se*, although fibronectin was secreted by macrophages cultured for 3 days in medium containing 0.5% FCS. This concurs with observations by other investigators who demonstrated that human [Alitalo K *et al,* 1980] and mouse macrophages [Werb Z and Chin JR, 1981]) also secrete fibronectin. The function of macrophage fibronectin may be to aid binding of macrophages to mesangial cells since Dubois CH *et al* [1982] have shown that *in vitro* peritoneal macrophages preferentially bind to mesangial cells via surface fibronectin interactions. However, these data would indicate that macrophage:mesangial cell contact is not required for macrophages to induce mesangial cell fibronectin production.

Fibronectin accumulation was not due to endotoxin contamination since addition of LPS directly to mesangial cells or in combination with MPCM had no additional effects on basal or stimulated fibronectin production respectively.

Taken together, the metabolic inhibitor studies, biosynthetic labelling and Northern blot analysis of fibronectin gene transcription suggest that the MPCM-mediated upregulation of mesangial cell fibronectin production occurs, at least in part, as a result of increased mRNA transcription and protein synthesis. A contribution to fibronectin accumulation from a decrease in degradation rate however, is suggested by the observation that MPCM causes an upregulation of TIMP-1 gene transcription. Although upregulation of message does not

always imply translation of protein, a number of reports have suggested that TIMP-1 mRNA expression and protein secretion are tightly coupled [Jones CL *et al*, 1991., Carome MA *et al*, 1993., Tang WW *et al*, 1994]. Therefore, if the magnitude of translated TIMP-1 equals or exceeds that of the matrix metalloproteinase stromelysin, given the stoichiometry of enzyme:inhibitor complexing, then this would result in an overall decrease in fibronectin degradation.

The observed increase in fibronectin levels was not secondary to a stimulation of cell proliferation. When fibronectin production was corrected for the DNA or protein content of the cell monolayer, the effect of MPCM was still observed. The DNA content of mesangial cell cultures was slightly increased (as assessed by DNA assay) at MPCM concentrations of 10 and 20%. However, ^{3}H-thymidine incorporation assays showed that MPCM suppressed mesangial cell proliferation at concentrations of 100 and 50%, an effect which was not due to cytotoxicity. Although the results of the two assay techniques do not concur exactly, taken together, the data suggest that the MPCM effects on mesangial cell proliferation are dose dependent and reversible. These results would tend to support those of other investigators who found that macrophage supernatants had both stimulatory and suppressive effects on mesangial cell proliferation, depending on the concentration of supernatant used [Mattana J and Singhal PC, 1993]. The demonstration that the protein content of MPCM-stimulated mesangial cell cultures was reduced compared to controls suggests that MPCM, at the 50% dose used, has a suppressive effect on proliferation. TGFß for example, is known to act as a bifunctional regulator of mesangial cell proliferation showing opposing effects dependent on concentration [MacKay K *et al*, 1989]. The suppressive effect of MPCM on proliferation is in agreement with the findings of Ooi

YM *et al* [1983] who demonstrated suppression of mesangial cell proliferation by murine MPCM derived from endotoxin treated mice. These observations contrast with those of other groups who have found that macrophage conditioned media can stimulate mesangial cell proliferation [Lovett DH *et al,* 1983a., Mattana J and Singhal PC, 1994., Singhal PC *et al*, 1996., Weissgarten J *et al*, 1993]. As well as differences in MPCM concentration, the disparities between the observations may be due to the particular experimental conditions used. This is best illustrated in work carried out by Oite's group who found that macrophage derived factors caused proliferation in subconfluent mesangial cells grown in medium containing 0.5% FCS [Morioka T *et al*, 1991], and a decrease in cell number when mesangial cells were confluent and were grown in medium containing 5% FCS [Narita I *et al,* 1991]. The current experiments were carried out on confluent cells in medium containing 0.5% FCS.

At present it is not clear whether the factor(s) suppressing proliferation and inducing fibronectin production are identical. Many *in vivo* studies have reported that cell proliferation precedes matrix accumulation. This has led to the formulation of the hypothesis that glomerular proliferation leads to accumulation of extracellular matrix ultimately progressing to glomerulosclerosis [Sriker LJ *et al*, 1989]. Eng E *et al* [1994], on the other hand, were able to demonstrate that the two processes could be partially dissociated. They showed that administration of interferon γ to anti-Thy-1 nephritic rats significantly reduced cell proliferation whilst increased levels of matrix deposition were maintained. These *in vivo* experiments thus concur with the findings reported in this thesis that proliferation can be dissociated from matrix production.

Of interest is the fact that the glycosaminoglycan heparan sulphate has been shown to

increase the production of laminin, fibronectin and type V collagen whilst decreasing cell number by 23% in rat mesangial cells [Groggel GC and Hughes ML, 1995] and light chains isolated from the urine of patients with biopsy-proven light chain deposition disease were shown to increase mesangial cell fibronectin, laminin and collagen IV in the absence of cell proliferation [Zhu L *et al*, 1995].

The combined expression of fibronectin, laminin and collagen IV in mesangial cells has previously been reported in a number of experimental conditions including high glucose exposure [Ayo SH *et al*, 1990], following stimulation with thromboxane [Bruggeman LA *et al,* 1993] and in response to morphine-stimulated macrophages [Singhal PC *et al*, 1996]. However, differential production of the three matrix proteins has also been described [Wagner C *et al*, 1994]. Whether one factor induces production of the three matrix proteins in a coordinated manner through the activation of a common transcriptional element or whether the matrix proteins are independently regulated in this system is yet to be elucidated. TGFß for example, has been shown to directly stimulate the activity of the mouse α2(I) collagen promoter [Rossi P *et al*, 1988] and the fibronectin promoter [Dean DC *et al*, 1988] in human cell lines. It has been suggested that matrix genes expressed in response to TGFß might be activated by a common transcriptional factor such as nuclear factor 1 [Roberts AB *et al*, 1988]. Although TGFß has been implicated in glomerulosclerosis it is worthy of note that Border *et al* [1990a] have reported that expression of fibronectin, collagen and laminin by rat mesangial cells is not substantially affected by TGFß as demonstrated by immunoprecipitation of metabolically labelled tissue culture supernatants.

MPCM upregulated glomerular fibronectin production. Analysis of the effects of MPCM on glomerular cores to some extent eliminates the somewhat artificial effects of a plastic substratum since the cells are maintained in a conformation similar to the one *in vivo*. In this case the relative contribution of each cell type to the observed increase in fibronectin levels cannot be assessed and therefore the observations must be taken as a cumulative effect of all the cell types.

Mesangial cells were found to upregulate MCP-1 gene transcription in response to MPCM. If the MCP-1 message is translated, either directly or in response to a second signal, it would suggest that mesangial cells could play an effector role in reponse to an initial influx of macrophages by activating and recruiting a further influx of these cells in the manner of a positive feed-back loop. A counter-regulatory mechanism to such a feed back loop may be provided by IL-10 and TGFß, cytokines which have been shown to be involved in the suppression macrophage cytokine secretion [Fiorentino DF *et al,* 1991., De Waal MR *et al*, 1991., Niiro H *et al*, 1994., Kitamura M *et al*, 1995]. The data in the current study have suggested that exposure of macrophages to TGFß can significantly inhibit their ability to stimulate matrix production by mesangial cells.

In the subsequent chapters, endeavours to characterise the factor(s) in MPCM involved in mediating fibronectin accumulation by rat mesangial cells are described.

CHAPTER FOUR

Characterisation of MPCM. I

4.1 Introduction

Much research has recently been carried out on various cytokines and growth factors known to be produced by macrophages, describing their roles in the modulation of glomerular cell proliferation, matrix synthesis and immunoinflammatory regulation [reviewed in Abboud HE, 1993., Striker LJ *et al*, 1991]. More often than not the work has focused on the properties and actions of a single cytokine within any one particular disease model. To date, the roles of cytokines and growth factors in renal progression have been correlative; their precise pathobiological functions have yet to be fully determined. Given the nature of the potential interactions within the glomerulus which are likely to include paracrine stimulation and autocrine loops, it is unlikely that the sclerotic process will involve a single cytokine acting in isolation.

There is limited data on the characterisation of macrophage derived factors in conditioned media with respect to their pro-fibrogenic and/or mitogenic properties. Lovett DH *et al* [1983a] tentatively identified IL-1ß as the mitogenic factor in MPCM derived from endotoxin treated rats. However, this factor was only active in the presence of serum. Mosquero JA [1993] described the upregulation of fibronectin production by proliferating mesangial cells in response to MPCM derived from macrophages elicited using various agents. His data suggested that IL-1ß was not the cytokine responsible for the matrix accumulation mediated by his conditioned media and suggested that IL-6 and TGFß could be possible candidates. Narita and co-workers [1991] reported that the glycosaminoglycan stimulatory activity of their monocyte derived factor had an apparent molecular weight of 10-17kD ruling out TGFß and PDGF as possible stimulatory factors. Furthermore, the glycosaminoglycan stimulatory activity could not be reproduced by IL-6 or IL-1ß.

Investigators from the same laboratory had earlier shown that the mitogenic factor(s) in human monocyte conditioned medium were more like PDGF than IL-1ß or IL-6 [Morioka T *et al*, 1991]. The anti-mitogenic activity in mouse MPCM reported by Ooi YM *et al*, [1983] was narrowed to two fractions having different molecular weights (14.6-29kD and 29-68kD), each mediating their actions via different mechanisms.

The experiments described in this and the subsequent chapters aimed to characterise the component(s) of MPCM responsible for the upregulation of fibronectin production by cultured rat mesangial cells.

4.2 Effect of treatment of MPCM on mesangial cell fibronectin production

In order to determine the characteristics of the biologically active component(s) of MPCM neat MPCM was subjected to a number of treatments and its ability to stimulate fibronectin production by mesangial cells was subsequently analysed.

4.2.1 Materials and Methods

a) trypsin digestion

Trypsin type IX (Sigma) was added to 3.5ml neat MPCM at a final concentration of 450μg/ml and incubated overnight at 37°C. Proteolytic digestion was terminated using an excess of soya bean trypsin inhibitor (200μl of 5mg/ml solution, Sigma). Trypsin acts on the lysyl and arginyl bonds of peptide chains and hydrolyses esters and amides.

b) protease digestion

Protease V8 (Sigma) was added to 3.5ml neat MPCM at a final concentration of 10μg/ml and incubated overnight at 37°C. Proteolytic digestion was terminated using an excess of soyabean trypsin inhibitor (200μl of 5mg/ml solution). Protease V8 cleaves peptide bonds on the carbonyl side of aspartic and glutamic acid residues.

c) heat treatment

3.5ml MPCM was placed into a 5ml glass volumetric flask and placed into a boiling water bath for 5min.

d) charcoal adsorption

1g activated charcoal (Sigma) was wetted with 2ml serum free RPMI in a sterile 30ml universal tube. The tube was then centrifuged at 2000rpm and the supernatant removed. 4ml MPCM was then added to the wet charcoal mixed thoroughly and incubated at 4°C for 30min. The tube was centrifuged again and the supernatant was removed. Activated carbon adsorbs small molecules on to its surface.

Following treatment of neat MPCM, it was diluted to a 50% solution (FCS at 0.5%). This was added to confluent, quiescent mesangial cells for 7 days. The supernatants were subsequently assayed for fibronectin as described in chapter 2 section 2.6.

4.2.2 Results

Results are expressed as % of untreated MPCM (Fig 4.1).

No significant effect on fibronectin stimulatory activity was observed following trypsinisation (95.0 $\pm$ 20.8%) although protease V8 treatment reduced activity by approximately 50% (51.7 $\pm$ 10.3%). Boiling for 5 min decreased the biological activity by about a third (69.6 $\pm$ 19.3%) whilst adsorption on to charcoal almost completely abolished its activity (5.2 $\pm$ 1.7%).

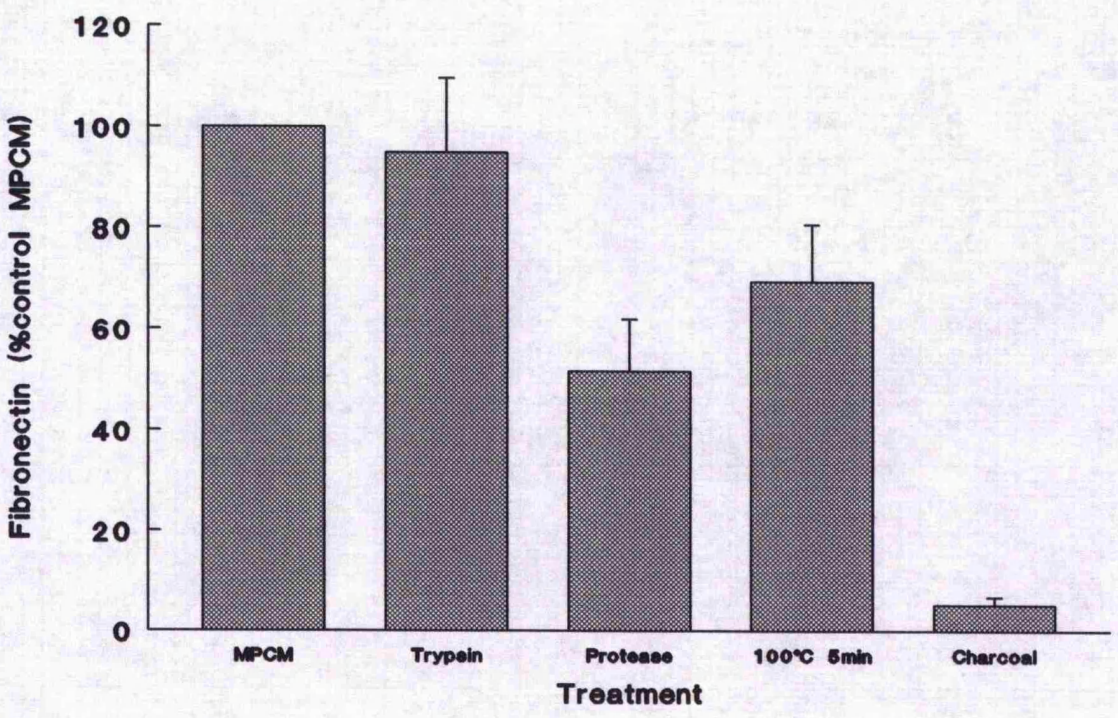


Fig 4.1 Effect of treatment of MPCM on fibronectin production.

MPCM was subjected to a number of treatments. After 7 days of culture the ability of MPCM to induce fibronectin production in mesangial cells was analysed. Fibronectin production is expressed as a percentage of that produced with untreated MPCM on day 7. Values are means ± SEM (n=3-4) each carried out in quadruplicate.

4.3 Fractionation of MPCM

In order to try and isolate the biologically active component(s) of MPCM and to determine its molecular weight, concentrated MPCM was fractionated by HPLC on a calibrated superose 6 gel filtration column.

4.3.1 Materials and Methods

15ml of MPCM was concentrated 20-30 fold using the Centriprep 3 concentrators. 200μl of the concentrate was fractionated on a superose 6 gel filtration column. The fractions were then added to mesangial cells in a 1:1 dilution with medium containing 1% FCS such that the final concentration of FCS remained at 0.5% (described in chapter 2 sections 2.9-10). After 7 days of culture supernatants and cell lysates were assayed for fibronectin.

4.3.2 Results

A major peak of fibronectin stimulatory activity was observed in the fractions corresponding to an apparent molecular weight of 12-20kD with a shoulder of activity to the leading edge of the main peak at approximately 20-30kD (Fig 4.2a). A similar activity profile was found for stimulation of cell associated fibronectin production (Fig 4.2a). The biologically active component(s) did not co-elute with any of the major protein peaks of MPCM as determined by the A_{280} protein elution profile (Fig 4.2b).

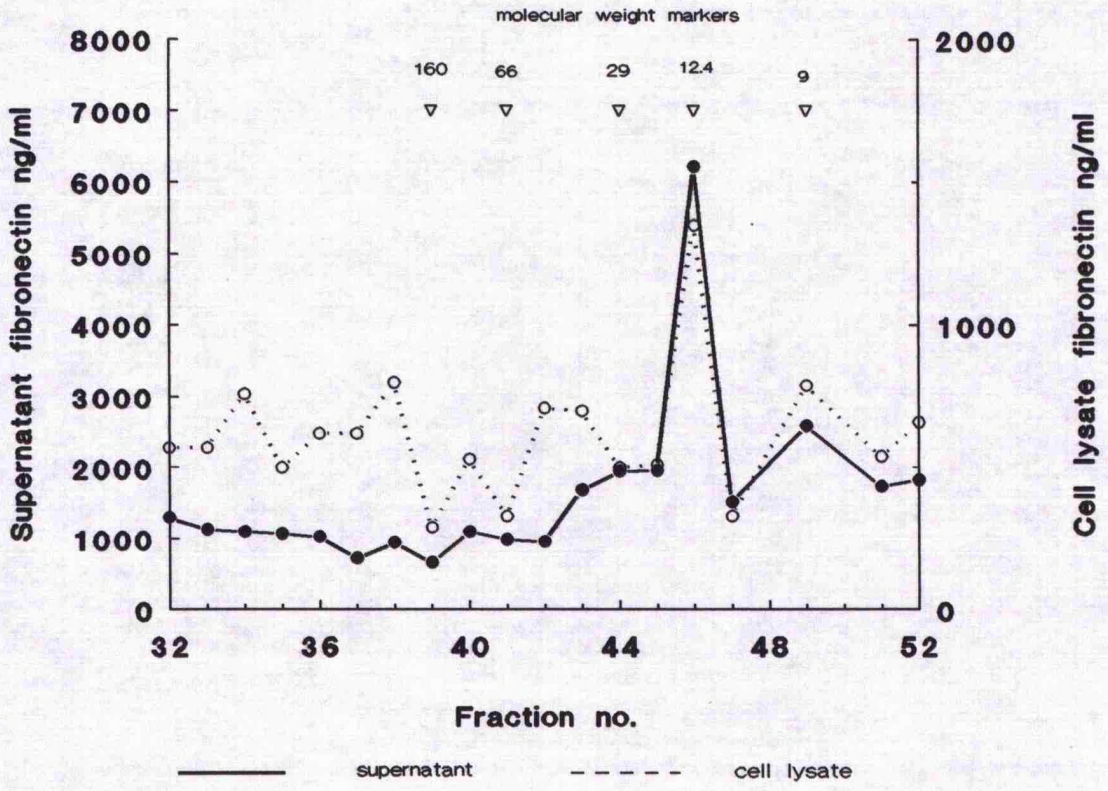


Fig 4.2a Effect of fractionation of MPCM by HPLC on fibronectin production. HPLC fractions of MPCM were added to confluent, quiescent mesangial cells. Day 7 supernatants (—) and cell lysates (---) were assayed for fibronectin. A representative experiment of three runs is presented.

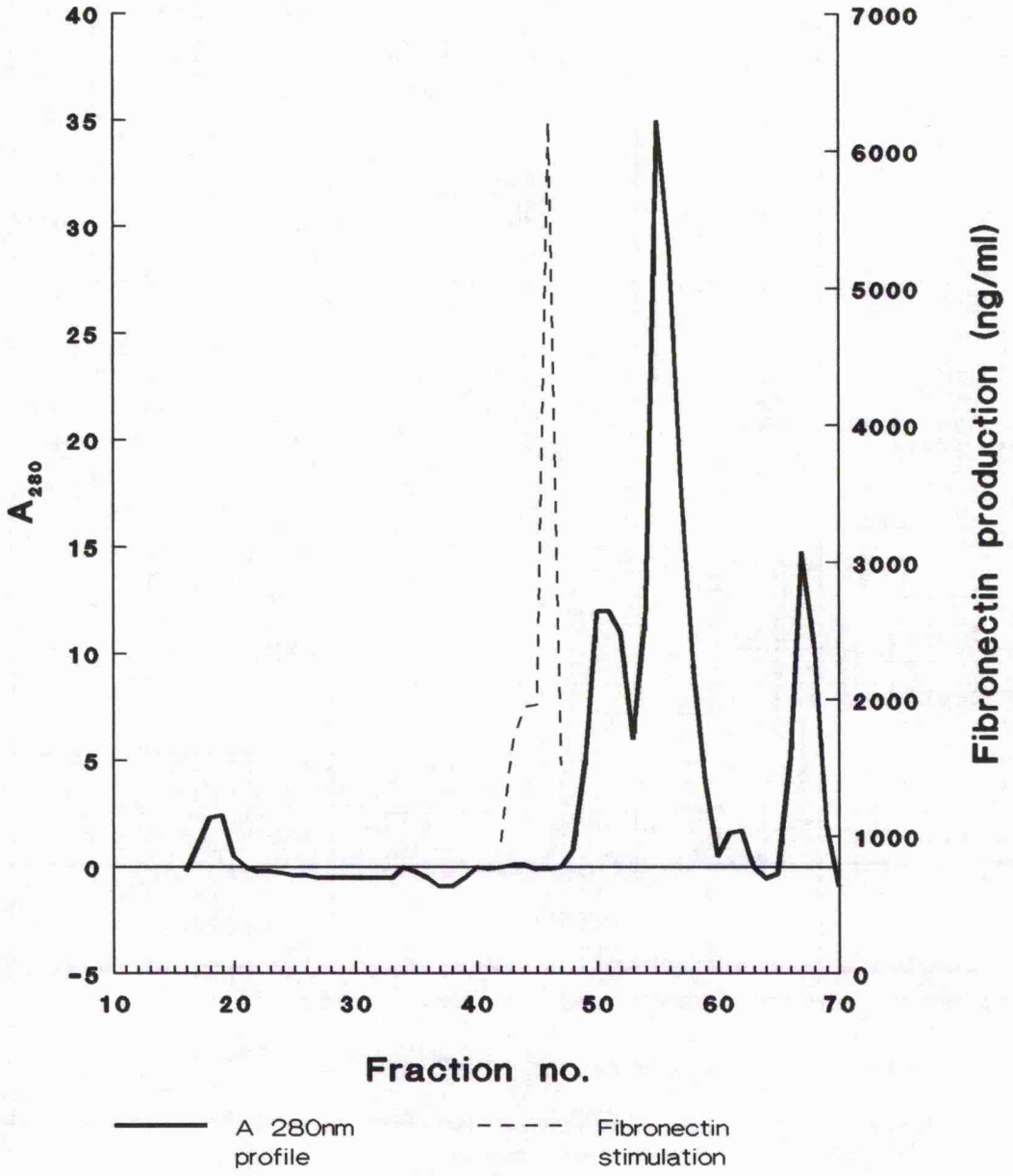


Fig 4.2b A_{280} protein elution profile.

HPLC A_{280} protein elution profile of MPCM (—). The position of the fibronectin stimulating fractions is also shown (---).

4.4 SDS-PAGE of MPCM

Since the concentration of the fibronecting stimulating factor(s) in MPCM is too low to be detected by the HPLC uvicord monitor, an attempt was made to "visualise" the active component(s) by SDS-PAGE and silver staining.

4.4.1 Materials and Methods

MPCM was concentrated in a centriprep 3 concentrator and lyophilised in a freeze drier (Edwards modulyo). The lyophilised powder was reconstituted in 250μl of sample buffer and electrophoresed, along with molecular weight markers (BioRad) and a TGFß standard (R&D Systems), on a 15% polyacrylamide gel. The gel was subsequently fixed and silver stained.

Fractions 42-47 from the HPLC fractionation (broadly those fractions which induced fibronectin production by mesangial cells) were also analysed by SDS-PAGE and the gels stained with silver (described in chapter 2 sections 2.9-12).

4.4.2 Results

It can be seen from the 15% gel (Fig 4.3) that MPCM is a complex mixture of many compounds spanning a wide range of molecular weights but with major bands corresponding to molecular weights of >102, 69, 60, 36, 32, 26.3, 21.4, 14.5 and 6kD.

No protein could be detected in the gels of HPLC fractions 42-47 stained with silver.

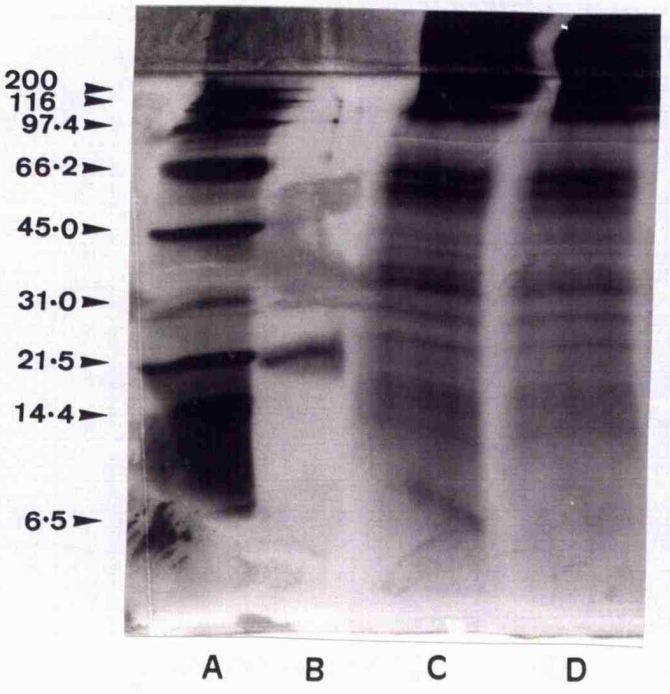


Fig 4.3 Silver-stained SDS-PAGE of MPCM

Concentrated MPCM was fractionated by SDS-PAGE (15% gel) and stained with silver. Lane A = mol wt markers, lane B = position of TGFß, lanes C and D = MPCM.

4.5 Discussion

Preliminary characterisation studies suggest that the biologically active component(s) of MPCM appears to be a low molecular weight protein or peptide. Its resistance to trypsinisation may suggest that the protein contains few aspartic or glutamic acid residues around its active site or, more likely, a carbohydrate moiety affords the molecule some protection from trypsin digestion. The ability of MPCM to retain a large part of its biological activity following boiling would suggest that the active component(s) contains a high proportion of disulphide bonds; the acid and heat stability of TGFß has been attributed to its high number of disulphide bonds which are essential for its biological activity [Roberts AB *et al,* 1983].

Gel filtration chromatography has narrowed the molecular weight of the biological activity to the region of 12-30kD, a molecular weight range that encompasses a number of cytokines and growth factors. The concentration of the biologically active factor in MPCM is very low since it does not register a peak on the A_{280} MPCM protein elution profile with the most sensitive full scale deflection setting and no protein could be detected in the biologically active HPLC fractions which had been fractionated by SDS-PAGE and stained with silver.

Additional experiments were therefore carried out in order to further characterise the biologically active factor(s) in MPCM responsible for induction of fibronectin production in cultured rat mesangial cells.

CHAPTER FIVE

Characterisation of MPCM II

The role of cytokines

5.1 Introduction

Preliminary characterisation studies indicated that the biologically active component(s) of MPCM is a small peptide(s) of a size comparable to most growth factors and cytokines.

The growth factor TGFß is known to be secreted by activated macrophages and has been shown to play a major role in the stimulation of matrix production by glomerular cells [Massague J, 1990]. Border and associates have reported that the level of TGFß mRNA and the number of cells producing the protein was higher in glomeruli isolated from Thy-1 nephritic rats than in glomeruli from normal rats [Okuda S *et al,* 1990]. It was later shown in the same model that administration of a neutralising antibody to TGFß resulted in a significant suppression of extracellular matrix production with a concommitant reduction in the degree of histological damage [Border WA *et al,* 1990b]. Similarly, treatment of nephritic rats with the proteoglycan decorin, which binds and neutralises the biological activity of TGFß, reduced the extent of glomerular damage [Border WA *et al,* 1992a]. TGFß expression has also been reported in other models of glomerulonephritis including Habu snake venom-induced mesangioproliferative glomerulonephritis [Barnes JL and Abboud HE, 1993], anti-GBM nephritis [Coimbra T *et al,* 1991] and in PAN nephrosis [Ding G *et al*, 1993]. Further support for a pathogenetic role of TGFß in renal scarring has been provided by the observation that *in vivo* transfection of the TGFß gene increases the production of extracellular matrix deposition in the kidney [Isaka Y *et al*, 1993].

PDGF is another growth factor that plays an important role in the progression of renal disease. In the $^{5}/_{6}$ nephrectomy model in rats, glomerulosclerosis is preceded by early, low level mesangial cell proliferation associated with both an upregulation of PDGF B-chain

mRNA, PDGF protein and an influx of platelets [Floege J *et al*, 1992b]. Proteinuria, macrophage infiltration and accumulation of matrix follow the phase of cell proliferation. Other investigators have demonstrated an increase in PDGF A and B chain mRNA and B chain protein in isolated glomeruli of Thy-1 glomerulonephritic rats, particularly during the phase of mesangial cell proliferation [Iida H *et al*, 1991]. Administration of a neutralising antibody to PDGF has been shown to result in a significant reduction in matrix deposition and cell proliferation in anti-Thy-1 nephritic rats [Johnson RJ et al, 1992]. Floege and associates [1993] have demonstrated that intravenous infusion of PDGF induces glomerular mesangial cell proliferation and matrix accumulation. Intraglomerular transfection of the PDGF gene has also been shown to induce intrinsic glomerular cell proliferation [Isaka Y *et al,* 1993].

The secretion of TNFα is strongly associated with macrophage infiltration. Isolation of glomerular macroghages from rabbits with nephrotoxic nephritis has demonstrated that they secrete TNFα [Tipping PG *et al,* 1991b]. Furthermore, anti-TNFα antibodies can dose dependently reduce proteinuria and reduce glomerular necrosis in this model [Hruby ZW *et al,* 1991]. Macrophage secretion of IL-1ß has also been demonstrated in nephrotoxic nephritis [Matsumoto K and Hatano M, 1989., Tipping PG *et al*, 1991a]. In addition, IL-1 receptor antagonist treatment has proved beneficial in suppressing crescentic glomerulonephritis [Lan HY *et al*, 1993b]. Using immunohistological staining techniques Diamond and Pesek [1991b] demonstrated the presence of TNFα and IL-1ß within the glomerulus of rats with acute PAN nephrosis. When animals were placed on an EFAD diet to reduce macrophage infiltration, the number of TNFα and IL-1ß positive cells decreased in parallel with the number of ED1 positive cells [Diamond JR and Pesek I, 1991b].

The aim of this chapter, therefore, was to define whether known cytokines were involved in MPCM-mediated stimulation of mesangial cell fibronectin production, particularly focusing on TGFß, PDGF, TNFα and IL-1ß and to define the potential cytokine interactions involved.

5.2 Effect of cytokines on mesangial cell fibronectin production

In order to examine whether $TGF\beta_1$, PDGF, TNFα or IL-1ß could reproduce the actions of MPCM on fibronectin production, mesangial cells were cultured in the presence of these cytokines.

5.2.1 Materials and Methods

Confluent, quiescent mesangial cells were treated with:

a) 10ng/ml of either:

- human $TGF\beta_1$,
- human PDGF (predominantly AB isoform),
- murine TNFα or
- murine IL-1ß (R&D Systems).

b) 10ng/ml of human $TGF\beta_1$ in combination with 10ng/ml of either:

- TNFα,
- IL-1ß or
- PDGF

c) 10ng/ml of either:

- TNFα,
- PDGF or

· IL-1ß

in combination with increasing dilutions of TGFß$_1$ (10, 1.0, 0.1, 0.01, 0.001ng/ml).

d) MPCM in combination with 10ng/ml of either:

· TGFß$_1$,

· TNFα,

· PDGF or

· IL-1ß.

The incubations were carried out for:

a) 7 days after which the supernatants and cell lysates were assayed for fibronectin (chapter 2, section 2.6) or

b) 24hr following which RNA was extracted from the cells and subjected to Northern blot analysis (chapter 2, section 2.25).

The cytokine dose of 10ng/ml was used in these experiments since it falls within the range found by other investigators to be appropriate for use in tissue culture (Suzuki S *et al*, 1993., Kohno M *et al*, 1994., Floege J *et al*, 1990., Martin J *et al*, 1994].

5.2.2 Results

Exposure of mesangial cells directly to 10ng/ml of TGFß$_1$, PDGF, TNFα or IL-1ß resulted in small increases in secreted and cell associated fibronectin levels (Fig 5.1). With the exception of PDGF stimulated fibronectin secretion, all increases in fibronectin were significantly greater than control levels ($p < 0.04$). However, none of the cytokines individually were able to reproduce the magnitude of MPCM-induced fibronectin production. In all cases MPCM-induced fibronectin levels were significantly greater than those induced by any individual cytokine ($p < 0.001$).

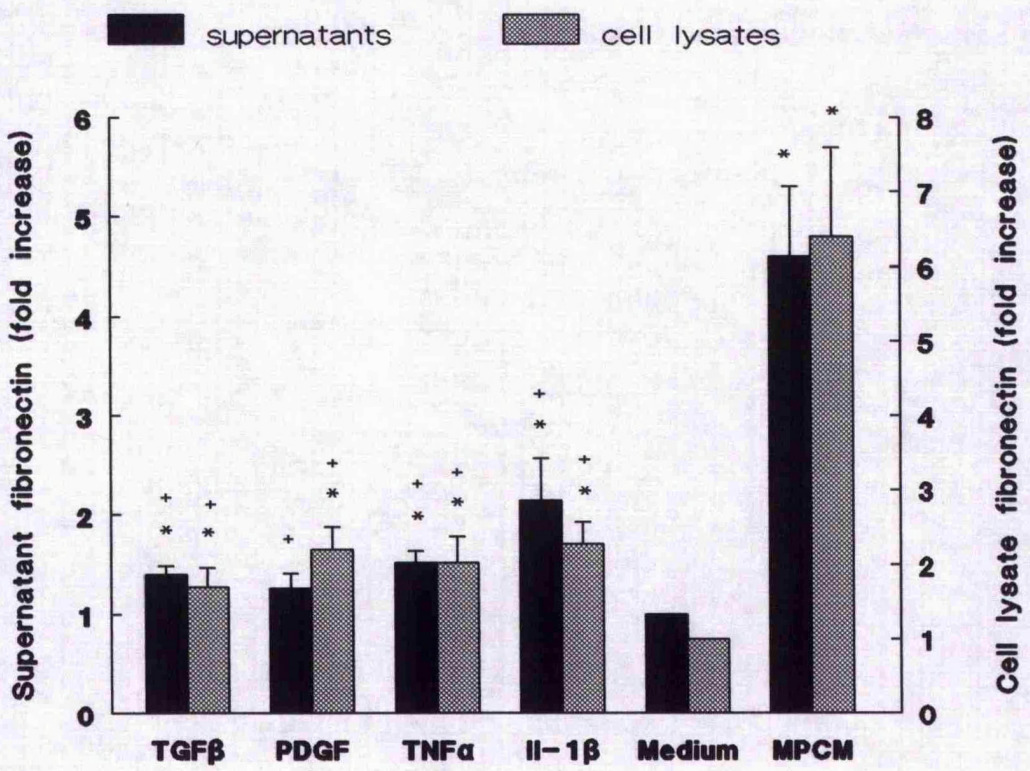


Fig 5.1 Effect of individual cytokines on mesangial cell fibronectin production. Mesangial cells were exposed to 10ng/ml of either TGFß$_1$, PDGF, TNFα, IL-1ß or MPCM for 7 days. Supernatants and cell lysates were assayed for fibronectin. Results are expressed as fold increase in fibronectin over medium alone (ng Fibronectin/μg cell protein). Values are means $\pm$ SEM (n=5-22) each carried out in quadruplicate. *$p < 0.04$ vs medium, +$P < 0.001$ vs MPCM.

When mesangial cells were exposed to 10ng/ml TGFß$_1$ added in combination with 10ng/ml of TNFα, IL-1ß or PDGF, fibronectin levels were synergistically increased to a degree comparable with that observed with MPCM (Fig 5.2). There was no significant difference between fibronectin levels induced by cytokine combinations and MPCM.

Northern blot analysis demonstrated that individually each cytokine significantly upregulated fibronectin gene transcription above control levels ($p < 0.05$, n=3-10) (Table 5.1). However, the TGFß$_1$:TNFα and TGFß$_1$:IL-1ß combinations resulted in a synergistic augmentation of fibronectin message to a level greater than the sum of the individual cytokines respectively (Fig 5.3 and Fig 5.4). The results from the TGFß$_1$:PDGF combination were equivocal, although, the combination did not appear to exhibit the synergistic increase in the fibronectin transcript at the 24hr time point suggesting that with this particular combination the increase in fibronectin protein production may occur via a different mechanism.

Table 5.1 Effect of individual cytokines on fibronectin gene transcription.
Mesangial cells exposed to 10ng/ml cytokine for 24hr. RNA was extracted and analysed by Northern blotting. Results are expressed as fold increase in densitometric units over medium alone. The effect of MPCM is shown for comparison.

	TGFß	PDGF	IL1ß	TNFα	MPCM
Fn mRNA	2.04±0.54	2.31±0.31	4.79±0.03	3.1±0.66	2.9±0.2

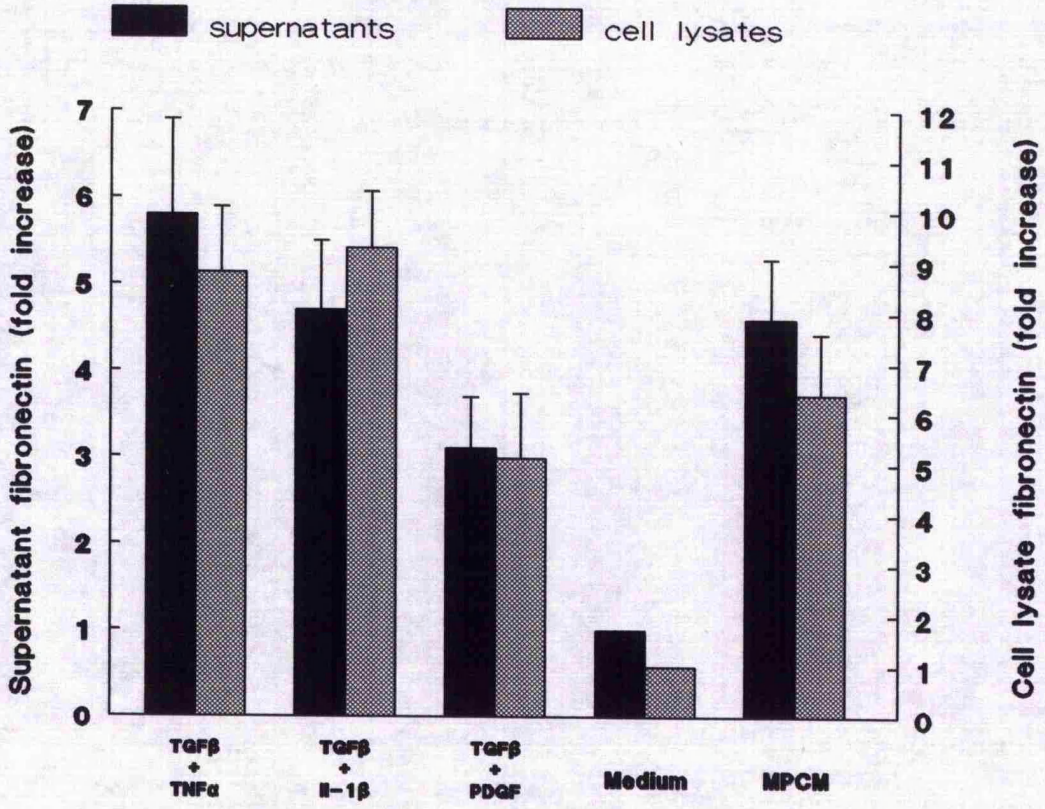
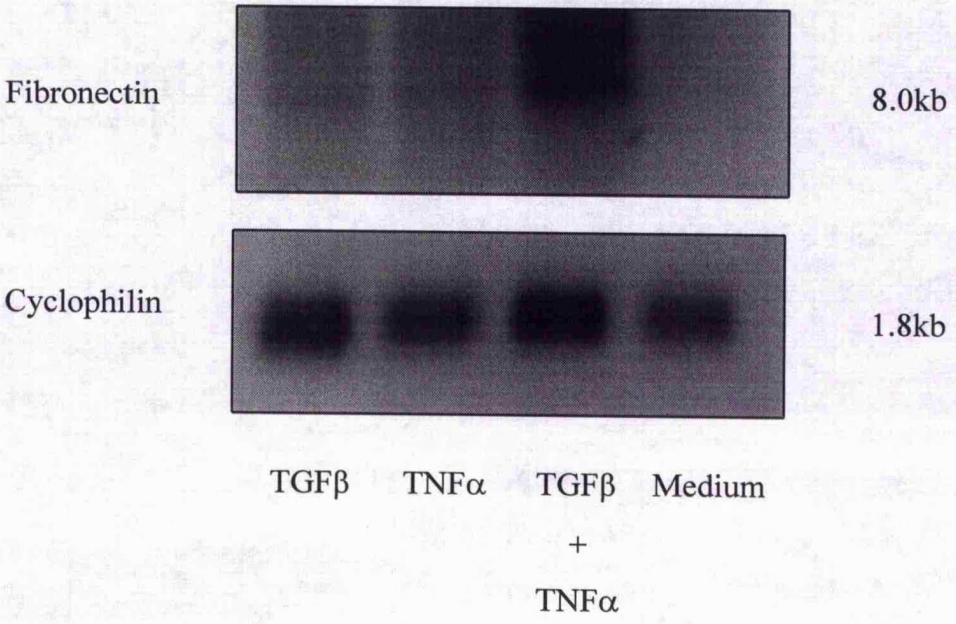


Fig 5.2 Effect of combinations of cytokines on fibronectin production.

Mesangial cells were exposed to 10ng/ml TGFß$_1$ in combination with 10ng/ml of either TNFα, IL-1ß or PDGF for 7 days. Supernatants and cell lysates were assayed for fibronectin. Results are expressed as fold increase in fibronectin over medium alone (ng Fibronectin/μg cell protein). All cytokine combinations significantly increased fibronectin levels compared to medium alone $p<0.001$. Values are means $\pm$ SEM (n=5-8) each carried out in quadruplicate. MPCM induced fibronectin levels are shown as a comparison.

Fig 5.3 Effect of TGFß$_1$ and TNFα combination on fibronectin gene transcription. Northern blot shows the effect of TGFß$_1$, TNFα, TGFß$_1$+TNFα and medium on fibronectin gene transcription. Representative blot of three experiments is presented. Densitometric analysis of the 8kb fibronectin bands, expressed as fold increase over medium alone, from all three experiments is shown normalised for RNA loading.



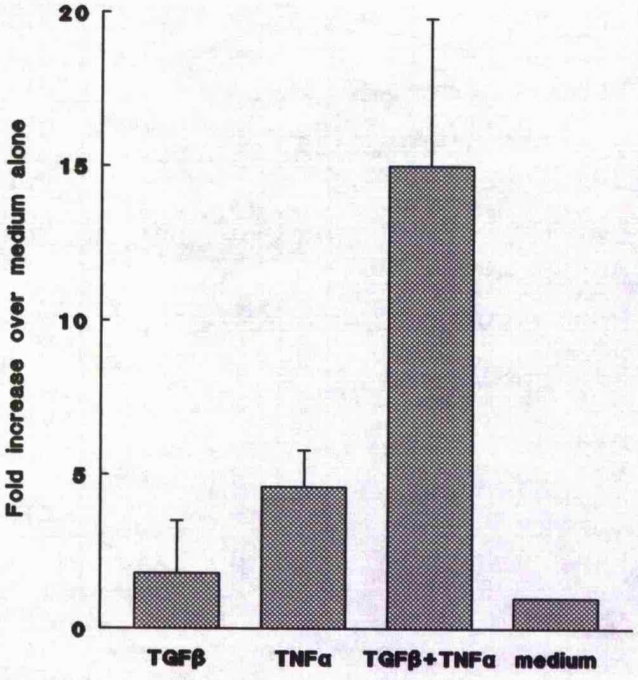
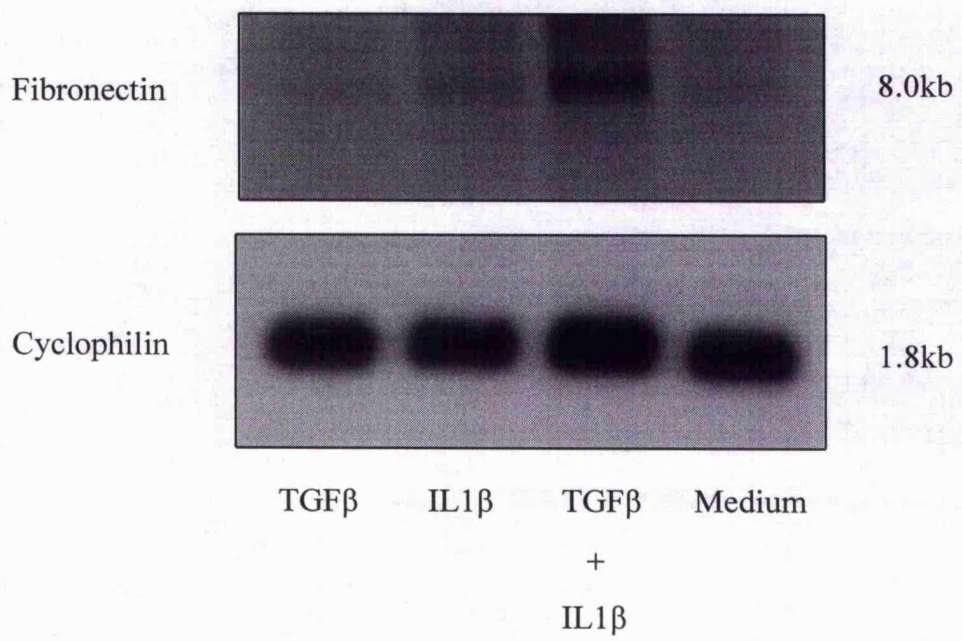
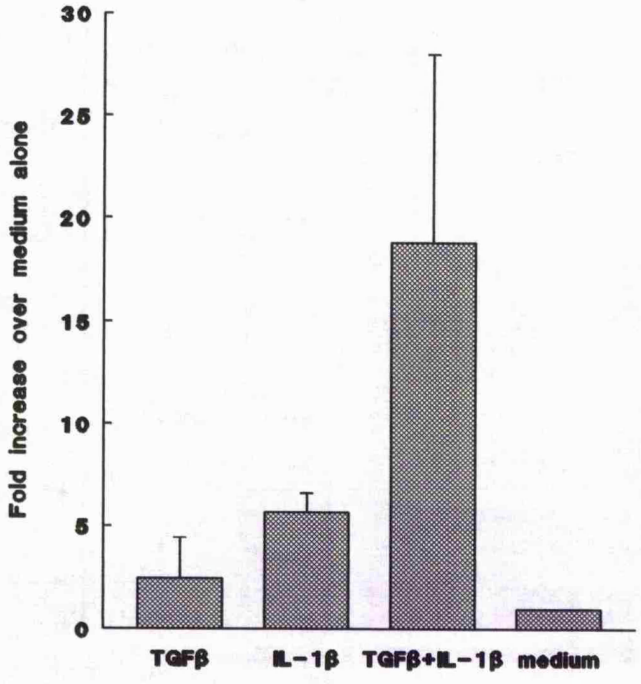


Fig 5.4 Effect of TGFß$_1$ and IL-1ß combination on fibronectin gene transcription. Northern blot shows the effect of TGFß$_1$, IL-1ß, TGFß$_1$+IL-1ß and medium on fibronectin gene transcription. Representative blot of two experiments is presented. Densitometric analysis of the 8kb fibronectin bands, expressed as fold increase over medium alone, from the two experiments is shown normalised for RNA loading.





The synergistic effect of combinations of cytokines on fibronectin production was titratable; increasing the dilution of TGFß$_1$ against a constant concentration of either TNFα, PDGF or IL-1ß decreased the degree of fibronectin secretion (Fig 5.5).

When 10ng/ml of TGFß$_1$, TNFα, PDGF or IL-1ß were added to mesangial cells in combination with MPCM, TNFα and PDGF significantly increased both secreted and cell associated fibronectin levels over those observed with MPCM alone ($p < 0.03$). Addition of TGFß$_1$ increased fibronectin in the cell associated fraction only ($p = 0.002$) (Fig 5.6). The increases in fibronectin observed were greater than the predicted additive values of MPCM and cytokines alone, indicating that the cytokines may be synergistically interacting with other factors in MPCM.

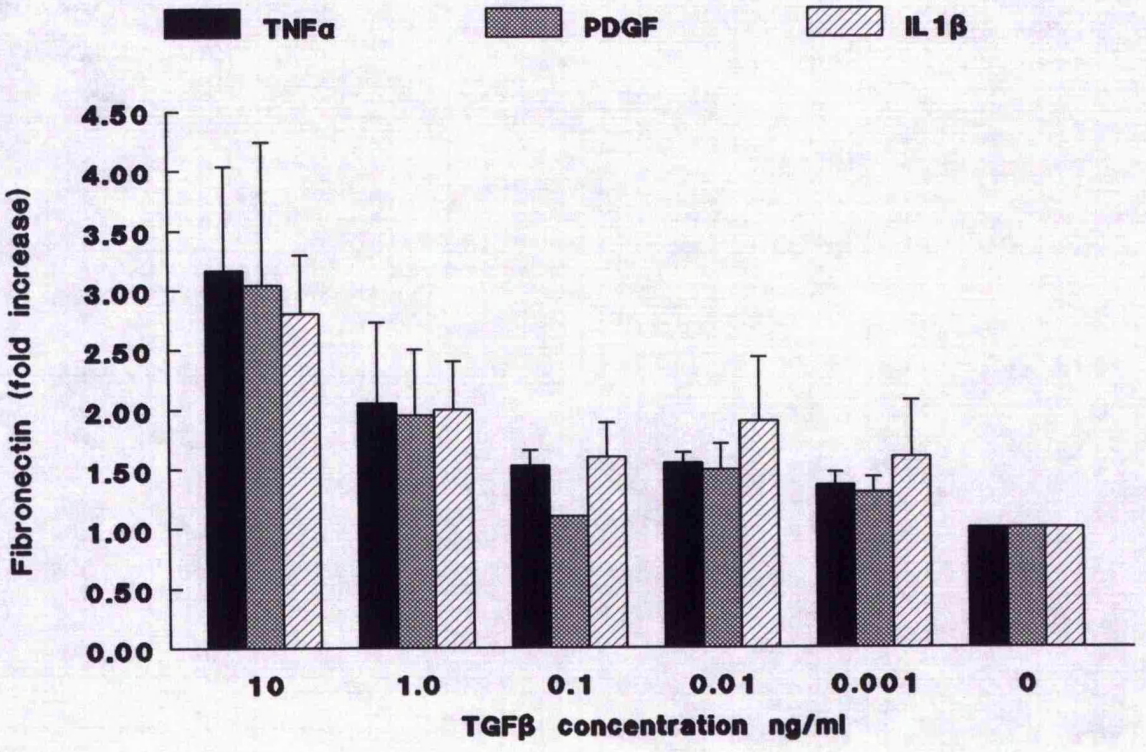


Fig 5.5 Effect of TGFß$_1$ titration on cytokine combination-induced fibronectin production. 10ng/ml of either TNFα, PDGF or IL-1ß were co-incubated with 10, 1, 0.1, 0.01, 0.001, 0 ng/ml TGFß$_1$ for 7 days. Supernatants were assayed for fibronectin. Results are expressed as fold increase over medium alone (ng fibronectin/μg cell protein). Values are means $\pm$ SEM (n=3) each carried out in triplicate.

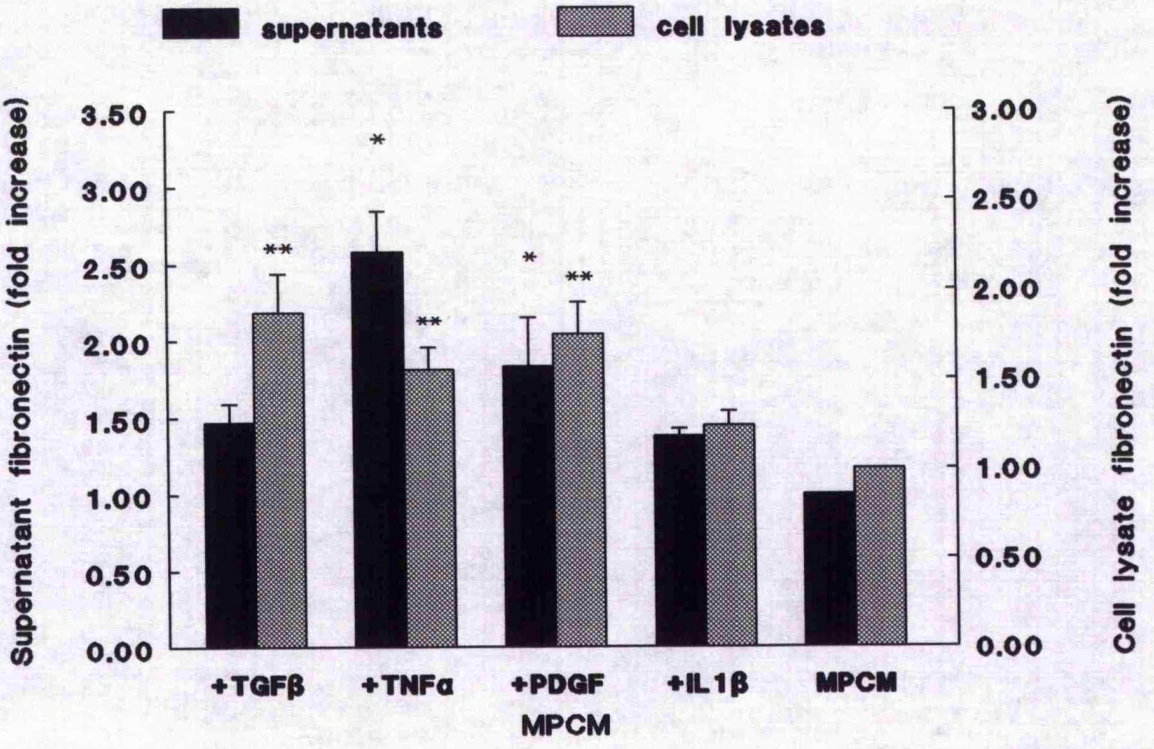


Fig 5.6 Effect of addition of cytokines in combination with MPCM on fibronectin production. Mesangial cell were treated with 10ng/ml of either TGFß$_1$, TNFα, PDGF or IL-1ß in combination with MPCM for 7 days. Supernatants and cell lysates were assayed for fibronectin. Results are expressed as fold increase over MPCM alone (ng fibronectin/μg cell protein). Values are means $\pm$ SEM (n=3) each carried out in quadruplicate.
*$p<0.03$, **$p=0.002$ vs to MPCM.

5.3 Effect of cytokines on the gene transcription of laminin, collagen IV, TIMP-1 and MCP-1 by mesangial cells.

It was demonstrated in chapter 3 that, as well as the message for fibronectin, MPCM upregulated the gene transcription of the matrix proteins laminin B1 chain and α1 (IV) collagen as well as the inhibitor of matrix metalloproteinases TIMP-1 and the chemokine MCP-1. The effects of TGFß$_1$, PDGF, TNFα and IL-1ß on the above mentioned genes were also assessed and compared to those of MPCM.

5.3.1 Materials and Methods

RNA was extracted from mesangial cells exposed to 10ng/ml TGFß$_1$, PDGF, TNFα and IL-1ß for 24hr. Northern blot analyses were carried out using ^{32}P-labelled probes for laminin B1 chain, collagen IV α1 chain, TIMP-1 and MCP-1 (as described in chapter 2, section 2.25).

5.3.2 Results

Only IL-1ß was able to reproduce the effect of MPCM on α1 (IV) collagen, laminin B1 messages and TIMP-1 mRNA. Both IL-1ß and TNFα induced MCP-1 message to levels comparable with MPCM whilst PDGF appeared to have no effect. The results are summarised in table 5.2 and compared to MPCM.

Table 5.2 Effect of individual cytokines on the gene transcription α1 (IV) collagen (Col IV), laminin B1 chain (LM), TIMP-1 and MCP-1 by mesangial cells. Mesangial cells were exposed to 10ng/ml TGFß$_1$, PDGF, TNFα or IL-1ß for 24hr. RNA was extracted and analysed by Northern blotting. Results are expressed as fold increase in densitometric units over medium alone. The effect of MPCM is shown for comparison.

	Col IV mRNA	LM mRNA	TIMP-1 mRNA	MCP-1 mRNA
TGFß	1.4±0.4	1.8±0.9	2.3±0.6	1.8±0.5
PDGF	1.6±0.4	1.8±0.7	3.8±0.8	0.7±0.04
TNFα	1.6±0.1	1.9±0.3	6.8±1.5	4.8±0.1
IL-1ß	4.2±0.04	4.2±0.04	18.7±9.4	5.5±0.2
MPCM	3.1±0.3	4.9±0.2	15.2±2.5	5.8±0.8

5.4 Detection of cytokines in MPCM

In order to ascertain whether TGFß$_1$, PDGF, TNFα or IL-1ß or combinations thereof, might be responsible for the observed effects of MPCM, assays were performed to try and detect them in the MPCM.

5.4.1 Materials and Methods

The presence of these cytokines in standard MPCM or concentrated MPCM was measured by:

a) Western blotting

b) Commercial cytokine ELISA assays

using the methods described in chapter 2, sections 2.14 and 2.19.

5.4.2 Results

TGFß$_1$, PDGF-AB and IL-1ß could not be detected in neat or concentrated MPCM using Western blotting or by ELISA, at least not at the sensitivity levels of the assays. TNFα however, was detected at 90pg/ml in concentrated MPCM using the rat TNFα ELISA assay. Of note is the fact that the intermediate product of MPCM production (the medium in contact with the macrophages containing LPS before it is washed away) contained high levels of TNFα (2471 $\pm$ 132 pg/ml) however, none of the other cytokine were detected in this medium.

5.5 Detection of cytokines in mesangial cell tissue culture supernatants

Cytokine ELISA assays were carried out on mesangial cell tissue culture supernatants in order to ascertain whether TGFß$_1$, PDGF-AB, TNFα or IL-1ß were secreted by mesangial cells in response to MPCM or to individual cytokines.

5.5.1 Materials and Methods

TGFß$_1$, PDGF-AB, TNFα and IL-1ß assays were carried out on day 7 tissue culture supernatants of mesangial cells exposed to:

a) 50% MPCM

b) medium alone

c) 10ng/ml of TGFß$_1$, PDGF, TNFα or IL-1ß

Assays were carried out as described in chapter 2, section 2.19.

Northern blot analyses probing for TGFß$_1$ and PDGF B chain mRNA were carried out on RNA extracted from mesangial cells exposed MPCM or individual cytokines for 24hr.

5.5.2 Results

Assay of tissue culture supernatants from mesangial cells exposed to medium alone demonstrated that mesangial cells constitutively secreted TGFß$_1$ and PDGF-AB into their culture medium. This autocrinal secretion was upregulated 1.69 ± 0.16 and 2.29 ± 0.28 fold respectively ($p < 0.001$) in respose to MPCM (Fig 5.7a and b). Furthermore the secreted TGFß$_1$ was in the latent or inactive form since it could not be measured in the assay unless the samples had been activated by acidification.

TNFα was only detected in tissue culture supernatants from mesangial cells exposed to

MPCM but only just at the limits of assay sensitivity (35pg/ml) and even then only in some samples and not others. IL-1ß did not appear to be secreted (or at least could not be detected at the sensitivity levels of the assays).

Northern analyses confirmed that TGFß$_1$ and PDGF B chain gene transcription were upregulated 2.2 $\pm$ 0.4 and 5.67 $\pm$ 1.2 fold respectively ($p < 0.001$, $p = 0.004$) in response to MPCM (Figs 5.8 and 5.9).

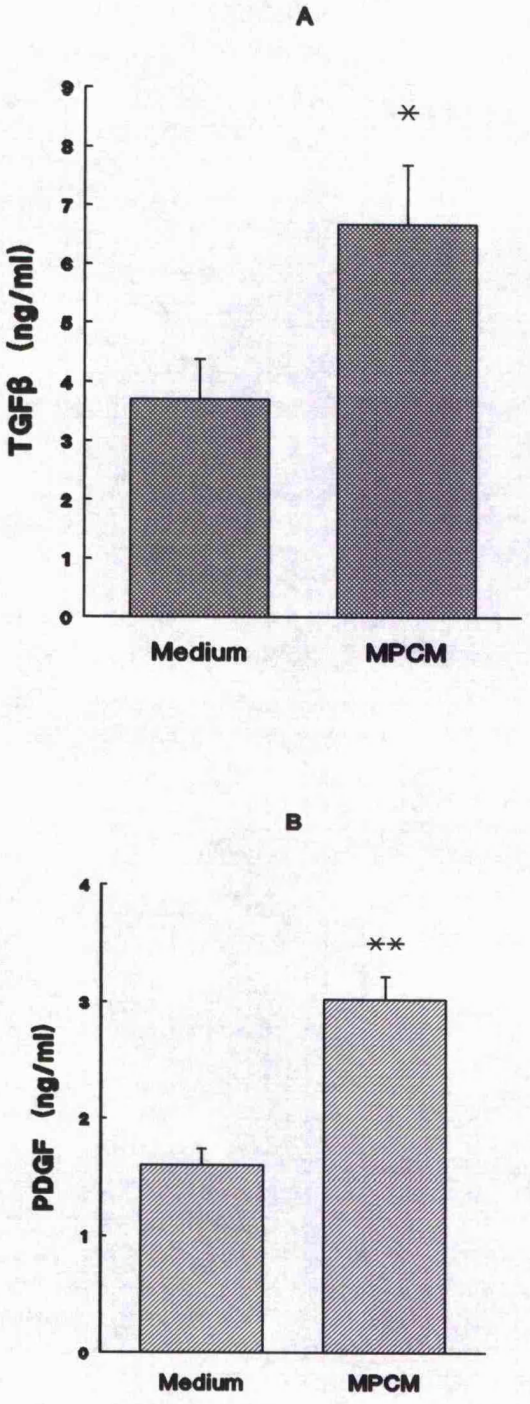
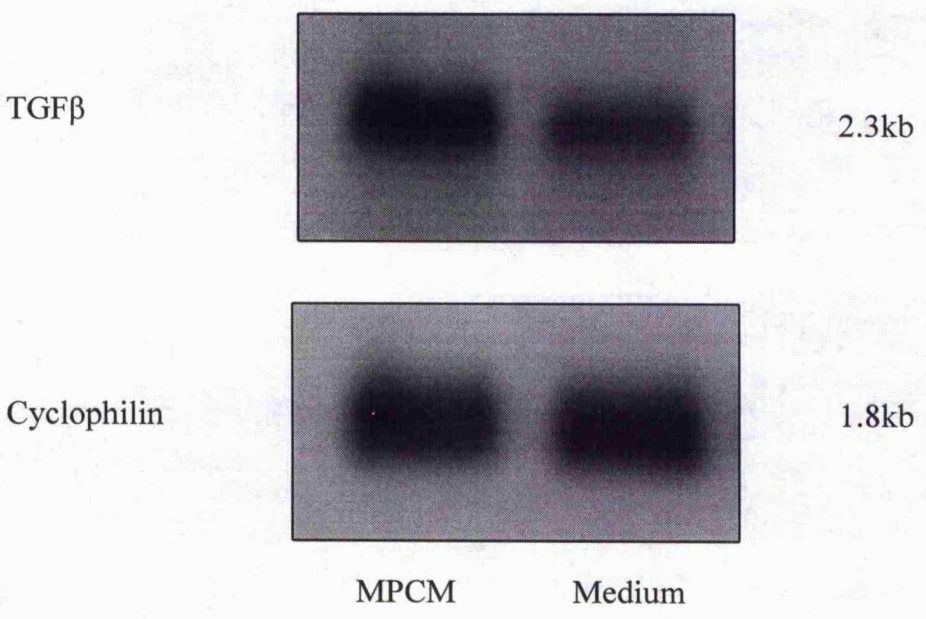


Fig 5.7 TGFß$_1$ and PDGF-AB secretion by mesangial cells in response to MPCM. Mesangial cells were exposed to MPCM or medium alone for 7 days. Supernatants were assayed for A) TGFß$_1$ and B) PDGF-AB secretion. Values are means ± SEM (n=5-9). *p=0.041 and **p<0.001 vs medium alone.

Fig 5.8 Effect of MPCM on TGFß$_1$ gene transcription.

Mesangial cells were exposed to MPCM or medium alone for 24hr. RNA was extracted and analysed by Northern blotting. One representative blot of four is shown. Densitometric analysis is presented as fold increase over medium alone and includes data from all four blots normalised for RNA loading. *$p < 0.001$



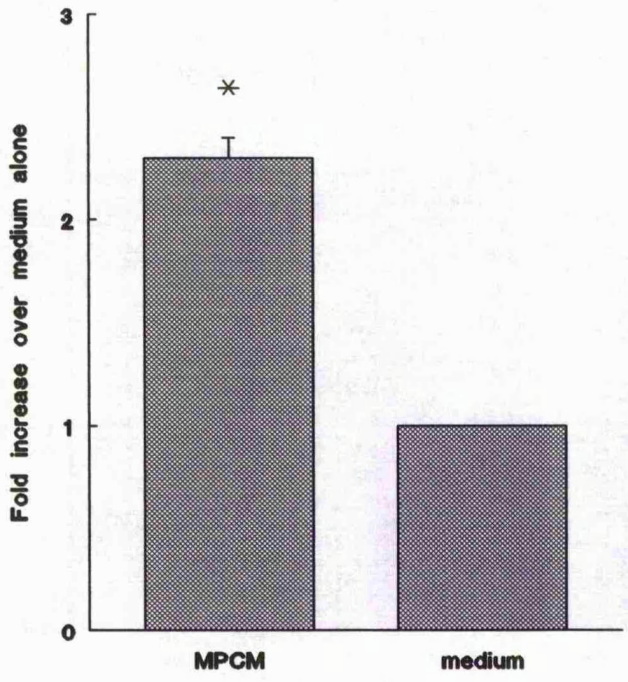
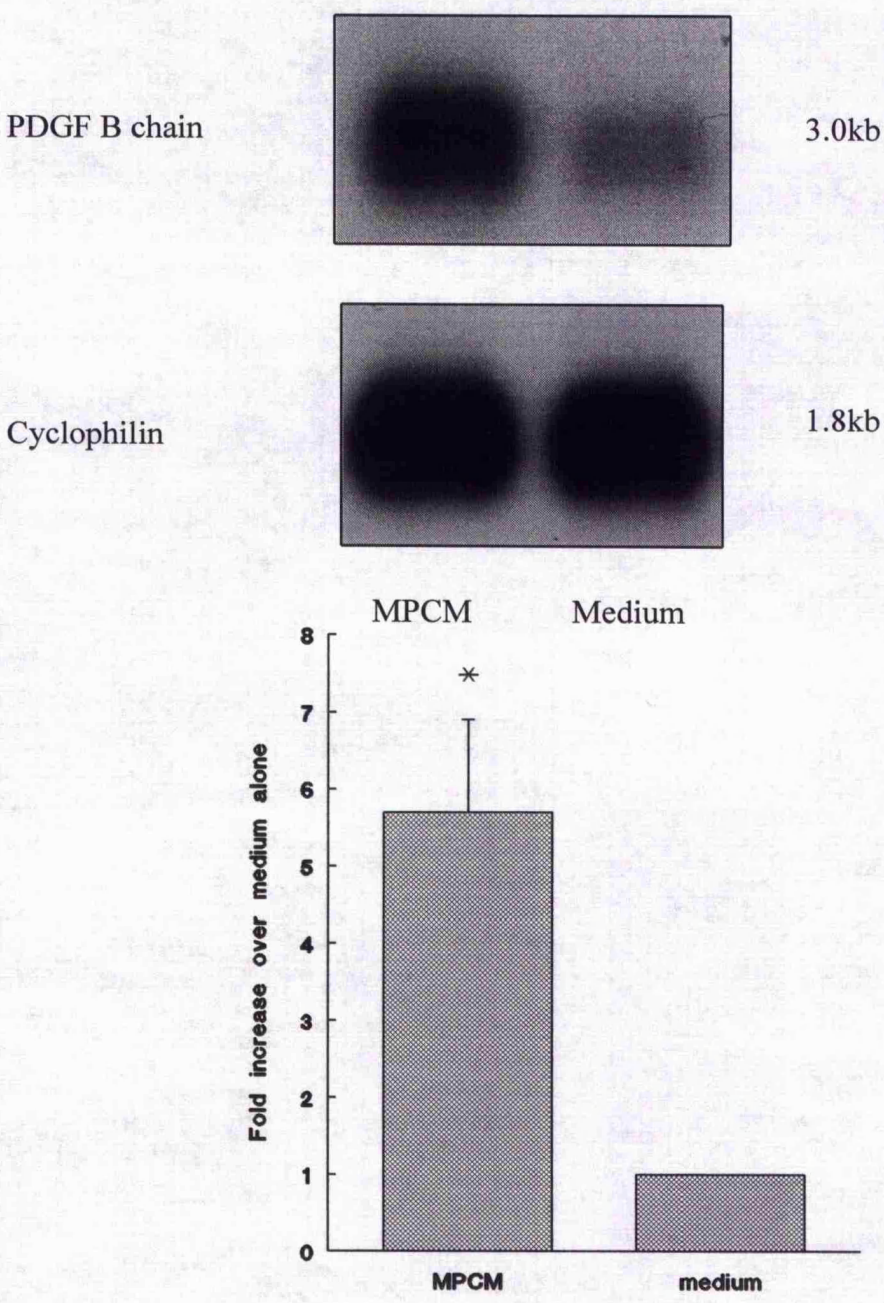


Fig 5.9 Effect of MPCM on PDGF B chain gene transcription.

Mesangial cells were exposed to MPCM or medium alone for 24hr. RNAs were extracted and analysed by Northern blotting. One representative blot of five is shown. Densitometric analysis is presented as fold increase over medium alone and includes data from all five blots normalised for RNA loading. *p=0.004.



The secretion of TGFß$_1$ and PDGF-AB in response to individual cytokines was also analysed and the profile of secretion compared to that of MPCM. Mesangial cells exposed to 10ng/ml TGFß$_1$ secreted high levels of PDGF-AB into their culture supernatants. However, 10ng/ml TNFα or IL-1ß appeared not to stimulate any PDGF-AB secretion over that of medium alone. (Fig 5.10a).

10ng/ml PDGF, TNFα or IL-1ß induced similar levels of TGFß$_1$ secretion into culture supernatants (Fig 5.10b). These data suggest that secretion of TGFß$_1$ can be induced by a number of cytokines, whilst PDGF-AB secretion appears to be largely dependent on the actions of TGFß$_1$.

Northern blot analysis of RNA from mesangial cells exposed to TGFß$_1$, PDGF, TNFα or IL-1ß confirmed that TGFß$_1$ gene transcription was upregulated in response to these cytokines (Table 5.3). However, in contrast to the results from measurement of secreted cytokine protein, TNFα and IL-1ß induced high levels of PDGF B chain mRNA. This disparity could be due to the fact that the production of high levels of PDGF B chain message may reflect the secretion of either PDGF-AB or PDGF-BB isoforms; since only the former isoform was measured in these experiments, secretion of the BB isoform by mesangial cells cannot be excluded. An alternative explanation may be that the message for PDGF B chain is inefficiently translated.

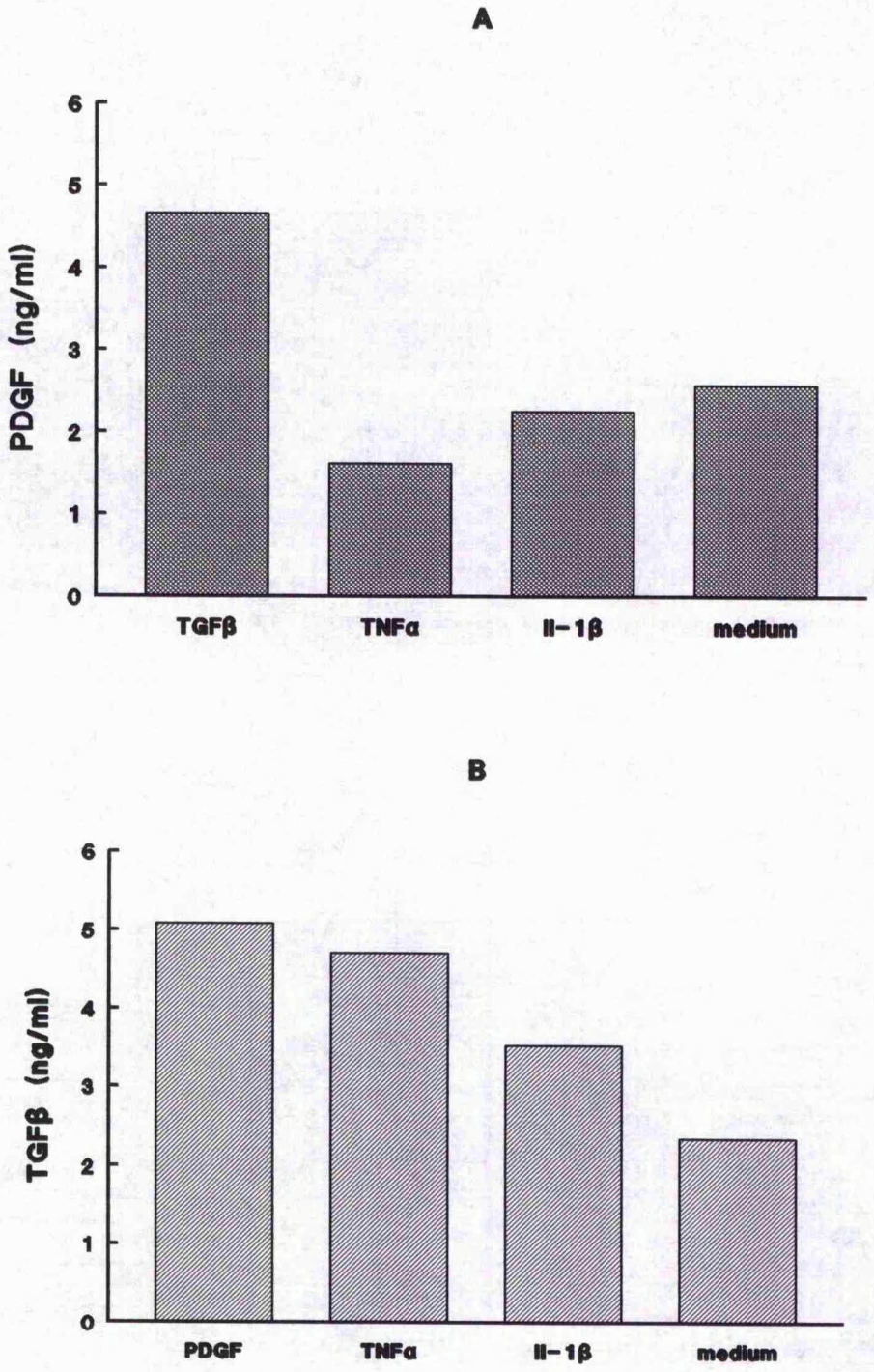


Fig 5.10 TGFß$_1$ and PDGF-AB secretion in response to TGFß$_1$, TNFα, IL-1ß or PDGF. Tissue culture supernatants were assayed for A) PDGF-AB or B) TGFß$_1$. Representative of 4 and 3 experiments respectively are presented.

Table 5.3 Effect of cytokines on TGFß$_1$ and PDGF B chain mRNA transcription.
Mesangial cells were exposed to 10ng/ml TGFß$_1$, PDGF, TNFα or IL-1ß for 24hr. Northern blots were carried out on extracted RNA. mRNA transcription is expressed as fold increase in densitometric units over medium alone.
MPCM-induced TGFß$_1$ and PDGF B chain transcription are included as a comparison.

Added Cytokine	TGFß mRNA	PDGF mRNA
TGFß	1.7±0.2	2.03±0.4
PDGF	2.3±0.5	0.7±0.1
TNFα	1.7±0.2	9.2±2.7
IL-1ß	3.5±0.8	13.0±3.6
MPCM	2.2±0.4	5.67±1.2

5.6 Relevance of TGFß$_1$ and PDGF-AB secretion

There is a marked increase in secretion of TGFß$_1$ and PDGF-AB in response to MPCM. The relevance of the secretion of these cytokines to the observed increases in fibronectin production in response to MPCM was determined in the next set of experiments.

Suramin (a generous gift from Bayer) is a polyanionic, anti-helminthic drug known to antagonise a variety of growth factors, including PDGF and TGFß, binding to their receptors [Coffey RJ *et al*, 1987., Betsholtz C *et al*, 1986]. The method of inhibition of receptor ligation is incompletly understood but it is presumed to involve direct binding of the drug to the growth factor itself rather than its receptor [Hosang M, 1985]. On the basis of studies carried out on bFGF it has been suggested that suramin exerts its effects by either sterically occluding the receptor binding region or inducing a conformational change in the growth factor [Eriksson *et al*, 1991]. Other studies have also reported that suramin induces microaggregation of growth factors [Middaugh CR *et al*, 1992]. Suramin was therefore used in this study as a tool to define the relevance of the autocrinal secretion of TGFß and PDGF-AB in the upregulation of fibronectin production.

5.7 PDGF and TGFß$_1$ cell binding assays

In order to confirm that suramin could antagonise the ligation of TGFß$_1$ and PDGF to their respective mesangial cell receptors, cell binding assays in the presence and absence of the drug were carried out.

5.7.1 Materials and Methods

Gluteraldehyde-fixed mesangial cells were exposed to 10ng/ml PDGF or TGFß$_1$ in the presence or absence of 150μg/ml suramin. Growth factor binding was subsequently assessed *in situ* using immunostaining as described in chapter 2 section 2.20.

5.7.2 Results

Significantly less PDGF and TGFß$_1$ was bound to mesangial cells (observed as a reduction in optical density) in the presence of suramin (Figs 5.11a and b, $p < 0.001$) confirming that the drug was indeed physically preventing growth factor:receptor binding.

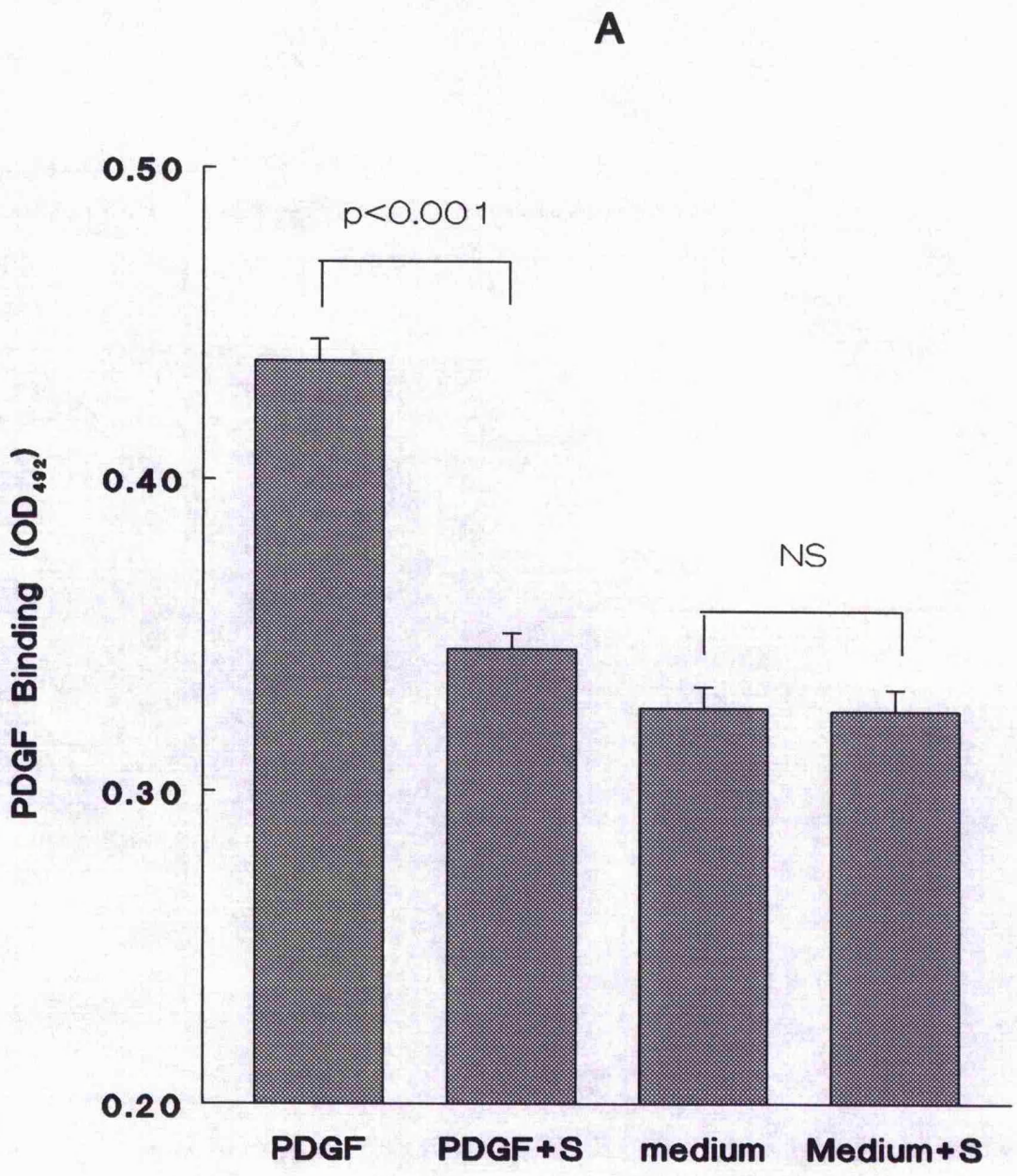


Fig 5.11a Effect of Suramin on PDGF cell binding. Mesangial cells were incubated with PDGF ± suramin. Bound PDGF was detected using goat anti-PDGF followed by an HRP-conjugated second antibody. Each condition represents 6 wells assayed in duplicate (representative of three experiments).

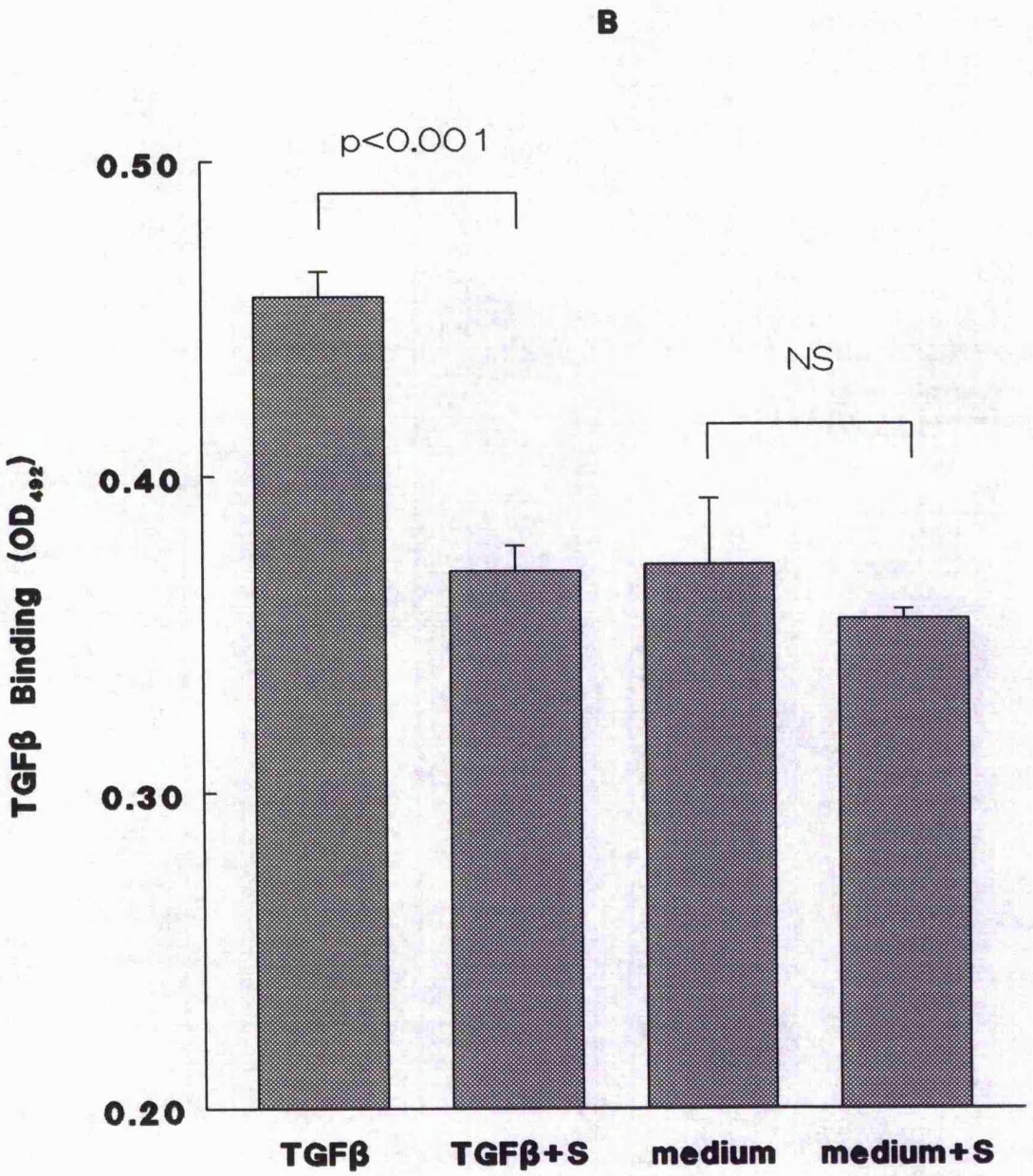


Fig 5.11b Effect of suramin on TGFß$_1$ cell binding. Mesangial cells were incubated with TGFß$_1$ ± suramin. Bound TGFß$_1$ was detected using rabbit anti-TGFß followed by an HRP-conjugated second antibody. Each condition represents 6 wells assayed in duplicate (representative of three experiments).

5.8 Effect of suramin on MPCM-mediated PDGF-AB, TGFß$_1$ and fibronectin secretion.

Having confirmed that 150μg/ml suramin could inhibit PDGF and TGFß$_1$ mesangial cell binding, the effects of this drug on MPCM-induced PDGF-AB, TGFß$_1$ and more importantly fibronectin secretion were investigated.

5.8.1 Materials and Methods

Confluent quiescent mesangial cells were exposed to 50% MPCM or to a dose range (100, 50, 20, 10, 1, 0 %) in the presence or absence of 150μg/ml suramin for 24hr. Supernatants were then assayed for TGFß$_1$, PDGF-AB and fibronectin as described in chapter 2, sections 2.19 and 2.6.

5.8.2 Results

MPCM stimulated the secretion of TGFß$_1$ and PDGF-AB in a dose dependent manner (Fig 5.12 and 5.13).

TGFß$_1$ levels in the tissue culture supernatants of mesangial cells exposed to MPCM in the presence of suramin were apparently dramatically reduced as assessed by ELISA (Fig 5.12). TGFß$_1$ levels were reduced to 36.0 $\pm$ 9% of untreated 50% MPCM (n=2). However, PDGF-AB levels, were dramatically elevated in the presence of the drug (Fig 5.13). PDGF-AB levels were increased by a further 66.4 $\pm$ 10% ($p<0.001$, n=5) over untreated 50% MPCM. Fibronectin levels under the same conditions were only marginally reduced (Fig 5.14). A summary of the effects of suramin on a 50% MPCM solution with respect to fibronectin production are summarised in table 5.5. Suramin reduced supernatant fibronectin levels by 16.2% while its effects on cell associated fibronectin were more

marked reducing levels by 40.5%. The apparent reduction in basal fibronectin production in response to suramin did not reach statistical significance.

Table 5.4 Effect of Suramin on MPCM mediated fibronectin production.
Results are means ± SEM expressed as % of MPCM or medium alone (ng fibronectin/μg cell protein).

		Control(%)	+ Suramin(%)	P value
MPCM	Supernatant	100	83.8±4.4	0.02
	Cell lysate	100	59.5±3.0	0.005
Medium	Supernatant	100	99.0±5.9	NS
	Cell lysate	100	83.6±23.0	NS

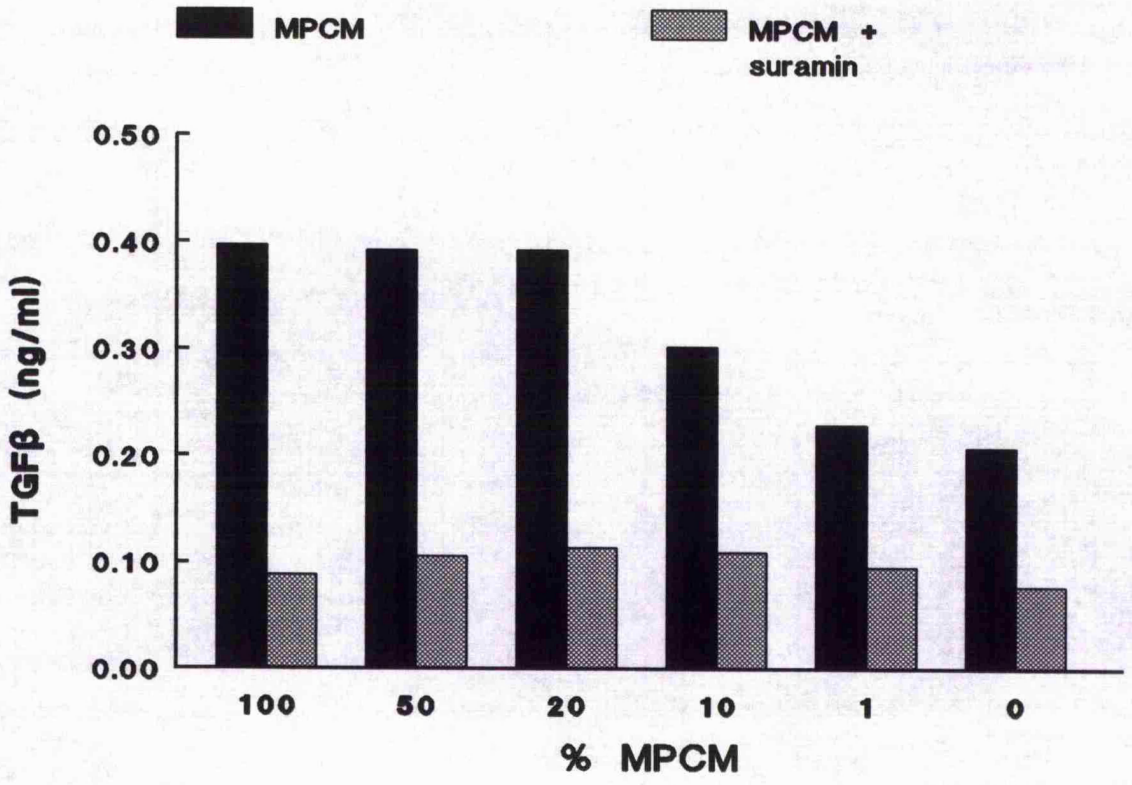


Fig 5.12 Effect of Suramin on TGFß$_1$ secretion in response to MPCM.
Mesangial cells were exposed to 100, 50, 20, 10, 1, 0% MPCM ± 150μg/ml suramin for 24hr. Supernatants were assayed for TGFß$_1$ secretion.

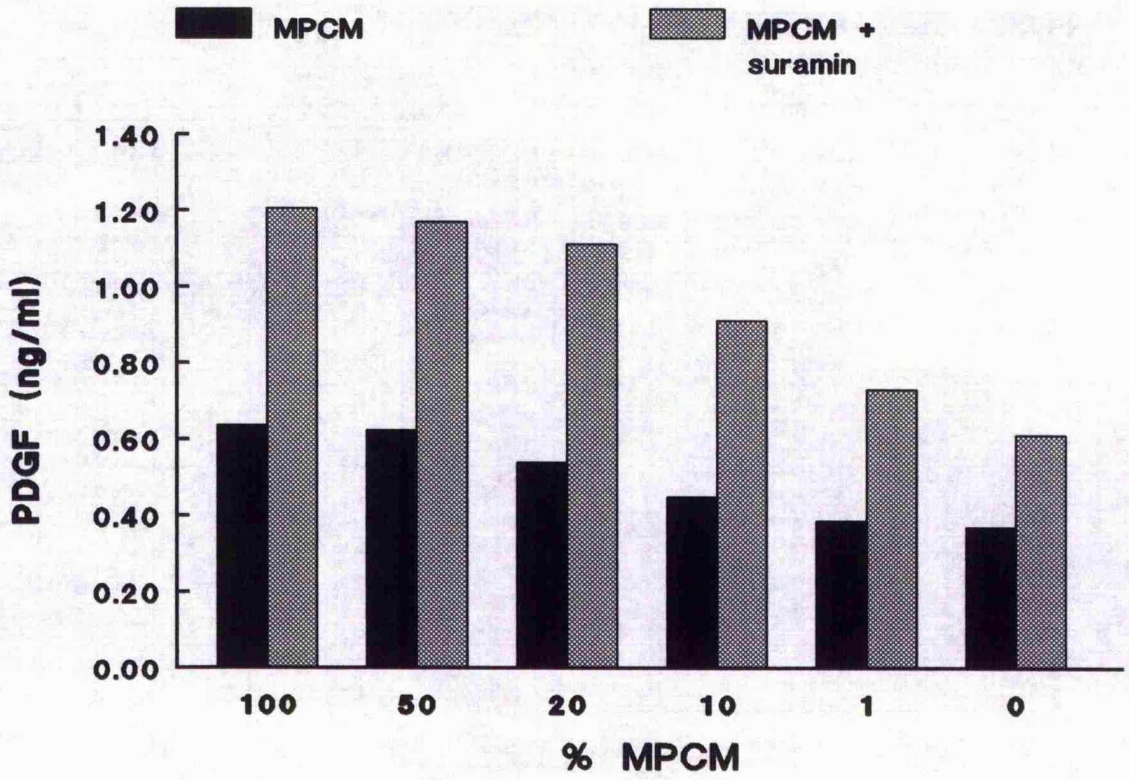


Fig 5.13 Effect of Suramin on PDGF-AB secretion in response to MPCM.
Mesangial cells were exposed to 100, 50, 20, 10, 1, 0% MPCM ± 150μg/ml suramin for 24hr. Supernatants were assayed for PDGF-AB secretion.

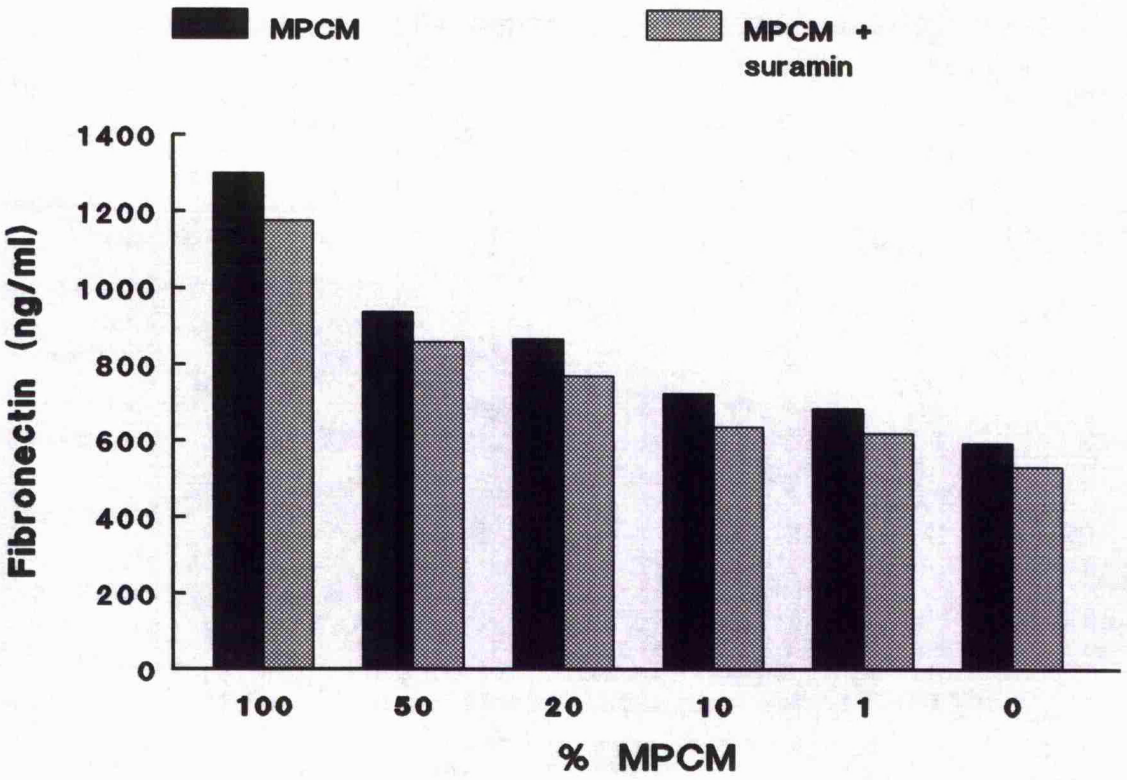


Fig 5.14 Effect of Suramin on fibronectin secretion in response to MPCM.
Mesangial cells were exposed to 100, 50, 20, 10, 1, 0% MPCM ± 150μg/ml suramin for 24hr. Supernatants were analysed for fibronectin secretion.

5.9 Effect of suramin in the TGFß and PDGF-AB ELISAs.

As suramin is able to induce conformational changes in $TGF\beta_1$ and PDGF and in so doing is able to antagonise the binding of these growth factors to their respective receptors, it is possible that such a conformational change may also affect cytokine:antibody interactions in the ELISA assays which may result in misinterpretation of the data. For this reason the effect of exogenously added suramin on the ability to detect $TGF\beta_1$ and PDGF-AB in tissue culture supernatants was examined.

5.9.1 Materials and Methods

Suramin to a final concentration of 150μg/ml was added to tissue culture supernatants from mesangial cells exposed to MPCM. The culture supernatants were then assayed for $TGF\beta_1$ and PDGF-AB and the values compared with control tissue culture supernatants which had had no suramin but an equivalent volume of HBSS added.

5.9.2 Results

Measurement or recovery of $TGF\beta_1$ was severely impaired in the presence of exogenously added suramin (3.01 $\pm$ 0.6 ng/ml detected in MPCM vs 0.65 $\pm$ 0.24 ng/ml in MPCM + suramin $p=0.02$, $n=3$). Suramin had no effect on the recovery of PDGF-AB. These data suggest that the suramin-induced changes to $TGF\beta_1$ render it, for the most part, non-recognisable by its receptor and anti-TGFß antibody.

Taken together, the suramin experiments would suggest that the drug causes marked perturbations in the $TGF\beta_1$ and PDGF-AB receptor binding and secretion resulting in small but significant effects on MPCM-mediated mesangial cell fibronectin production.

5.10 Neutralisation of putative cytokine activity.

An alternative approach to identify the putative cytokine activity in MPCM, was to expose mesangial cells to conditioned medium in combination with a panel of neutralising anti-cytokine antibodies.

5.10.1 Materials and Methods

Confluent, quiescent mesangial cells were exposed to:

a) MPCM in combination with 10μg/ml of either:

- pan specific rabbit anti-human TGFß,
- goat anti-human PDGF,
- goat anti-murine TNFα or
- goat anti-murine IL-1ß (R&D Systems)

b) MPCM in combination with 10μg/ml of each of:

- rabbit anti-TGFß,
- goat anti-PDGF,
- goat anti-TNFα and
- goat anti-IL-1ß

c) A "cocktail" of cytokines containing 2.5ng/ml of each of TGFß$_1$, PDGF, TNFα and IL-1ß in combination with 10μg/ml of each of:

- rabbit anti-TGFß,
- goat anti-PDGF,
- goat anti-TNFα and
- goat anti-IL-1ß.

Supernatants and cell lysates were assayed for fibronectin (chapter 2, section 2.6).

5.10.2 Results

Addition of anti-TGFß to mesangial cells in combination with MPCM reduced its ability to stimulate fibronectin; supernatant fibronectin levels were reduced to 72.9 ± 3.7% and cell associated fibronectin levels to 66.3 ± 6.8% of untreated MPCM (Fig 5.15). Antibodies to PDGF, TNFα or IL-1ß had no effect on either supernatant or cell associated fibronectin levels implying that these cytokines did not contribute, at least individually, to the observed effects. Increasing the antibody levels to 50μg/ml had no additional effect. There was an apparent reduction in basal supernatant fibronectin (medium + anti-TGFß) levels to 88.1% but this did not reach statistical significance. No reduction was observed in basal cell lysate fibronectin.

Exposure of mesangial cells to MPCM in combination with a "cocktail" of anti-cytokine antibodies, reduced secreted fibronectin levels to 77.9% of untreated MPCM. This reduction is comparable to that seen with anti-TGFß alone. However, the same "cocktail" of anti-cytokine antibodies was able to effectively eliminate the cytokine-stimulated fibronectin levels to 6.7% of untreated MPCM levels (Fig 5.16).

The magnitude of the reduction in fibronectin levels produced by the neutralising antibodies is reminiscent of the reduction observed following suramin treatment. These observations would suggest that these cytokines, and particularly TGFß, do play a role, albeit a minor one, in the stimulation of MPCM-induced fibronectin production in rat mesangial cells.

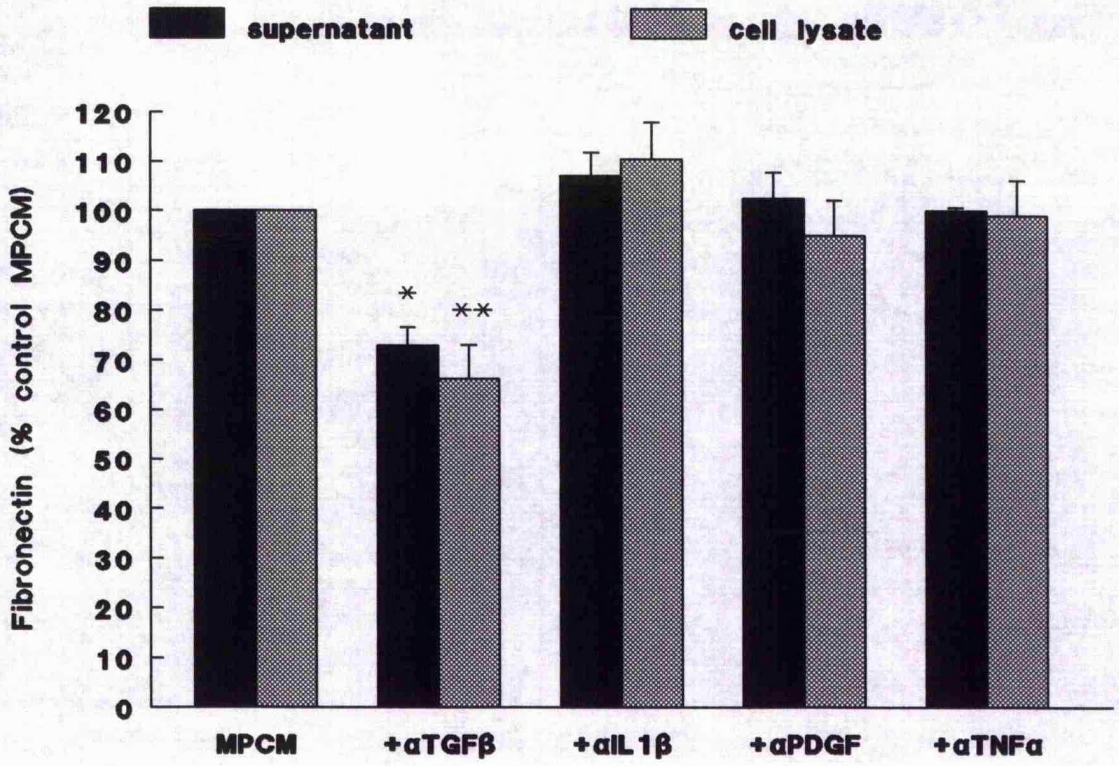


Fig 5.15 Effect of neutralising antibodies on MPCM mediated fibronectin secretion. Mesangial cells were exposed to MPCM in combination with antibodies to TGFß, PDGF, TNFα or IL-1ß. Supernatants and cell lysates were assayed for fibronectin. Values are means ± SEM (n=3-5) each carried out in quadruplicate, *$p < 0.001$, **$p = 0.003$ vs MPCM.

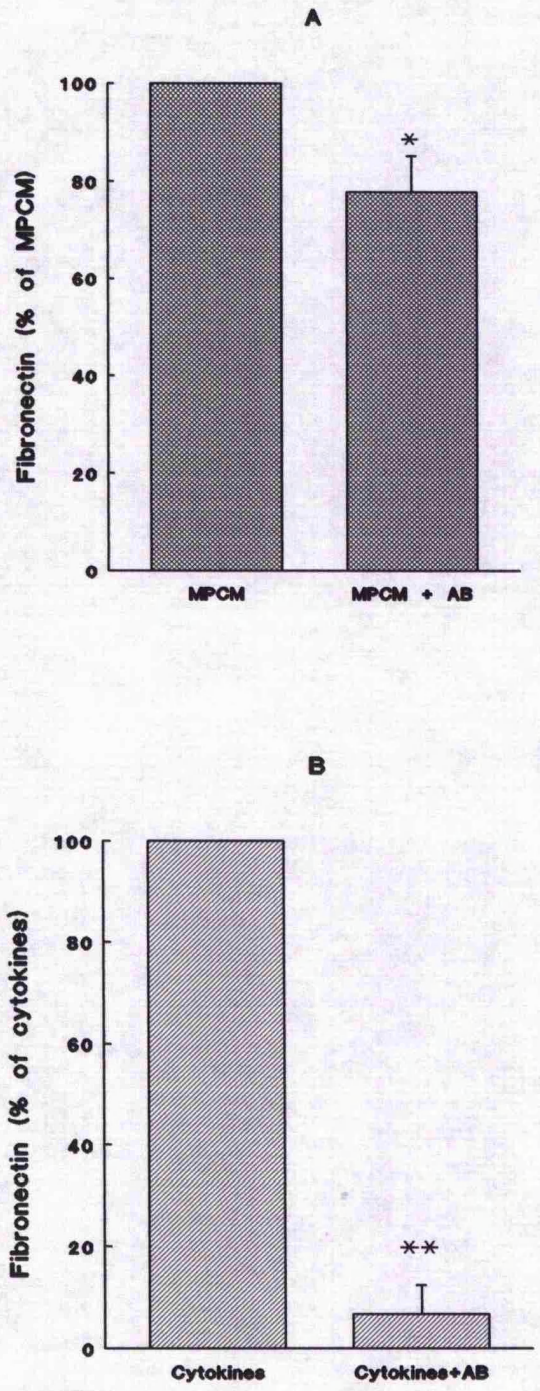


Fig 5.16 Effect of a "cocktail" of neutralising antibodies on MPCM and cytokine-mediated fibronectin production. Mesangial cells exposed to MPCM or cytokines ± a cocktail of anti-cytokine antibodies. Supernatants were assayed for fibronectin. Values are means ± SEM (n=3) each carried out in quadruplicate. *p=0.04, **p<0.001 vs MPCM.

5.11 Discussion

TGFß, PDGF, TNFα and IL-1ß have all been reported to be present at some stage in the diseased glomerulus, and are all known to be secreted by activated macrophages. The experiments in this chapter demonstrate that individually these cytokines cannot reproduce the effects of MPCM on mesangial cell fibronectin production. However, in combination (as may be found in macrophage secretions) they synergistically upregulate the production of this matrix protein. Synergistic interactions between bioactive peptides is not an uncommon phenomenon; TNFα has previously been shown to synergise with interferon γ to greatly enhance fibronectin production by human melanocytes and malignant melanoma cells [Varani J *et al*, 1989]. However, this is the first report of synergistic interactions between cytokines in glomerular mesangial cells with respect to fibronectin production. The mechanism of these synergistic interactions probably occurs at the level of transcription where the message for the fibronectin gene product is augmented over and above the levels expected for an additive effect. Whether the mechanism for the enhanced message is due to increased message stability or increased rate of transcription is yet to be investigated. The TGFß:PDGF combination did not exhibit such augmentation in fibronectin message. The mechanism of action in this case may lie at the post transcriptional or translational level. The role of metalloproteinases and their inhibitors in the synergistic accumulation of fibronectin in the cytokine combinations cannot be excluded.

Although $TGF\beta_1$, PDGF and IL-1ß could not be detected in the MPCM at the sensitivity levels of Western blotting or the commercial ELISA assays (TNFα was detected in concentrated MPCM), mesangial cells were shown to constitutvely secrete $TGF\beta_1$ and

PDGF-AB into their culture media and this secretion was upregulated in response to MPCM. Secreted TGFß$_1$ was found to be in the latent or inactive form in accord with the findings of other investigators [Kaname S *et al*, 1992].

The experiments with suramin demonstrated that the drug could induce an accumulation of secreted PDGF-AB in the tissue culture medium whilst reducing binding of the growth factor to mesangial cells. These observations concur with those of Huang and Huang [1988] who described an increased secretion of v-*sis* gene product (a protein indistinguishable from PDGF B chain) as well as inhibition of binding to the PDGF receptor in response to suramin in renal carcinoma cells. The effects of suramin on TGFß$_1$ secretion are more difficult to interpret. The drug is capable of altering the conformation of TGFß$_1$ in such a way as to reduce its binding to mesangial cell TGFß receptors and its recognition by the anti-TGFß antibody in the ELISA. This would indicate that suramin renders TGFß$_1$, largely, physiologically non-functional. These results are consistent with those previously reported regarding suramin's interactions with other protein growth factors and particularly the observations of Wade TP *et al* [1992] who suggested that the suramin-mediated reversal of TGFß-induced growth inhibition was due to inactivation of TGFß by suramin. It is clear, that regardless of the mechanism of the effects of suramin, neither inactivation of MPCM or mesangial cell derived-derived TGFß$_1$ nor an upregulation of PDGF-AB secretion has a major effect on MPCM-mediated fibronectin production in rat mesangial cells. Since the growth factors that are inhibited by suramin are primarily heparin binding proteins [La Rocca *et al*, 1990] it would suggest that the major fibronectin inducing factor(s) in MPCM does not fall into this category. It is worthy of note that the actions of suramin are not specific and are largely dependent on the polyanionic nature of

the molecule. As well as preventing growth factor interaction with cell surface receptors suramin has also been shown to inhibit a variety of enzymes non competitively, consistant with intermolecular crosslinking as an inactivating event [La Rocca RV *et al*, 1990., Nakajima M *et al*, 1991., Spigelman Z *et al*, 1987]. Some proteins have been reported to be precipitated by high doses of the drug [Town B *et al*, 1950]. Therefore, when interpreting the data one cannot exclude the possible effects of suramin on other cellular functions.

The fact that TGFß$_1$ and PDGF play a relatively minor role in MPCM mediated fibronectin production was further illustrated by the experiment wherein a "cocktail" of neutralising anti-cytokine antibodies was able to abrogate most of the cytokine-induced fibronectin production but was only able to reduce MPCM-stimulated fibronectin levels by about 22%.

When the ability of TGFß$_1$, PDGF, TNFα and IL-1ß to induce fibronectin, laminin, collagen IV, TIMP-1 and MCP-1 gene transcription was compared, it was clearly seen that of the four cytokines studied, IL-1ß exhibited properties closest to those of MPCM. However, the similarities of the effects at the mRNA level were not reflected at the level of protein translation or more precisely protein secretion; IL-1ß alone could not induce high levels of fibronectin secretion. Furthermore, it could not be detected in MPCM or in tissue culture supernatants and neutralising antibodies could not abrogate MPCM-induced accumulation of fibronectin. Although IL-1ß has been shown to upregulate collagen production [Torbohm I *et al,* 1989] and to modulate matrix remodelling via its activities on metalloproteinases and TIMPs [Shingu M *et al,* 1993], these data would support the proposition of Atkins' group that IL-1 mediates renal fibrosis through injury rather than

via direct stimulation of extracellular matrix synthesis [Nikolic-Paterson DJ *et al,* 1996]. This proposition was based on the observations that IL-1 receptor antagonist (IL-1ra) treatment of rats with anti-GBM disease inhibited renal fibrosis largely by inhibition of macrophage-mediated tissue injury [Lan HY *et al*, 1993., Nikolic-Paterson DJ *et al*, 1994] and that IL-1ra treatment of anti-Thy-1 nephritic rats reduced mesangial cell proliferation but did not abrogate deposition of the matrix proteins laminin and fibronectin [Nikolic-Paterson DJ *et al*, 1996]. It is however, surprising that IL-1ß, with its high profile association with macrophages, was not detected in MPCM. Cytokine mRNA levels following LPS stimulation are highly regulated. Different LPS doses exhibit variable patterns of cytokine response. These responses are both time and dose dependent are not necessarily associated with the secretion of protein. In LPS stimulated human macrophages for example, secretion of IL-1α and ß is hardly detected although their gene activation is markedly upregulated [Zhong WW *et al*, 1993]. This may be an explanation for the lack of detectablity of IL-1ß in this system. On the other hand, other authors have suggested that non-macrophage derived IL-1 may be antigenically different from macrophage-derived IL-1 [Dinarello CA, 1985]. This may also explain why IL-1ß was not detected in MPCM with antibodies raised against recombinant murine IL-1ß.

In the current study TNFα also induced effects comparable to those of MPCM on mesangial cells at the message level, and like IL-1ß, was unable, independently, to reproduce the effects of MPCM. As with anti-IL-1ß, anti-TNFα antibodies were unable to neutralise the effects of MPCM on fibronectin production. In experimental animals, manouevres to antagonise the effects of TNFα using neutralising antibodies or recombinant soluble receptors have been shown to reduce the degree of histological damage in nephrotoxic nephritis [Mulligan MS *et al*, 1993] and in anti-Thy-1 nephritis [Pan CG *et*

al, 1993]. However, as with IL-1ß, the effects of TNFα on renal fibrosis are probably mediated via tissue injury rather than by any direct pro-fibrogenic activity.

In this thesis induction of message in response to a particular stimulus was analysed after 24 hours of stimulation. Message can obviously be induced much earlier and transiently so that by 24 hours mRNA levels may have returned to baseline. An example of this in the current study was the observation that PDGF appeared to have no effect on MCP-1 mRNA at the 24 hour time point. However, Goppelt-Strube and Stroebel [1995] demonstrated that MCP-1 message in response to PDGF reached a maximum between 2 and 4 hours and returned to baseline by 6 to 8 hours. The mRNA data at 24 hours serves only as a point of comparison between the effects of MPCM and other cytokines and is not meant as an absolute measure of the effects of a particlar cytokine on the expression of specific mRNA.

The effects of MPCM are clearly very complex, involving $TGFß_1$ and PDGF secretion. The contribution of these secreted growth factors to cultured mesangial cell fibronectin production appear relatively small in comparison to the overall effects of MPCM *per se*. However, *in vivo*, these cytokines could also exert paracrine effects on other local glomerular cells inducing matrix production. For example, mesangial cell $TGFß_1$ could paracrinally stimulate glomerular epithelial cells to produce fibronectin, laminin and collagen IV [Nakamura T *et al*, 1992], whilst autocrinally stimulating mesangial cells to synthesise proteoglycans [Border WA *et al*, 1990a].

The data so far indicate that although $TGFß_1$, PDGF, TNFα and IL-1ß play a role in matrix deposition, another, as yet undefined, factor(s) is responsible for the majority of the observed increase in MPCM-mediated fibronectin production by rat mesangial cells.

CHAPTER SIX

Characterisation of MPCM III

6.1 Introduction

The cytokines TGFß, PDGF, TNFα and IL-1ß are known to be involved in the progression of glomerular disease. This study has confirmed that these cytokines have the potential to contribute to the sclerotic process. However, none of these cytokines are able, at least individually, to reproduce the effects of MPCM. Despite the fact that combinations of these cytokines are able to induce synergistic upregulation of fibronectin, induction of this matrix protein cannot be abrogated using neutralising antibodies. Characterisation studies thus far have not been able to identify the fibronectin inducing factor(s) responsible for the observed effect of MPCM.

The aim of this chapter therefore, was to try and shed more light on the component(s) of MPCM responsible for inducing fibronectin in mesangial cells.

6.2 Fractionation of MPCM

In order to define the molecular weight of the biologically active component of MPCM more precisely, the resolution of MPCM fractionation was improved. To this end a superose 12 gel filtration column with a resolution range of $1x10^3$ - $1x10^5$ D was employed.

6.2.1 Materials and methods

15ml aliquots of MPCM were concentrated 20-30 fold using Centriprep 3 concentrators. 200μl of the concentrate was fractionated on a superose 12 column by HPLC. The column had been previously calibrated with αamylase (200kD), BSA (66kD), carbonic anhydrase (29kD), cytochrome oxidase (12.4kD) and aprotinin (6.5kD). The fractions were sterile filtered using 13mm, 0.2μm acrodiscs (Gelman Science). 250μl of each fraction were

added to confluent, quiescent mesangial cells and made up to 1ml with medium such the final concentration of FCS remained at 0.5% (section 2, chapter 2.10). After 7 days the supernatants were assayed for fibronectin.

6.2.2 Results

The major peak of fibronectin stimulating activity eluted in fractions 37, 38 and 39 with fraction 38 containing the highest activity (Fig 6.1a). A shoulder of activity to the leading edge of the main peak was again observed. As with the superose 6 column the major peak of biological activity did not co-elute with any of the major protein peaks of MPCM as determined by the A_{280} protein elution profile (Fig 6.1b). However, it did co-elute almost exactly in the same fractions as the 12.4 kD molecular weight marker cytochrome C (Fig 6.1c).

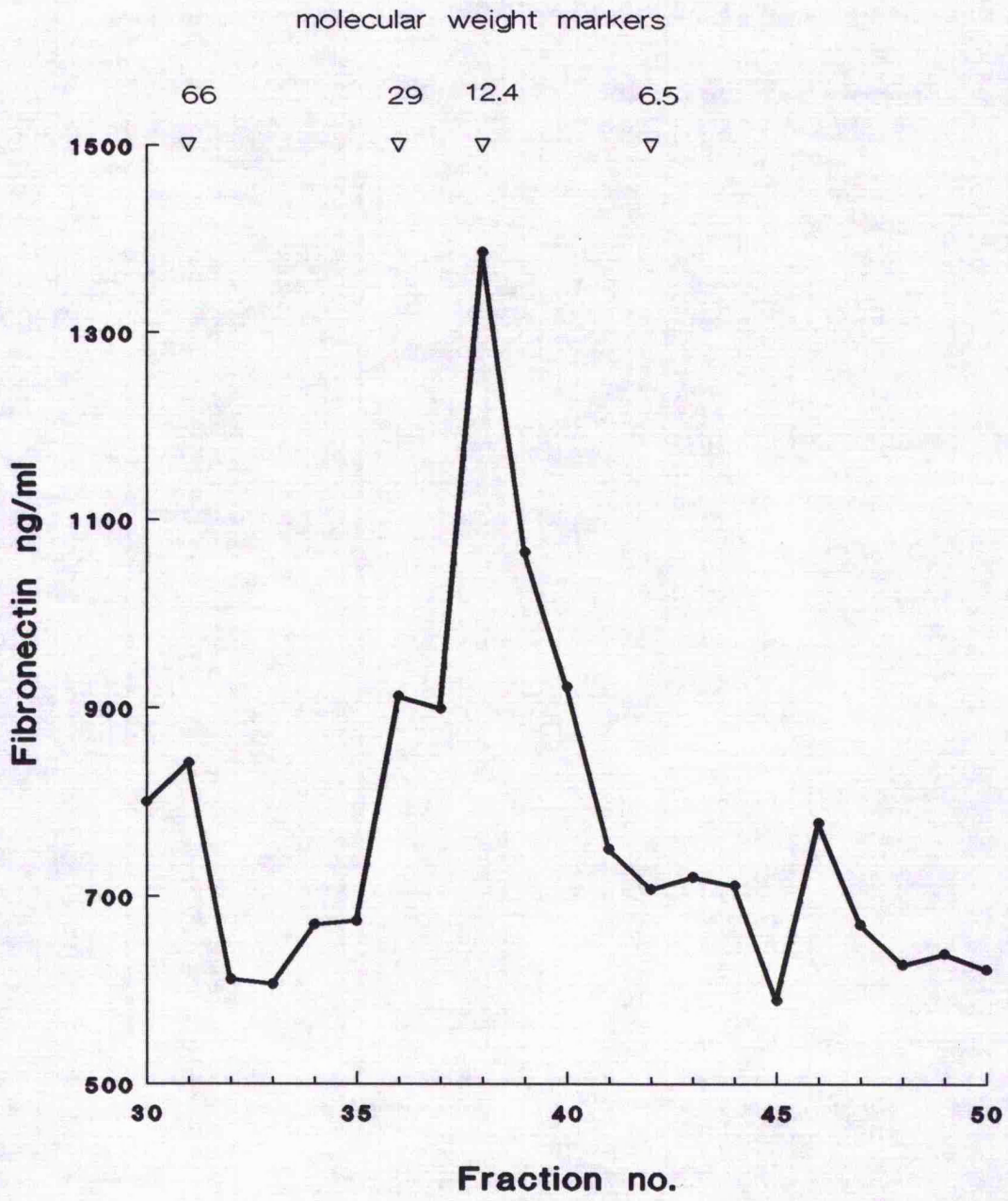


Fig 6.1a Fractionation of MPCM by Superose 12 gel filtration.

Mesangial cells were exposed to column fractions 30-50. After 7 days of culture supernatants were assayed for fibronectin. A representative experiment from three runs is presented.

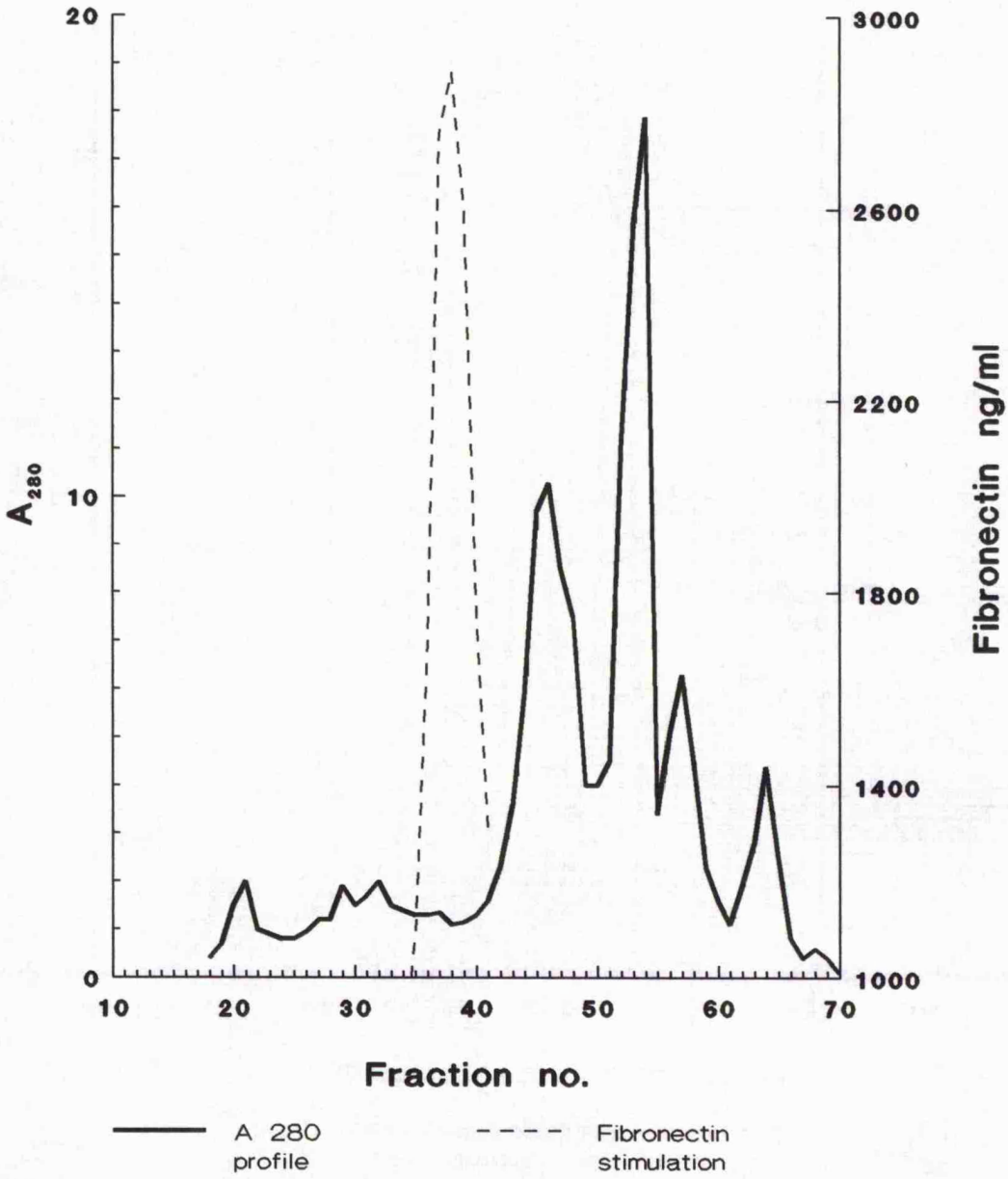


Fig 6.1b Fractionation of MPCM by Superose 12 gel filtration.

Position of fibronectin stimulating activity (---) superimposed on the A_{280} protein profile of MPCM (—).

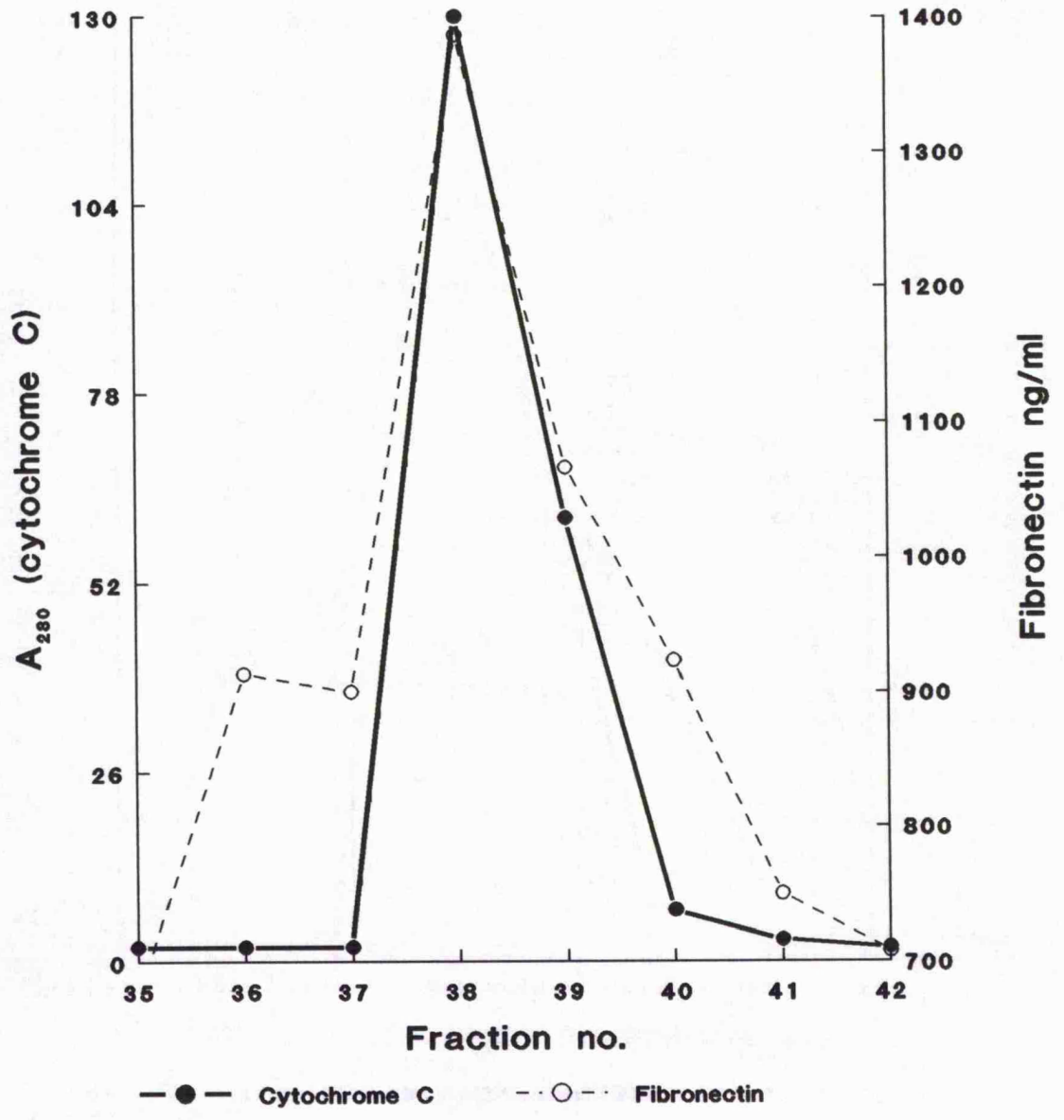


Fig 6.1c Fractionation of MPCM by Superose 12 gel filtration.

Position of fibronectin stimulating activity (---) superimposed on the elution profile of 12.4kD cytochrome C molecular weight marker (—).

6.3 Secretion of TGFß$_1$ and PDGF-AB induced by MPCM fractions.

Experiments in chapter 5 demonstrated that MPCM was able to upregulate TGFß$_1$ and PDGF-AB secretion. Secretion of these growth factors appears to play a minor but significant role in the upregulation of fibronectin production. The following experiment aimed to investigate whether the upregulation of secretory activity can be attributed to a particular fraction of MPCM.

6.3.1 Materials and Methods

Tissue culture supernatants from mesangial cells which had been exposed to MPCM fractions 35-42 (ie those fractions containing the fibronectin stimulating activity) were assayed for TGFß$_1$ and PDGF-AB as described in chapter 2, section 2.19.

6.3.2 Results

The ability to stimulate TGFß$_1$ was found in all the fractions containing fibronectin stimulating activity. The peak of TGFß$_1$ stimulating activity however, coincided with fraction 39 - one fraction to the right of the main fibronectin stimulating fraction and corresponding to a molecular weight of $\approx$11.7kD (Fig 6.2). PDGF-AB stimulating activity was also found in the all the fractions tested. However, the PDGF-AB profile differed from that of TGFß$_1$ in that two additional peaks of PDGF-AB stimulating activity were found. These peaks ocurred in fraction 36 - two fractions to the left of the main fibronectin peak and corresponding to a molecular weight of $\approx$24.7 (cf TGFß which has a molecular weight of 25kD) with a smaller peak in fraction 41 - corresponding to a molecular weight of $\approx$7kD (Fig 6.3). These data suggest that the factor(s) in MPCM which stimulates the upregulation of fibronectin is also able to stimulate TGFß$_1$ and PDGF-AB secretion.

factors in MPCM which can stimulate TGFß$_1$ and particularly PDGF-AB secretion independently of fibronectin.

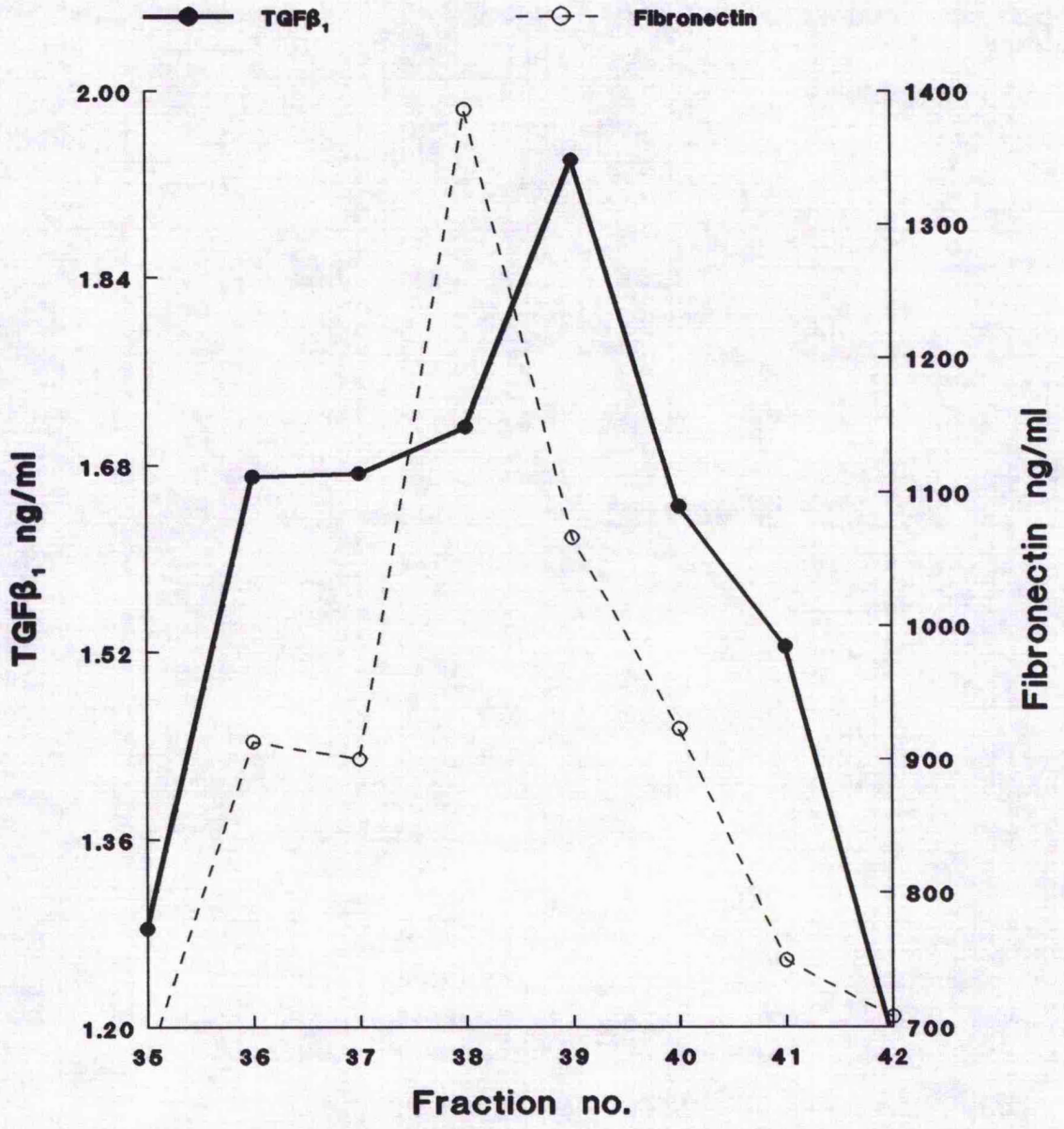


Fig 6.2 TGFß$_1$ secretion by mesangial cells in response to column fractions.
Tissue culture supernatants from mesangial cells exposed to column fractions were assayed for TGFß$_1$. The TGFß$_1$ stimulating activity (—) is superimposed on the corresponding fibronectin stimulating activity (---).

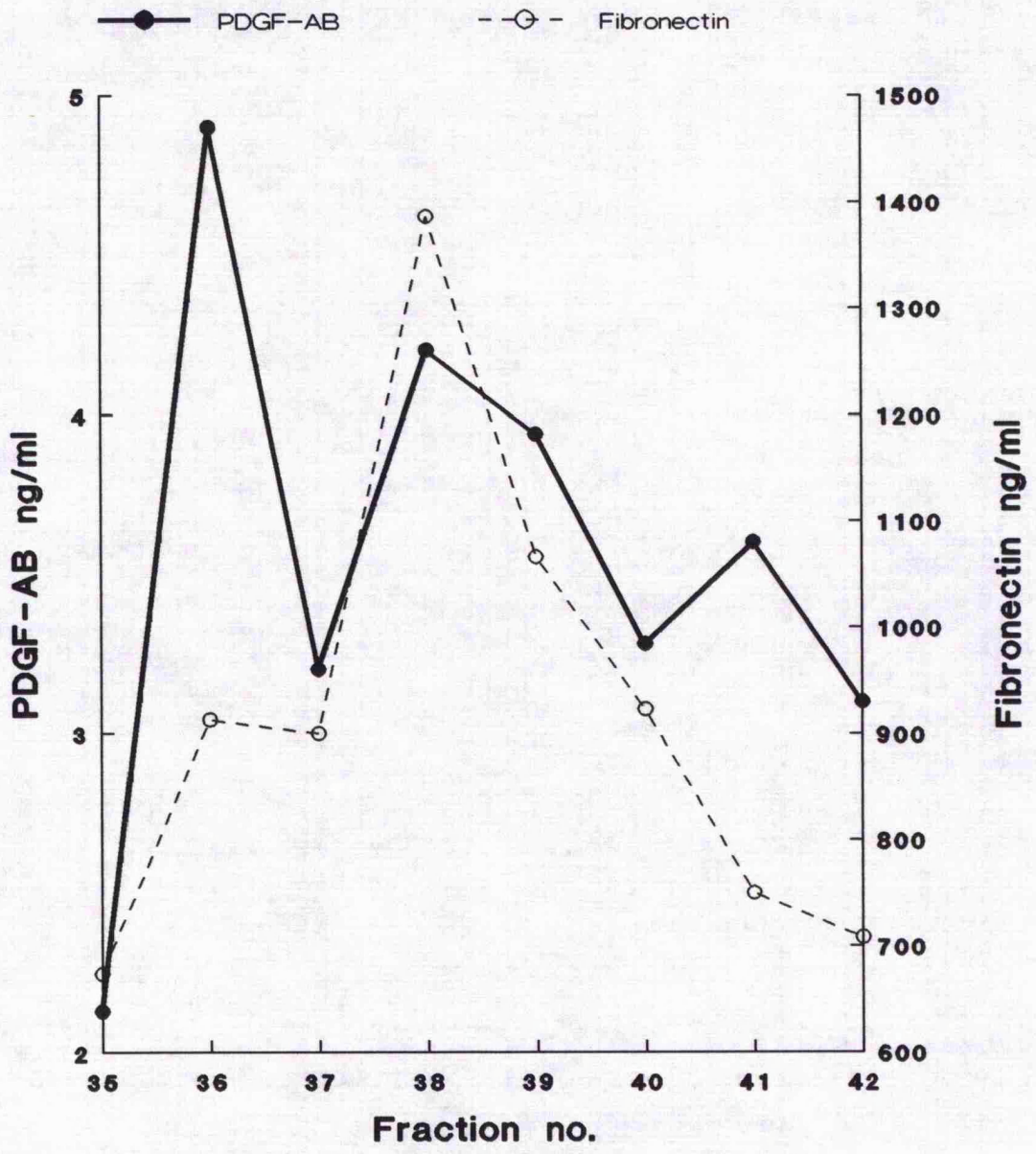


Fig 6.3 PDGF-AB secretion by mesangial cells in response to column fractions. Tissue culture supernatants from mesangial cells exposed to column fractions were assayed for PDGF-AB. The PDGF-AB secretion profile (—) is superimposed on the corresponding fibronectin secretion profile (---).

6.4 Metabolic labelling of MPCM

To date, the fibronectin inducing factor(s) in MPCM has not been detected by any means other than by measurement of its fibronectin stimulating activity. Biological assays are known to be up to 100 fold more sensitive than chemical detection of protein by electrophoresis and silver staining. In order to be able to "visualise" this factor(s) in MPCM, it was metabolically labelled with ^{35}S-methionine. The rationale being that a factor present in very small amounts may be monitored or detected more easily when radiolabelled, particularly during sequential concentration and purification steps.

6.4.1 Materials and Methods

MPCM was metabolically labelled with ^{35}S-methionine during the LPS stimulation stage (chapter 2, section 2.18). The ^{35}S-MPCM was concentrated, fractionated by SDS-PAGE and autoradiographed to obtain a profile of the radioactively labelled components present in MPCM.

Concentrated ^{35}S-MPCM was fractionated on the superose 12 HPLC column. A 200μl aliquot of each fraction was counted on a scintillation counter. Fractions 34-40 were then further fractionated by SDS-PAGE on a 12.5% gel. The gel was dried and autoradiographed for 6 weeks.

6.4.2 Results

Fig 6.4 shows an autoradiograph of a gel following SDS-PAGE of ^{35}S-MPCM. It illustrates that MPCM is made up of a complex mixture of many components which can readily be labelled with ^{35}S-methionine.

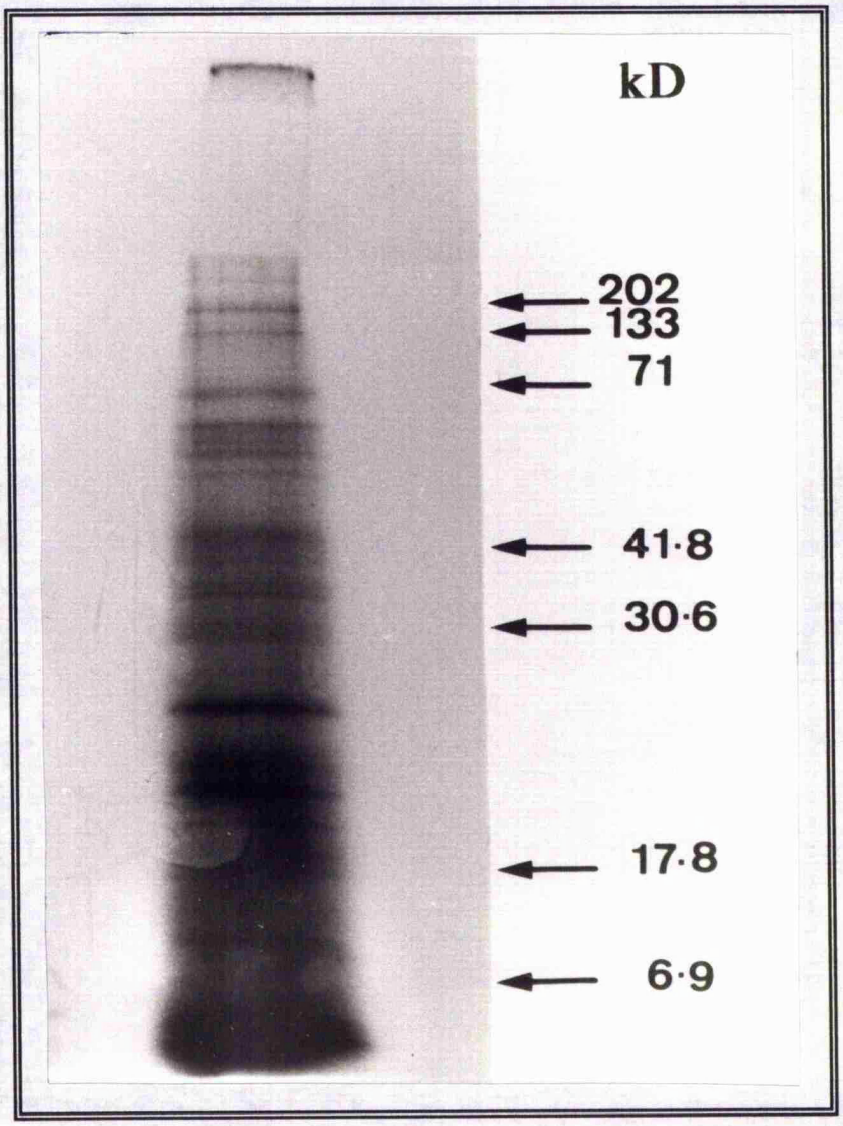


Fig 6.4 Autoradiograph of biosynthetically labelled ^{35}S-MPCM, fractionated by SDS-PAGE. Concentrated ^{35}S-MPCM was fractionated by SDS-PAGE (%T=12.5).

The radioactive profile of ^{35}S-MPCM fractionated on a superose 12 column is shown in Fig 6.5. It can be seen that two major peaks were isolated corresponding to molecular weights of around 6.5kD and 1.2kD. No peaks of activity were detected in the fractions containing the fibronectin stimulating activity.

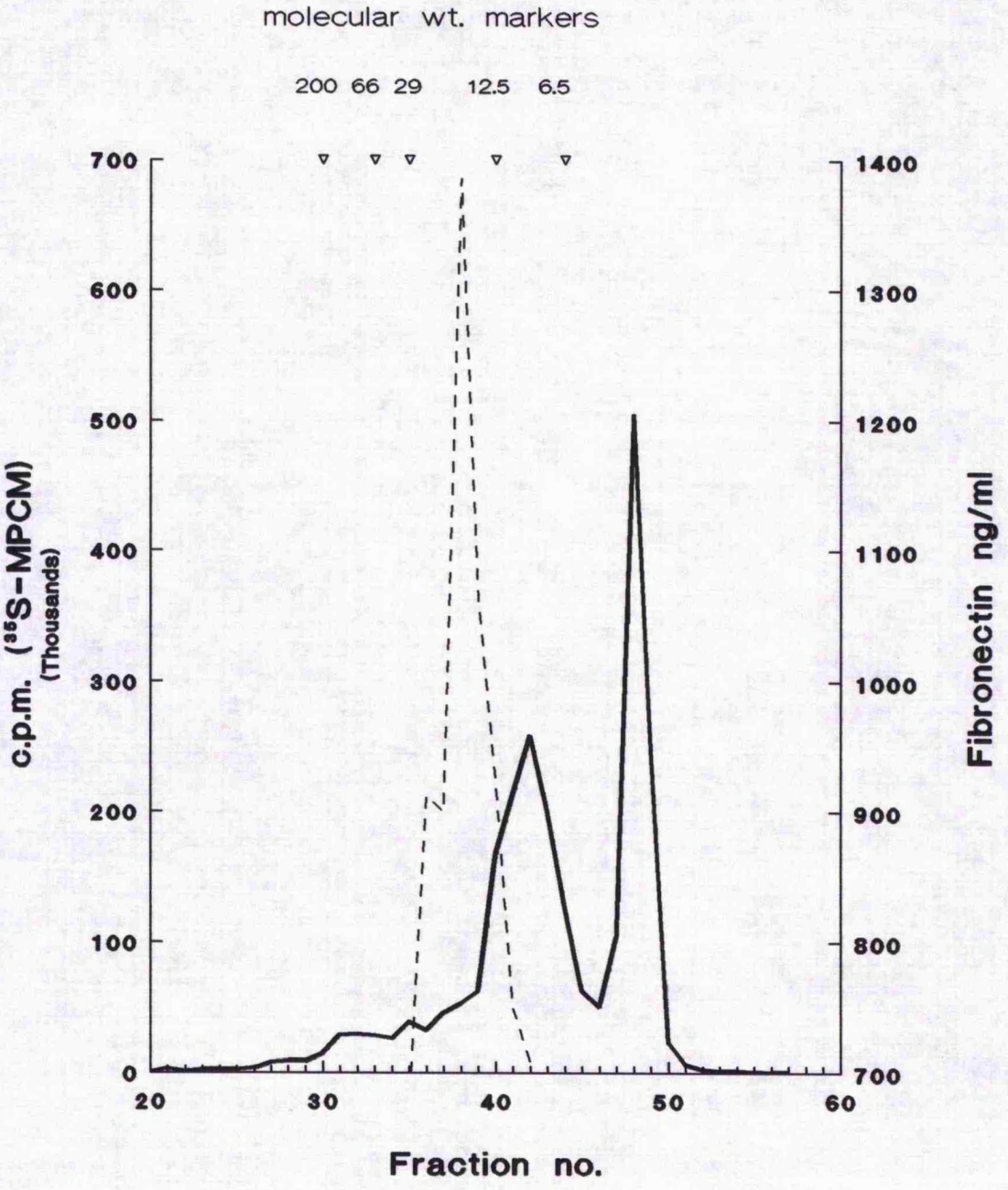


Fig 6.5 Superose 12 HPLC fractionation of ^{35}S-MPCM. The radioactive profile of MPCM fractions (—) is shown superimposed on the position of the fibronectin stimulating activity (---). A representative experiment of 2 runs is presented.

Autoradiography of the gel following SDS-PAGE of fractions 34-40 showed a single faint band in fractions 36, 37 and 38 (Fig 6.6). Densitometric analysis of these bands showed that the band in fraction 37 was the most intense (0.162, 0.277, 0.067 arbitary densitometric units for fractions 36-38 respectively). Fraction 37 by gel filtration corresponds to a molecular weight of ≈ 19.2kD. The position of the bands identified in fractions 36-38 on the 12.5% gel suggest a molecular weight of ≈ 26.3kD - higher than predicted by gel filtration chromatography. These data would suggest that the protein detected on the gel may not correspond with the fibronectin stimulating factor that is being sought.

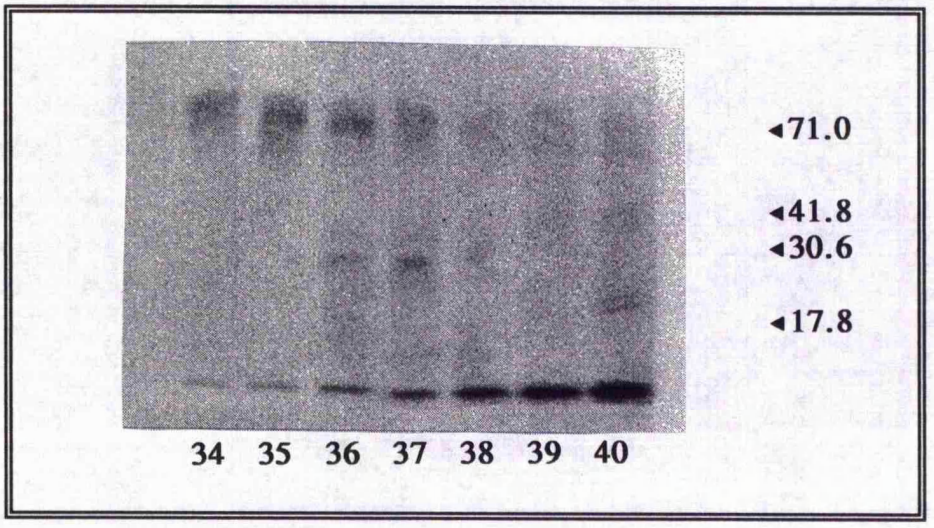


Fig 6.6 Autoradiograph of SDS-PAGE fractionation of superose 12 column fractions. Superose 12 column fractions 34-40 of ^{35}S-MPCM were fractionated on a 12.5% SDS polyacrylamide gel. The gel was dried and autoradiographed.

6.5 Discussion

The higher resolution of the superose 12 column has narrowed the molecular weight of the major fibronectin inducing component to an apparent molecular weight of about 12.5 kD with a shoulder of activity corresponding to a molecular weight of 25kD.

Biosynthetic labelling of the macrophage secretory products has again demonstrated that MPCM is composed of a complex mixture of different molecules. However, the major labelled products which elute off the superose 12 column have apparent molecular weights of 6.5 and 1.2kD.

Autoradiography of superose 12 fractionated and SDS-PAGE fractionated MPCM has shown up one band in three fractions, the most intense of which does not appear to coincide with the peak fraction of fibronectin stimulating activity (one fraction out) and having a higher molecular weight than predicted by gel filtration. These data would indicate that the protein detected on the gel may not correspond with the fibronectin stimulating factor of fraction 38 that is being sought.

Occasionally the physical properties of a polypeptide are such that its molecular weight cannot always be determined using certain chromatographic techniques. TGFß is one such example. When transforming growth factors were first being isolated and characterised platelet derived TGFß was found to have an apparent molecular weight of 15-18kD [Assoian RK *et al*, 1983] whilst placental TGFß eluted at an apparent molecular weight of 3-5kD [Frolick CA *et al*, 1983]. Other workers found that TGFα and TGFß could co-elute together despite their molecular weights being 6 and 25kD respectively [Anzano MA *et al*, 1983]. It is possible that similar physical properties may apply to the biologically active component(s) of MPCM therefore giving rise to erroneous molecular weight determinations. It is also possible that the active component of fraction 38 (in terms of

fibronectin stimulating activity) does not contain many methionine residues; TGFß for example, only contains 2 methionine residues/mol, the least frequently occuring amino acid in this molecule [Assoian RK *et al*, 1983]. Therefore the use of a different labelled amino acid would have to be considered.

Further experiments to define the nature of the fibronectin inducing component(s) of MPCM are beyond the scope of this thesis. However, possible further approaches for its analysis will be addressed in the general discussion.

CHAPTER SEVEN

General Discussion

7.1 General discussion

Matrix deposition and hypercellularity are the histological hallmarks of glomerulosclerosis [Klahr S *et al,* 1988]. The aetiology of glomerulosclerosis is multifactorial and could result from a combination of several processes which individually may represent an adaptive or perhaps even a beneficial response to injury. Notwithstanding, progression of chronic renal scarring is an inevitable consequence once sufficient renal damage has occurred [Holvey J and Hayslet JP, 1986]. One of the initial events that occurs during progression of renal injury to glomerulosclerosis in many experimental and human clinical disorders is the influx of mononuclear cells into the glomeruli (and the tubulointerstitium), although the precise cellular and molecular mechanisms governing this process are yet to be fully understood. Since macrophages are a rich source of many regulatory molecules and cytokines it is conceivable that the factors elaborated by these infiltrating cells could alter the phenotypic expression of resident glomerular cells with respect to their mitogenic and pro-fibrogenic capacities. Macrophages may additionally alter the cell biology of resident glomerular cells such that they then act as effector cells secreting other peptide growth factors which act in both autocrine and paracrine fashion, exacerbating the initial injury and initiating the sclerotic process.

It is now well appreciated that cytokines are important mediators in macrophage associated glomerular injury; indeed there is a wealth of literature describing the effects of individual cytokines and growth factors, on the upregulation of matrix gene transcription or protein secretion or induction of cell proliferation both *in vivo* and *in vitro*. It has been claimed that some cytokines such as TGFß and PDGF have satified "Koch's Postulate" with regard to their role in the development of glomerulosclerosis [Koch R, 1882]. The criteria

establishing a role for a particular cytokine in mediating a specific biological effect include the demonstration that:

a) the cytokine exhibits the desired effect on target cells *in vitro*,

b) the cytokine is secreted or expressed in disease states,

c) inhibition of the cytokine *in vivo* abbrogates the proposed effect of the cytokine in disease and

d) administration of the cytokine *in vivo* (or overexpression in transgenic animals) reproduces the biological effect.

However, it is unlikely that a single cytokine acting alone will be solely responsible for the progression of glomerulosclerosis, given the potential complex network of autocrine loops and paracrine pathways. To date, the majority of the evidence supporting a role for macrophage derived cytokines in glomerular disease has been correlative.

The work presented in this thesis goes some way to provide direct evidence for the role of macrophage derived factors contributing to the progression of glomerulosclerosis.

This study has demonstrated that MPCM can cause cultured mesangial cells to secrete the matrix protein fibronectin in a dose and time dependent manner independently of cell proliferation. In the system used mesangial cell proliferation was reversibly suppressed in response to MPCM. The degree of cell proliferation observed in response to a particular stimulus will be dependent on a number of parameters including cell density, state of cellular activation/quiescence, concentration of serum and the presence of other factors.

An important point to note is the fact that matrix production can, at least in part, be dissociated from cell proliferation, an observation that concurs with the findings of others [Eng *et al,* 1994., Zhu L *et al*, 1995., Groggel GC and Hughes ML, 1995] prior to which

the two processes were considered inextricably linked [Striker LJ et al, 1989].

Biosynthetic labelling studies together with Northern blot analyses have shown that the MPCM-mediated accumulation of fibronectin was due, at least in part, to increased gene transcription and protein synthesis. In addition, the genes for α1 (IV) collagen and laminin B1 chain were also found to be upregulated in response to MPCM. Although the exact composition varies among diseases, the extracellular matrix in the sclerosing mesangial areas generally contains these three matrix proteins.

Matrix production in general is a balance between the processes of synthesis and degradation. Matrix accumulation therefore occurs as a consequence of the net synthesis and/or inhibition of degradation of a given protein. In the current study, as well as stimulating matrix synthesis, MPCM was found to augment the gene transcription of TIMP-1, thus predicting that, if message was translated and exceeded the production of matrix metalloproteinases such as transin/stromelysin, accumulation of matrix via inhibition of degradation may also occur.

One of the primary stimuli initiating macrophage infiltration into the glomerulus is the secretion, by injured glomerular cells, of chemotactic factors such as the chemokine MCP-1. Expression of MCP-1 message was found to be upregulated by mesangial cells in response to MPCM. If MCP-1 message is translated then this would suggest that not only do injured or activated mesangial cells stimulate an influx of macrophages into the glomerulus but that once present, the macrophages themselves may mediate a further influx by stimulating mesangial cells to release chemokines. Thus a positive feed back loop is initiated. This observation not only supports but further elaborates on the findings of other

investigators who demonstrated that IL-1ß and TNFα could stimulate mesangial cells to express MCP-1 [Largen PJ *et al,* 1995., Zoja C *et al,* 1991., Rovin BH *et al,* 1992] thereby supporting a role for mesangial cells in macrophage recruitment and activation. A positive feed back loop such as this would have to be counter-regulated if macrophage accumulation with concomitant secretion of pro-fibrogenic, pro-inflammatory and mitogenic factors is not to get out of control. Investigations by others have demonstrated that both IL-10 and TGFß may play a counter-regulatory role by suppressing the secretion of macrophage-derived cytokines. The data in this thesis have further indicated that fibronectin stimulating factors are suppressed by $TGF\beta_1$. TGFß therefore, not only attracts monocytes and stimulates them to produce other cytokines but also induces macrophage deactivation, an event which provides an essential counter-regulatory mechanism during the course of an inflammatory response [Roberts AB *et al,* 1990a].

The cytokines most often cited in the literature to be associated with glomerular damage were unable, individually, to reproduce the potent effects of MPCM with respect to fibronectin production (chapter 5). Of the four cytokines studied, IL-1ß had the most similar characteristics to MPCM with respect to its effect on mesangial cell mRNA upregulation for extracellular matrix proteins and cytokines. However, the effects of IL-1ß were dissimilar to MPCM at the level of translation, when matrix or cytokine protein production was measured. This latter observation emphasises that conclusions cannot always be extrapolated from findings at the molecular level and stresses the importance of being able to detect the protein.

The concentration of cytokines used in this thesis was 10ng/ml. This dose falls in the range

most commonly used in tissue culture. However, the effects of certain cytokines are mediated at different doses in different cells. For example TGFß stimulates the synthesis of the 72kD type IV collagenase at concentrations greater than 1ng/ml whilst at lower concentrations (0.1-1ng/ml) TIMP-1 synthesis predominates [Marti HP *et al*, 1994]. Similarly the effect of TGFß on proliferation has been shown to be bifunctional depending on the concentration and cell line used [MacKay K *et al*, 1989]. It is therefore possible that the optimal dose for a particular effect by a particular cytokine was not used.
Nevertheless, this dose was appropriate for cytokine combinations which were able to synergistically upregulate the production of fibronectin to levels comparable with those observed with MPCM. Synergy between cytokines is a common phenomenon, although this is the first report of such interactions in the upregulation of fibronectin production in mesangial cells. The mechanism of the synergistic interactions probably occurs at the level of transcription with the augmentation of the message for fibronectin. The precise regulatory mechanisms for this phenomenon however, await further investigation. The observed synergistic interactions again illustrate the flaw in looking at one cytokine in isolation as interactions with other cytokines are missed and the role of one cytokine may possibly be misinterpreted.

Macrophages have been shown to have very high steady-state levels of TGFß mRNA even in the unactivated state but have been shown to secrete the protein only once the cells have been activated [Assoian RK *et al*, 1987]. The relevance of these findings to renal pathology have been demonstrated by Ding G *et al* [1994] who showed that glomerular macrophages isolated from PAN nephritic or dietary-hypercholesterolaemic rats stained positively for TGFß whereas macrophages from normal animals were negative. In this thesis, however,

neither TGFß$_1$, PDGF, TNFα nor IL-1ß could be detected in standard MPCM using conventional western blotting and cytokine ELISA techniques, possibly as a result of the limits of sensitivity of these assays.

TGFß$_1$ and PDGF-AB were, however, shown to be secreted in large amounts by mesangial cells and secretion was increased in response to MPCM. The actions of TGFß and PDGF are often described as being interelated particularly with respect to cell proliferation. Haberstroh U *et al,* [1993] have demonstrated that TGFß-stimulated mesangial cell proliferation was associated with increased PDGF ß receptor and PDGF-BB expression and it is the activation of this autocrine loop which is responsible for stimulating DNA synthesis. Similar observations have been noted in fibroblasts [Gronwald RGK *et al,* 1989., Soma Y and Grotendorst GR, 1989] and smooth muscle cells [Battegay EJ *et al,* 1990]. In fact, it has been suggested that the mitogenic effects of a number of peptide mitogens are mediated via the production and subsequent actions of PDGF [Silver BJ *et al,* 1989]. In contrast, in human proximal tubular cells, a converse relationship between TGFß and PDGF has been described [Phillips AO *et al,* 1995]. In these cells PDGF is involved in modulating glucose mediated TGFß synthesis at both the levels of transcription and translation.

In the current study suramin was shown to significantly interfere with TGFß$_1$ and PDGF receptor binding; it caused such conformational changes in TGFß$_1$ as to render it largely unrecognisable by an anti-TGFß antibody. The consequential effects of suramin on TGFß$_1$ and PDGF were to cause only a small, though significant, reduction in rat mesangial cell fibronectin production. Similarly, treatment with anti-TGFß neutralising antibodies was

able to induce only a small reduction in fibronectin production. In further experiments a "cocktail" of neutralising anti-cytokine antibodies was able to abrogate only 22% of MPCM stimulated fibronectin production. This reduction was no greater than that seen with anti-TGFß alone. This was despite the fact that the antibody "cocktail" abolished the fibronectin that was stimulated by the corresponding cytokine combination.

Although this study has shown that $TGF\beta_1$ only has a small effect upon rat mesangial cell fibronectin production, an observation that concurs with the findings of others [Border *WA et al,* 1990a], TGFß clearly does play an important role in fibrogenesis. Treatment of anti-Thy-1 nephritic rats with anti-TGFß or the proteoglycan decorin has been shown to ameliorate renal injury and reduce the degree of histological damage [Border WA *et al*, 1990b., Border WA *et al,* 1992a]. Most recently Border's group has demonstrated that gene transfer of decorin cDNA into skeletal muscle results in a significant reduction in levels of glomerular TGFß mRNA and protein, matrix deposition and proteinuria in nephritic rats [Isaka Y *et al*, 1996].

In any event, a reduction of 20% in matrix protein production (as seen with anti-TGFß in this thesis) may still be biologically significant in a process that takes weeks or years to develop. Moreover, the current studies do not exclude an effect of TGFß on other glomerular cells.

Preliminary characterisation studies suggested that the biologically active component of MPCM was a 12-30kD, heat stable protein/peptide resistant to trypsinisation although susceptible to some degree of proteolytic digestion. Further characterisation studies were able to narrow the molecular weight of the biologically active component to around 12.5

kD with some activity being attributed to a component with a higher molecular weight. Analysis of the MPCM fractions for their ability to induce TGFß$_1$ and PDGF-AB secretion demonstrated that their effect on the production of these cytokines was not limited to the fibronectin inducing fractions.

Although biosynthetic labelling of MPCM with ^{35}S methionine followed by sequential fractionation by gel filtration and SDS-PAGE resulted in the detection of a single band over three fractions, the molecular weight of the protein detected did not correspond with that predicted by gel filtration and did not correspond with the fraction containing peak fibronectin stimulating activity. At this point in time, the identity of the biologically active factor(s) remains unknown.

The experiments in this thesis used thioglycolate elicited, LPS stimulated peritoneal macrophages as a source of macrophage derived factors. Whether these truly reflect the properties of *bona fide* infiltrating glomerular macrophages under pathological conditions is open to argument. Macrophage activation is a multistep process and is a function of a number of criteria including cell origin, maturity, environment and immunologic factors [Hibbs JB *et al,* 1977., Chapman HA and Hibbs JB, 1977]. To-date the precise activation state of infiltrating glomerular macrophages has not been fully described. However, certain comparisons can be made between the present *in vitro* system and the pathophysiological *in vivo* conditions. Infiltrating macrophages are, in a broad sense, elicited into the glomerulus by the actions of chemokines etc. Macrophage adherence to a plastic surface has been shown to mimic the spreading of these cells on vascular surfaces [Doherty DE, 1987]. *In vitro,* the process of adhesion itself has been shown to upregulate the gene transcription of certain cytokines which are generally not translated until they have received

a second signal, from agents such as LPS [Haskill S *et al,* 1988]. Peritoneal macrophages were used in this study since glomerular macrophages could not be extracted from glomeruli in sufficient numbers to produce an adequately potent conditioned medium. Peritoneal macrophages on the other hand are obtained in reproducible yields with approximately 90% of the cells being macrophage in origin (as assessed by ED1 staining). In addition, it has been previously shown that peritoneal macrophages behave in a qualitatively similar way to glomerular macrophages. For example peritoneal and glomerular macrophages from diet induced hypercholesterolaemic and PAN nephrotic rats both exibit an upregulation of TGFß gene expression [Ding G *et al*, 1994].
Nevertheless, the most important aspect of these findings is the demonstration that MPCM can induce potent pro-fibrogenic characteristics in mesangial cells. One could argue that this approach is more representative of the *in vivo* situation than those studies which utilised heterologous, purified recombinant proteins (traditionally associated with macrophages) added in "industrial" or at least non-physiological concentrations to achieve similar effects.

The choice of fibronectin as a marker of glomerulosclerosis in this study was based upon several parameters including the fact that a) it is one of the major proteins of the mesangial matrix, b) it has been shown to accumulate in many of the conditions which lead to glomerulosclerosis [Dixon AJ *et al*, 1980] and c) it is not secreted as an artifact of cell culture conditions as is the case with interstitial collagens I and III and unlike the matrix proteins laminin and thrombospondin, its production is not upregulated in the presence of insulin, a common tissue culture additive [Abrass CK *et al*, 1994]. In addition, *in vivo* studies which simply demonstrate the presence of a particular matrix protein by

immunostaining do not confirm that mesangial cells have sythesised these proteins *de novo*. Intergrin and matrix assembly receptors upregulated on the cell surfaces in response to an insult could sequester circulating matrix molecules usually produced by other cell types and assimilate them into the surrounding matrix [Hewit T and Martin GR, 1984., M°Keown-Longo P, 1987., Ruoslahti E *et al*, 1985]. Mesangial cell culture is thus a more appropriate technique for examining which matrix molecules are produced by mesangial cells in response to particular factors.

The work in this thesis has illustrated that macrophages and particularly their secretory products provoke potent pro-sclerotic responses in mesangial cells causing them to upregulate the protein and/or genes for a) matrix proteins, b) an inhibitor of matrix metalloproteinases, c) pro-fibrogenic cytokines and d) a monocyte specific chemokine. These data provide the most compelling evidence to date that infiltrating glomerular macrophages could directly initiate the induction of glomerulosclerosis. However, as with all *in vitro* systems, caution must be taken when extrapolating tissue culture experiments to *in vivo* situations. *In vivo*, counter-regulatory mechanisms and interactions with other glomerular cells almost certainly exist and play a role in controlling the process of glomerulosclerosis.

A summary of the responses of mesangial cells to secreted macrophage factors described in this thesis and how they may play a role in the progression to glomerulosclerosis is presented diagramatically in Fig 7.1.

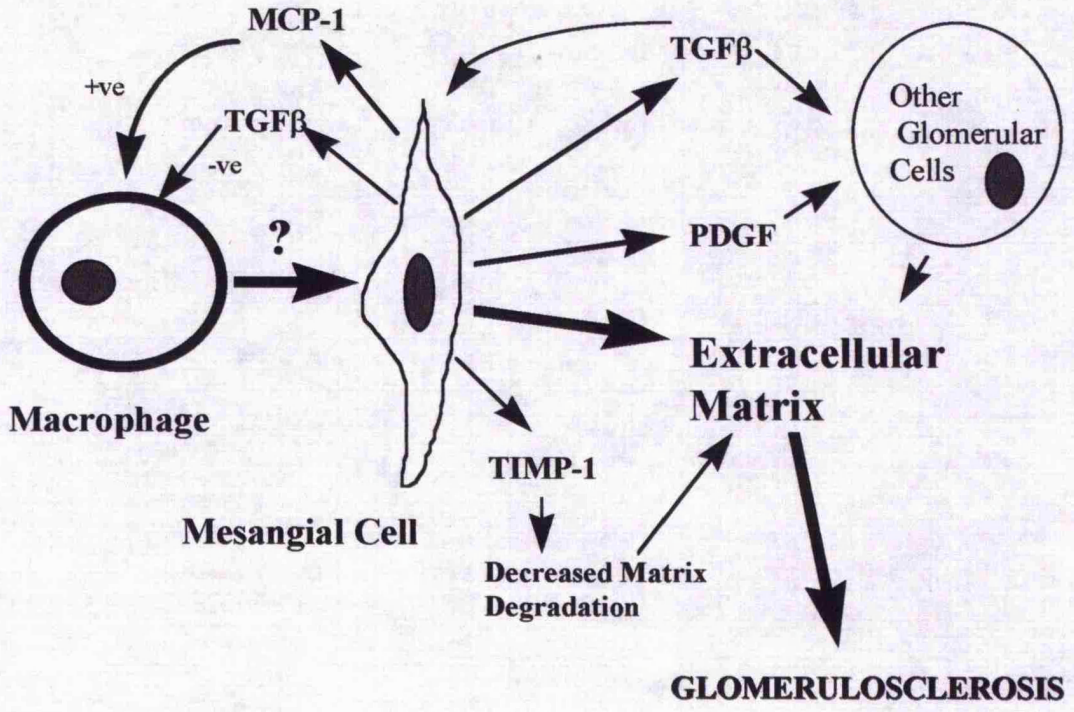


Fig 7.1 Diagramatic representation of mesangial cell responses to macrophage-derived factors.

7.2 The future?

As a result of the data presented in this thesis strategies are being developed to further characterise the factors involved. Large quantities of MPCM will be produced, concentrated and fractionated so that sufficient protein can be isolated to perform amino acid sequence analysis. If a positive identification cannot be made at this stage then oligonucleotide primers will be designed on the basis of the terminal protein sequences with a view to isolating the gene(s) of interest using the RT-PCR technique. The resultant cDNA could be sequenced and the complementary protein identified. The cDNA could then be utilised in Northern analysis to ascertain whether the gene was expressed in

glomeruli from animals with experimental renal disease and eventually to investigate whether infiltrating macrophages themselves express the gene. Furthermore, it would also be possible to insert the cDNA into an appropriate expression vector in order to produce large quantities of recombinant gene product. This could subsequently be used to raise specific antibodies. As well as having diagnostic potential such antibodies could also be used to suppress the fibrogenic potential of these inflammatory cells. In addition, stratagies using anti-sense probes to the gene of interest could prove useful in attenuating glomerulosclerosis.

APPENDIX I

GENERAL REAGENTS

Unless otherwise stated all buffer solutions were made up in deionised water (DI H_2O).

Phosphate buffered saline (PBS)

Per litre:

8g NaCl

0.2g KCl

1.15g Na_2HPO_4

0.2g KH_2PO_4

Tris buffered saline (TBS)

Per litre:

6.56g Tris base

8.52g NaCl

adjust pH to 7.2 with HCl

TISSUE CULTURE

Complete RPMI 1640

To 500ml RPMI 1640 (Gibco) add:-

100ml heat inactivated, batch tested foetal calf serum (FCS)

5ml Penicillin (10000IU/ml)/Streptomycin (10000μg/ml) in normal saline (Gibco)

5ml Glutamine (200mM) (Gibco)

500μl Bovine insulin (5mg/ml)(Sigma)

RPMI 1640 + 0.5% FCS

To 500ml RPMI 1640 add:-

2.5ml heat inactivated, batch tested FCS

5ml Penicillin (10000IU/ml)/Streptomycin (10000μg/ml)

5ml Glutamine (200mM)

HBSS-HEPES

To 500ml Hanks balanced salt solution (HBSS) (Gibco) add:-

10ml 1M HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Gibco)

MEM-D-valine

To 500 ml Minimum essential medium-D-valine (Gibco) add:-

50ml heat inactivated, batch tested FCS,

5ml Penicillin (10000IU/ml)/Streptomycin (10000μg/ml) in normal saline (Gibco)

5ml Glutamine (200mM) (Gibco)

500μl Bovine insulin (5mg/ml) (Sigma)

3% Thioglycollate Broth

Add 29.5g Thioglycollate (Sigma) to 1L H_2O and boil until dissolved.

Autoclave at 121°C

Hypnorm/Hypnoval anaesthesia

2ml Hyonoval (5mg/ml, Roche)

2ml Hypnorm (0.315mg/ml Fentanyl citrate, 10mg/ml Fluanisone, Roche)

4ml Sterile water

FIBRONECTIN ELISA

Coating buffer

0.05M carbonate pH 9.6

Mix 9 parts 1M $NaHCO_3$ (8.4g/100ml DI H_2O)

with 1 part 1M Na_2CO_3 (10.6g/100ml DI H_2O)

dilute 1:20 in DI H_2O and adjust to pH 9.6.

Wash buffer

PBS/0.3M NaCl/0.1% Tween 20

To 1L PBS add:-

20.75g NaCl

1ml Tween 20

BSA blocking solution

2% BSA in wash buffer

To 100ml washing buffer add:-

2g bovine serum albumin (fraction V)

Substrate buffer

0.03M citrate/phosphate pH 5.0

Per 100ml:

0.73g citric acid.H_2O

2.39g $Na_2HPO_4.12H_2O$

make up fresh each day

OPD/H_2O_2 substrate

To 12ml substrate buffer add:-

8mg OPD (4x2mg tablets, Dako)

5μl 30% H_2O_2

Add immediately before use and protect from light.

FOLINS PROTEIN ASSAY

Lowry A solution

Per litre:

20g Na_2CO_3 (anhydrous)

4g NaOH

0.2g K,Na-tartrate

store at 4°C.

Lowry B solution

Per litre:

5g $CuSO_4.5H_2O$

store at 4°C.

Lowry C solution

50 parts Lowry A + 1 part Lowry B

make fresh each day

SDS-PAGE

Gel buffers:

1.5M Tris HCl pH 8.8

To approx 80ml DI H_2O add:-

27.23g Tris base

adjust to pH 8.8 with 1N HCl. Make up to 150ml with DI H^2O, store at 4°C.

0.5 M Tris HCl pH 6.8

To 60 ml DI H_2O add:-

6g Tris base

adjust to pH 6.8 with 1N HCl. Make up to 100ml with DI H_2O, store at 4°C.

Polyacrylamide gels

	Stacking gel(ml)		*Resolving gel(ml)*	
	4%	*5%*	*12.5%*	*15%*
30% w/v acrylamide/N'N-bis-methylene-acrylamide	1.3	1.7	4.2	5.0
1.5M Tris-HCl pH 8.8	-	2.5	2.5	2.5
0.5M Tris-HCl pH 6.8	2.5	-	-	-
DI H_2O	6.1	5.65	3.15	2.65
Degass under vacuum for 15min,				
10% w/v SDS	0.1	0.1	0.1	0.1
0.05% APS	0.05	0.05	0.05	0.05
TEMED	0.01	0.005	0.005	0.005

Sample buffer	*reducing(ml)*	*non-reducing(ml)*
DI H_2O	8	8
0.5M Tris-HCl pH 6.8	2	2
Glycerol	1.6	1.6
10% SDS	3.2	3.2
2-ß-meraptoethanol	0.8	-
1% w/v bromophenol blue	0.1	0.1

5X Electrode running buffer pH 8.3

Per 600ml:

9g Tris base

43.2g glycine

3g SDS

SILVER STAINING

Fixing solutions

40% methanol/10% acetic acid (v/v)

Per litre:

400ml methanol

100ml glacial acetic acid

10% ethanol/5% glacial acetic acid

Per litre:

100ml absolute ethanol

50ml glacial acetic acid

Equilibration buffer prior to gel drying

40% methanol/10% acetic acid/3% glycerol

Per litre:

400ml methanol

100ml glacial acetic acid

30ml glycerol

WESTERN BLOTTING

Electrophoretic transfer buffer

25mM Tris/192mM glycine/20% methanol/0.05% SDS

Per litre:

3.03g Tris base

14.4g glycine

200ml methanol

5ml 10% SDS

Immunostaining buffers

Blocking buffer

TBS/1% BSA/0.05% Tween 20

To 100ml TBS add

1g BSA

50μl Tween 20

Washing buffer

TBS/0.05% Tween (TTBS)

To 1L TBS add

50μl Tween 20

Alkaline phosphatase buffer (AP)

100mM Tris-HCl/100mM NaCl/5mM $MgCl_2$ pH 9.5

To 800ml DI H_2O add

12.1g Tris base

5.84g NaCl

0.46g $MgCl_2$

adjust pH to 9.5, make up to 1L

IMMUNOPRECIPITATION

Immunoprecipitation wash buffer

PBS/0.5M NaCl/1% Triton/0.1% SDS/pH 7.4

To 800ml PBS add

29.2g NaCl

10ml Triton X 100

10ml 10% SDS

check pH, adjust to pH 7.4, make up to 1L

Sample buffer

8ml DI H_2O

2ml 0.5M Tris-HCL pH 6.8

1.6ml glycerol

4.7ml 10% SDS

1.6ml 2-ß-mecaptoethanol

AUTORADIOGRAPHY

Developer

To 103ml Kodak developer add

water to a volume of 473ml

Fixer

To 103ml Kodak developer add

water to a volume of 473ml

HPLC

Elution buffer

0.05M Na-phosphate/0.15M NaCl pH 7.4

Per litre:

69g/L NaH_2PO_4 H_2O (0.5M)

71g/L $NaHPO_4$ H_2O (0.5M)

mix the two solutions together to give pH 7.4, dilute 1:10 then add
8.7g NaCl
Filter before use.

MOLECULAR BIOLOGY

DEPC-treated water
Add 1ml DEPC to 1L nanopure H_2O
shake vigorously, leave to stand overnight then autoclave at 121°C.

DNA sample loading buffer
20% Ficol 400k/0.1M EDTA,pH 8.0/1% SDS/0.25% bromophenol blue
2g Ficol
2ml 0.5M EDTA pH 8.0
0.5ml 20% SDS
2.5ml 1% bromophenol blue
make up to 10ml with DEPC-H_2O

20X SSC
0.3M Na-citrate/3M NaCl, pH 7.0
Per litre:
175.3g NaCl
88.2g tri-sodium citrate
check pH 7.0

10X cDNA dilution buffer
100mM Tris-HCl, pH 7.4/50mM NaCl/1mM EDTA
1.21g Tris base
0.29g NaCl
200μl 0.5M EDTA
make up to 100ml

10X MOPS

200mM MOPS/50mM Na-acetate/10mM EDTA

Per lite:

41.9g MOPS

4.1g Na acetate

20ml 0.5M EDTA,pH 8.0

adjust to pH 7.0 with 1N NaOH

10X RNA loading buffer

30% Ficoll/10mM EDTA/0.05% bromophenolblue

3g Ficol 400K

200μl 0.5M EDTA

5mg bromophenol blue

RNA Sample loading buffer

750μl DI formamide

150μl 10X MOPS buffer

240μl formaldehyde

100μ DEPC H_2O

180μl 10X loading buffer

make fresh or freeze in aliquots

De-ionised (DI) formamide

Add MD-8 mixed bead resin (Sigma) to formamide to a final concentration of 10%.

Allow to shake for approx. 2hr at 4°C in the dark.

Methylene blue solution

0.5M Na-acetate, pH 5.2/0.04% methylene blue

Per 100ml:

4.1g Na-acetate

40mg methylene blue

Membrane stripping solution

Per litre:

5ml 20X SSPE

50ml 20% SDS

REFERENCES

Abboud HE, Poptic E, DiCorleto PE. Production of platelet-derived growth factor-like protein by rat mesangial cells in culture. J Clin Invest 80: 675-683, 1987.

Abboud HE, Pinzani M, Jaffer F, Knauss T, Grandaliano G, Pierce GF. Mitogenic signals for platelet-derived growth factor (PDGF) isoforms in human mesangial cells. J Am Soc Nephrol 1: 457, 1990 (abs)

Abboud HE, Woodruff KA, Synder SP, Bonewald LF. Polypeptide growth factors regulate the production of latent transforming growth factor ß in human mesangial cells. J Am Soc Nephrol 2: 434, 1991 (abs)

Abboud HE. Growth factors in glomerulonephritis. Kidney Int 43: 252-267, 1993.

Abrass CK, Peterson CV, Raugi GJ. Phenotypic expression of collagen types in mesangial matrix of diabetic and non diabetic rats. Diabetes 37: 1695-1702, 1988.

Abrass CK, Spicer D, Raugi GJ. Insulin induces a change in extracellular matrix glycoproteins synthesised by rat mesangial cells in culture. Kidney Int 46: 613-620, 1994.

Adams DO, Hamilton TA. Molecular basis of macrophage activation: Diversity and its origins. In: The natural Immune System; The Macrophage. Lewis CE, M^{c}Gee J O'D (eds), Oxford, Oxford University Press, 1992.

Adler S, Striker LJ, Striker GE, Perkinson DT, Hibbert J, Couser WG. Studies of progressive glomerular sclerosis in the rat. Am J Pathol 123: 553-562, 1986.

Affres H, Perez J, Hagege J, Fouqueray B, Kornprobst M, Ardaillou R, Baud L. Desferrioxamine regulates tumour necrosis factor release in mesangial cell. Kidney Int 39: 822-830, 1991.

Akiyama SK, Yamada KM. Fibronectin. Adv Enzymol 59: 1-57, 1987.

Alitalo K, Hovi T, Vaheri A. Fibronectin is produced by human macrophages. J Exp Med 151: 602-613, 1980.

Amore A, Cavallo F, Bocchietto E, Bussolino F, Gianoglio B, Peruzzi L, Porcellini MC, Coppo R. Cytokine mRNA expression by cultured rat mesangial cells after contact with environmental lectins. Kidney Int 43: 541-546, 1993.

Anderson S, Meyer TW, Rennke HG, Brenner BM. Control of glomerular hypertension limits glomerular injury in rats with reduced renal mass. J Clin Invest 76: 612-619, 1985.

Anderson S, Rennke HG, Brenner WM. Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat. J Clin Invest 77: 1993-2000, 1986.

Andrews PM, Coffey AK. Cytoplasmic contractile elements in glomerular cells. Federation Proc 42: 3046-3052, 1983.

Anzano MA, Roberts AB, Smith JM, Lamb LC, Sporn MB, DeLarco JE. Sarcoma growth factors from conditioned media of virally transformed cells is composed of both α and ß type transforming growth factors. Proc Natl Acad Sci USA 80: 6264-6268, 1983.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-ß in human platelets. J Biol Chem 258: 7155-7160, 1983.

Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB. Expression and secretion of type ß transforming growth factor by activated human macrophages. Proc Natl Acad Sci USA 84: 6020-6024, 1987.

Atkins RC, Holdsworth SR, Glasgow EF, Matthews FE. The macrophage in human rapidly progressive glomerulonephritis. Lancet 1: 830-832, 1976.

Auger MJ, Ross JA. The biology of the macrophage. In: The natural immune system; The Macrophage, Lewis CE, M^c Gee HO'D (eds) Oxford University Press, Oxford, 1-73, 1992.

Ausiello DA, Kreisberg JI, Roy C, Karnovsky MJ. Contraction of cultured rat glomerular cells of apparent mesangial origin after stimulation with angiotensin II and arginine vasopressin. J Clin Invest 65: 754-760, 1980.

Ayo SH, Radnik RA, Garoni J, Glass (II) WF, Kreisberg JI. High glucose causes an increase in extracellular matrix proteins in cultured mesangial cells. Am J Pathol 136: 1339-1348, 1990.

Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T, Harris RC. Mesangial cell, glomerular and renal vascular response to endothelin in the rat kidney: elucidation of signal transduction pathways. J Clin Invest 83: 336-342, 1989.

Badr KF. Non-cycloxygenase lipid mediators and regulation of renal function. In: Contempory Issues in Nephrology 24: Keane WF (ed) New York, Churchill-Livingstone, 85-107, 1991.

Bagchus WM, Hoedemaker P, Rozing J, Bakker WW. Glomerulonephritis induced by monclonal anti-Thy1.1 antibody. Lab Invest 55: 680-687, 1986.

Balza E, Borsi L, Allemanni G, Zardi L. Transforming growth factor ß regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett 228: 42-44, 1988.

Barlow DP, Green NM, Kurkinen M, Hogan BLM. Sequencing of laminin B chain cDNAs reveals C-terminal regions of coiled-coil alpha helix. EMBO J 3: 2355-2362, 1984.

Barnes JL, Hevey KA. Brief communication: Glomerular mesangial cell migration in response to platelet-derived growth factor. Lab Invest 62: 379-382, 1990.

Barnes JL, Abboud HE. Temporal expression of autocrine growth factors corresponds to morphological features of mesangial proliferation in Habu snake venom-induced glomerulonephritis. Am J Pathol 143: 1366-1376, 1993.

Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. TGFß induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63: 515-524, 1990

Baud L, Hagege J, Sraer J, Rondeau E, Perez J, Ardaillou R. Reactive oxygen production by cultured rat glomerular mesangial cells during phagocytosis is associated with stimulation of lipoxygenase activity. J Exp Med 158: 1836-1852, 1983.

Baud L, Ardaillou R. Reactive oxygen species: production and role in the kidney. Am J Physiol 251 (Renal Fluid Electrolyte Physiol 20): F765-F776, 1986.

Baud L, Perez J, Friedlander G, Ardaillou R. Tumour necrosis factor stimulates prostaglandin production and cyclic AMP levels in rat cultured mesangial cells. FEBS Lett 239: 50-54, 1988.

Baud L, Oudinet JP, Bens M, Noel L, Peraldi MN, Rondeau E, Etienne J, Ardaillou R. Production of tumour necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. Kidney Int 35: 1111-1118, 1989.

Berk BC, Alexander RW, Brock TA, Gimbone MA, Webb CR. Vasoconstriction: A new activity for platelet derived growth factor. Science 232: 87-90, 1986.

Beutler B and Cerami A. Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature 320: 584, 1986.

Betsholtz C, Johnsson A, Heldin CH, Westermark B. Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. Proc Natl Acad Sci USA 83: 6440-6444, 1986.

Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: A review. Crit Rev Oral Biol Med 4: 197-250, 1993.

Bomsztyk K, Sims JE, Stanton TH, Slack J, M^{c}Mahan CJ, Valentine MA, Dower SK. Evidence for different IL-1 receptors in murine B and T-cell lines. Proc Natl Acad Sci USA 86: 8034-8038, 1989.

Bonventre JV, Weber PC, Gronich JH. PAF and PDGF increase cytosolic [Ca^{2+}] and phospholipase activity in mesangial cells. Am J Physiol 254: F87-F94, 1988.

Border WA, Okuda S, Languino LR, Ruoslahti E. Transforming growth factor ß regulates production of proteoglycans by mesangial cells. Kidney Int 37: 689-695, 1990a.

Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E. Suppression of experimental glomerulonephritis by anti-serum against transforming growth factor ß. Nature 346: 371-374, 1990b

Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E. Natural inhibitor of transforming growth factor ß protects against scarring in experimental kidney disease. Nature 360: 361-364, 1992a

Border WA, Ruoslahti E. Transforming growth factor ß: The dark side of tissue repair. J Clin Invest 90: 1-7, 1992b.

Bornstein P, Savage H. Structurally distinct collagen types. Annu Rev Biochem 49: 957-1003, 1980.

Bowen-Pope DF, Hart CE, Seifert RA. Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which binds two different classes of PDGF receptors. J Biol Chem 264: 2502-2508, 1989.

Boyce NW, Tipping PG, Holdsworth SR. Glomerular macrophages produce reactive oxygen species in experimental glomerulonephritis. Kidney Int 35: 778-782, 1989.

Boyd FT, Massague J. Growth inhibitory response to transforming growth factor ß linked to expression of a 53 kDa cell surface TGFß receptor. J Biol Chem 264: 2272-2278, 1989.

Brady HR, Danton MD, Jimenez W, Takata S, Palliser D, Brenner BM. Chemoattractants provoke monocyte adhesion to human mesangial cells and mesangial cell injury. Kidney Int 42: 480-487, 1992.

Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease: The role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation and intrinsic renal disease. N Eng J Med 307: 652-659, 1982.

Brenner BM. Nephron adaptation to renal injury or ablation. Am J Physiol 249: F324-F337, 1985.

Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, Loetscher H. Identification of two types of tumour necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci USA 87: 3127-3131, 1990.

Brown DM, Michael AF, Oegema TR. Glycosaminoglycan synthesis by glomeruli in vivo and in vitro. Biochim Biophys Acta 674: 96-104, 1981.

Bruggeman LA, Horigan EA Horikoshi S, Ray PE, Klotman PE. Thromboxane stimulates extracellular matrix proteins in vitro. Am J Physiol 261 (Renal Fluid Electrolyte Physiol 30): F488-F494, 1991.

Bruggeman LA, Pellicoro JA, Horigan EA, Klotman PE. Thromboxane and prostacyclin differentially regulate murine extracellular matrix gene expression. Kidney Int 43: 1219-1225, 1993.

Buck CA, Horowitz AF. Cell surface receptors for extracellular matrix molecules. Annu Rev Cell Biol 3: 179-205, 1987.

Burgeson RE. New collagens, new concepts. Annu Rev Cell Biol 4: 551-577, 1988.

Carome MA, Striker LJ, Peten EP, Moore J, Yang CW, Stetler-Stevenson WG, Striker GE. Human glomeruli express TIMP-1 mRNA and TIMP-2 protein and mRNA. Am J Physiol 256: F923-F929, 1993.

Castellot JJ Jr, Hoover RL, Harper PA, Karnovsky MJ. Heparin and glomerular epithelial cell-secreted heparin-like species inhibits mesangial cell proliferation. Am J Pathol 120: 427-435, 1985.

Chapman HA Jr, Hibbs JB Jr. Modulation of macrophage tumorocidal capability by components of normal serum: A central role for lipid. Science 197: 282-285, 1977.

Choi ME, Kim EG, Huang Q, Ballerman BJ. Rat mesangial cell hypertrophy in response to transforming growth factor ß. Kidney Int 44: 948-958, 1993.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. Anal Biochem 162: 156-159, 1987.

Coffey RJ, Leof EB, Shipley GD, Moses HL. Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. J Cell Physiol 132: 143-148, 1987.

Coimbra T, Wiggins R,Noh JW, Merritt S, Phan SH. Transforming growth factor ß production in anti-glomerular basement membrane disease in the rabbit. Am J Pathol 138: 223-234, 1991

Conti FG, Striker LJ, Elliot SJ, Domenico A, Striker GE. Synthesis and release of insulin-like growth factor-1 by mesangial cells in culture. Am J Physiol 255: F1214-F1219, 1988.

Cook HT, Smith J, Salmon JA, Cattell V. Functional characteristics of macrophages in glomerulonephritis in the rat. Am J Pathol 134: 431-437, 1989.

Cook HT, Sullivan R. Glomerular nitrite synthesis in in situ immune complex glomerulonephritis in the rat. Am J Pathol 139: 1047-1052, 1991.

Cosio FG, Bakaletz AP. Role of fibronectin on the clearance and tissue uptake of antigens and immune complexes in the rat. J Clin Invest 80: 1270-1279, 1987.

Courtoy PJ, Kanwar YS, Hynes O, Farquar MG. Fibronectin localisation in the rat glomerulus. J Cell Biol 87: 691-696, 1980.

Courtoy PJ, Timpl R, Farquar MG. Comparative distribution of laminin, type IV collagen and fibronectin in the rat glomerulus. J Histochem Cytochem 30: 874-880, 1982.

Davies M, Thomas G, Martin J, Lovett DH. The purification and characterisation of a glomerular basement membrane degrading proteinase from rat mesangial cells. Biochem J 251: 419-425, 1988.

Dean DC, Newby RF, Bourgeois S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor ß and cAMP in human cell lines. J Cell Biol 106: 2159-2170, 1988.

Denton MD, Marsden PA, Luscinskas FW, Brenner BM, Brady HR. Cytokine-induced phagocyte adhesion to human mesangial cells. Role of CD11/CD18 intergrins and ICAM-1. Am J Physiol 261: F1071-F1079, 1991.

Derynk R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV. Human transforming growth factor ß complementary DNA sequence and expression in normal and transformed cells. Nature 316: 701-705, 1985.

Desmouliere A, Gabbiani G. Modulation of fibroblastic cytoskeletal features during pathological situations: The role of extracellular matrix and cytokines. Cell Motil Cytoskeleton 29: 195-203, 1994.

De Waal MR, Abrams J, Bennet B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL10 produced by monocytes. J Exp Med 174: 1209-1220, 1991.

Diamond JR, Karnovsky MJ. Non-anticoagulant protective effects in chronic aminonucleoside nephrosis. Renal Physiol 9: 366-374, 1986.

Diamond JR, Karnovsky MJ. Exacerbation of chronic aminonucleoside nephrosis by dietary cholesterol supplementation. Kidney Int 32: 671-677, 1987.

Diamond JR, Karnovsky MJ. Focal and segmental glomerulosclerosis. Analogies to atherosclerosis. Kidney Int 33: 917-924, 1988.

Diamond JR, Pesek-Diamond I, McCarter, Karnovsky MJ. Altered functional characteristics of rat macrophages during nephrosis: synergistic effect of hypercholesterolaemia. Am J Pathol 135: 711-718, 1989a.

Diamond JR, Pesek I, Ruggieri S, Karnovsky MJ. Essential fatty acid deficiency during acute puromycin nephrosis ameliorates late renal injury. Am J Physiol 257 (Renal Fluid Electrolyte Physiol 26): F798-F807, 1989b.

Diamond JR, Pesek-Diamond I. Sublethal X-irradiation during acute puromycin nephrosis prevents late renal injury: role of macrophages. Am J Physiol 260 (Renal Fluid Electrolyte Physiol 29) F779-F786, 1991a.

Diamond JR, Pesek I. Glomerular tumour necrosis factor and interleukin 1 during acute aminonucleoside nephrosis. An immunhistochemical study. Lab Invest 64: 21-28, 1991b.

Diamond JR, Ding G, Frye J, Pesek-Diamond IP. Glomerular macrophages and the mesangial proliferative response in the experimental nephrotic syndrome. Am J Pathol 141: 887-894, 1992.

Dijkstra CD, Dopp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognised by monoclonal antibodies ED1, ED2 and ED3. Immunology 54: 589-599, 1985.

Dinarello CA. An update on human interleukin 1: from molecular biology to clinical relevance. J Clin Immunol 5: 287, 1985.

Ding G, Pesek-Diamond I, Diamond JR. Cholesterol, macrophages and gene expression of TGFß1 and fibronectin during nephrosis. Am J Physiol 264: (Renal Fluid Electrolyte Physiol 33) F577-F584, 1993.

Ding G, Van Goor H, Frye J, Diamond JR. Transforming growth factor ß expression in macrophage during hypercholesterolaemic states. Am J Physiol 267 (Renal Fluid Electrolyte Physiol 36): F937-F943, 1994.

Dixon AJ, Burns J, Dunill MS, M°Gee JD. Distribution of fibronectin in normal and diseased kidneys. J Clin Pathol 33: 1021-1028, 1980.

Docherty AJP, Lyons A, Smith BJ, Wright EM, Stephens PC, Harris TJR. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 316: 701-705, 1985.

Doherty DE, Haslett C, Tonnesen MG, Henson PM. Human monocyte adherence: a primary effect of chemotactic factor on the monocyte to stimulate adherence to human endothelium. J Immunol 138: 1762-1771, 1987.

Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ. Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. Proc Natl Acad Sci USA 89: 2873-2877, 1992.

Dower SK, Urdal DL. The interleukin 1 receptor. Immunol Today 8: 46-51, 1987

Downer G, Phan SH, Wiggins RC. Analysis of renal fibrosis in a rabbit model of crescentic nephritis. J Clin Invest 82: 998-1006, 1988.

Dubois CH, Goffinet G, Foidart JB, Dechene CA, Foidart JM, Mahieu PR. Evidence for a particular binding capacity of rat peritoneal macrophages to rat glomerular mesangial cells in vitro. Eur J Clin Invest 12: 239-246, 1982.

Elema JD, Hoyer JR, Vernier RL. The glomerular mesangium: Uptake and transport of intravenously injected coloidal carbon in rats. Kidney Int 9: 395-406, 1976.

Egido J, Gomez-Chiarri M, Ortiz A, Bustos C, Alonso J, Gomez-Guerro C, Gomez-Garre D, Lopez-Armada MJ, Plaza J, Gonzalez E. Role of tumour necrosis factor in the pathogenesis of glomerular disease. Kidney Int 43: 559-564, 1993.

Eng E, Floege J, Young BA, Couser WG, Johnson RJ. Does exreacellular matrix expansion in glomerular disease require mesangial cell proliferation? Kidney Int 45 (sup 45) 44-47, 1994.

Eriksson AE, Cousens LS, Weaver LH, Matthews BW. Three dimentional structure of human basic fibroblast growth factor. Proc Natl Acad Sci USA 88: 3441-3445, 1991.

Falk RJ, Scheineman JI, Mauer SM, Michael AF. Polyantigenic expression of basement membrane consistant in diabetic nephropathy. Diabetes 32: suppl 2: 34-39, 1983.

Farquar MG. The glomerular basement membrane: a selective macromolecular filter. In: Cell Biology of the extracellular matrix. ED Hay (ed). New York. Plenum. 335-378, 1981.

Feinberg AP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13, 1983

Ferrario F, Castiglione A, Colasanti G, Di Belgioioso GB, Bertoli S, D'Amico G. The detection of monocytes in human glomerulonephtitis. Kidney Int 28: 513-519, 1985.

Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 147: 3815-3822, 1991.

Floege J, Topley N, Wessel K, Kaever V, Radeke H, Hoppe J, Kishimoto T, Resch K. Monkine and platelet-derived growth factor modulate prostanoid production in growth arrested mesangial cells. Kidney Int 37: 859-869, 1990.

Floege J, Alpers CE, Burns MW, Pritzl P, Gordon K, Couser WG, Johnson RJ. Glomerular cells, extracellular matrix accumulation and the development of glomerulosclerosis in the remnant kidney model. Lab Invest 66: 485-497, 1992a.

Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen-Pope DF, Couser WA, Johnson RJ. Glomerular cell proliferation and PDGF expression precedes glomerulosclerosis in the remnant kidney model. Kidney Int 41: 297-309, 1992b.

Floege J, Eng E, Young BA, Alpers CE, Barrett TB, Bowen-Pope DF, Johnson RJ. Infusion of platelet derived growth factor or basic fibroblast growth factor induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. J Clin Invest 92: 2952-2962, 1993.

Fogo A, Ichikawa I. Evidence for the central role of glomerular growth promoters in the development of sclerosis. Seminars in Nephrol 9: 329-342, 1989.

Foidart JB, Dechenne CA, Mahieu P, Creutz CE, De Mey J. Tissue culture of normal rat glomeruli: isolation and morphological characterisation of two homogeneous cell lines. Invest Cell Pathol 2: 15-26, 1979.

Foidart JB, Pirard YS, Winand RJ, Mahieu PR. Tissue culture of normal rat glomeruli: Glycosaminoglycan biosynthesis of homogeneous epithelial and mesangial cell populations. In: The glomerular basement membrane. 1st International Symposium, Vienna. Renal

Physiol 3: 169-173, 1980.

Fouqueray B, Boutard V, Philippe C, Kornreich A, Marchant A, Perez J, Goldman M, Baud L. Mesangial cell-derived interleukin 10 modulates mesangial cell response to lipopolysaccharide. Am J Pathol 147: 176-182, 1995.

Frolik CA, Dart LL, Meyers CA, Smith DM, Sporn MB. Purification and initial characterisation of a type ß transforming growth factor from human placenta. Proc Natl Acad Sci USA 80: 3676-3680, 1983.

Gibbons GH, Pratt RE, Dzau VJ. Vascular smooth muscle cell hypertrophy vs hyperplasia. J Clin Invest 90: 456-461, 1992.

Gilbert SF, Migeon BR. D-valine as a selective agent for normal human and rodent epithelial cells in culture. Cell 5: 11-17, 1975.

Giles KW, Myers A. An improved diphenylamine method for the esterification of deoxyribonucleic acid. Nature 206: 93, 1965.

Glasser RJ, Velosa JA, Michael AF. Experimental model of focal sclerosis I. Relationship to protein excretion in aminonucleoside nephrosis. Lab Invest 36: 519-526, 1977.

Gomez-Chiarri M, Hamilton TA, Egido J, Emancipator SN. Expression of IP-10, a lipopolysaccharide and interferon inducible protein in murine mesangial cells in culture. Am J Pathol 142: 433-439, 1993.

Goppelt-Struebe M, Stroebel M. Synergistic induction of monocyte chemoattractant protein-1 (MCP-1) by platelet-derived growth factor and interleukin-1. FEBS Lets 374: 375-378, 1995.

Grandaliano G, Valente AJ, Abboud HE. A novel biologic activity of thrombin: stimulation of monocyte chemotactic protein production. J Exp Med 179: 1737-1741, 1994.

Greenberg D, Barsh G, Ho T, Cunningham D. Serum-stimulated phosphate uptake and initiation of fibroblast proliferation. J Cell Physiol 90: 193-210, 1977.

Groggel GC, Hughes ML. Heparan sulphate stimulates extracellular matrix protein synthesis by mesangial cells in culture. Nephron 71: 197-202, 1995.

Grond J, Elema JD. Localisation and distribution of anionic charges in the glomerular mesangium of normal and nephrotic rats. Virchows Arch. [B] Cell Pathol 48: 135-143, 1985.

Grone E, Abboud HE, Hohne M, Walli AK, Grone HJ, Stucker D, Robenek H, Wieland E, Seidel D. Actions of lipoproteins in cultured human mesangial cells: modulation by mitogenic vasoconstrictors. Am J Physiol 263: F686-F696, 1992.

Gronwald RGK, Seifert RA, Bowden-Pope DF. Differential regulation of two platelet-derived growth factor receptor subunits by transforming growth factor ß. J Biol Chem 264: 8120-8125, 1989.

Gurner A, Smith J, Catell V. In vivo induction of Ia antigen in resident leukocytes in the normal rat renal glomerulus. Lab invest 55: 546-550, 1986.

Haberstroh U, Zahner G, Disser M, Thaiss F, Wolf G, Stahl RAK. TGFß stimulates rat mesangial cell proliferation in culture: role of PDGF ß-receptor expression. Am J Physiol 264 (Renal Fluid Electrolyte Physiol 33): F199-F205, 1993.

Handschumacher R, Harding M, Rice J, Drugge R. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 226: 544-547, 1984.

Harris KPG, Baker F, Walls J. Early increase in glomerular leukocyte number after a reduction in renal mass: implication for the pathogenesis of glomerulosclerosis. Clinical Science 85: 27-31, 1993.

Hart CE, Forstrom JW, Kelly JD, Seifert RA, Smith RA, Ross R, Murray MJ, Bowen-Pope DF. Two classes of PDGF receptor recognise different isoforms of PDGF. Science 240: 1529-1531, 1988.

Haskill S, Johnson C, Eierman D, Becker S, Warren K. Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. J Immunol 140: 1690-1694, 1988.

Hay ED. Cells and extracellular matrix, their organisation and mutual dependence. Mod Cell Biol 2: 509-548, 1983.

Hayman EG, Enguall E, Ruoslahti E. Concomitant loss of cell surface fibronectin and laminin from tranformed rat kidney cells. J Cell Biol 88: 352-357, 1981.

Heladin CH, Westermark B. Platelet-derived growth factor: mechanism of action and possible in vivo function. Cell Regulation 1: 555-566, 1990.

Hewitt T, Martin GR. Attachment proteins and their role in exracellular matrices. In: The Biology of glycoproteins. RJ Ivatt (ed), New York: Plenum, 65-93, 1984.

Hibbs JB Jr, Taintor RR, Chapman HA Jr, Weinberg JB. Macrophage tumour killing: Influence of local environment. Science 197: 279-282, 1977.

Hirano T, Morohoshi T. Treatment of hyperlipidaemia with probuscol suppresses the development of focal and segmental glomerulosclerosis in chronic aminonucleoside nephrosis. Nephron 60: 443-447, 1992.

Holdsworth SR, Neale TJ, Wilson CB. Abrogation of macrophage-dependent injury in experimental glomerulonephritis in the rabbit. J Clin Invest 68: 686-698, 1981.

Holdsworth SR, Neale TJ. Macrophage induced glomerular injury; cell transfer studies in passive autologous anti-glomerular basement membrane antibody-initiated experimental glomerulonephritis. Lab Invest 51: 172-180, 1984.

Holvey J, Hayslett JP. Clinical features and course of glomerulosclerosis. In: The progressive nature of renal disease. Mitch WE, Brenner WM, Stein JH (eds), New York, Churchill Livingstone, 189-201, 1986.

Hook M, Kjellen L, Johansson S. Cell surface glycosaminoglycans. Annu Rev Biochem 53: 847-869, 1984.

Hooke DH, Gee DC, Atkins RC. Leukocyte analysis using monoclonal antibodies in human glomerulonephtitis. Kidney Int 31: 964-972, 1987.

Horii Y, Muraguchi A, Iwano M, Matsuda T, Hirayama T, Yamada H, Fujii Y, Dohi K, Ishikawa H, Ohmoto Y, Yoshizaki K, Hirano T, Kishimoto T. Involvement of IL-6 in mesangial proliferative glomerulonephritis. J Immunol 143: 3949-3955, 1989.

Hosang M. Suramin binds to platelet-derived growth factor and inhibits its biological activity. J Cell Biochem 29: 265-273, 1985.

Hostetter TH, Olsen JL, Rennke HG, Venkatachalam MA, Brenner BM. Hyperfiltration in remnant nephrons. A potentially adverse response to renal ablation. Am J Physiol 241: F85-F93, 1981.

Houser MT, Scheineman JI, Basgen J, Steffes MW, Michael AF. Preservation of mesangium and immunohistochemically defined antigens in glomerular basement membrane isolated by detergent extraction. J Clin Invest 69: 1169-1175, 1982.

Hruby ZW, Shirota K, Jothy S, Lowry RP. Anti-serum against tumour necrosis factor production by glomerular macrophages in anti-glomerular basement membrane nephritis in rabbits. Kidney Int 40: 43-51, 1991.

Huang SS, Huang JS. Rapid turnover of platelet derived growth factor receptor in sis-transformed cells and reversal by suramin. J Biol Chem 263: 12608-12618, 1988.

Hurd ER, Johnston JM, Okita JR, MacDonald PC, Ziff M, Giliam JN. Prevention of glomerulonephritis and prolonged survival in New Zealand Black/ New Zealand White F1 hybrid mice fed an essential fatty acid-defficient diet. J Clin Invest 67: 476-485, 1981.

Hynes RO, Yamada KM. Fibronectins: Multifunctional modular glycoproteins. J Cell Biol 95: 369-377, 1982.

Hynes RO. Molecular Biology of fibronectin. Ann Rev Cell Biol 1: 67-90, 1985.

Hynes RO. Fibronectin. New York, Springer-Verlag, 1990.

Iida H, Seifert R, Alpers CE, Gronwald RGK, Phillips PE, Pritzl P, Gordon K, Gown AM, Ross R, Bowen-Pope DF, Johnson RJ. Platelet derived growth factor (PDGF) and PDGF receptor are induced in mesangial cell proliferative nephritis in the rat. Proc Natl Acad Sci USA 88: 6560-6564, 1991.

Ignotz RA, Massague J. Transforming growth factor-ß stimulates the expression of fibronectin and collagen and their incorporation into extracellular matrix. J Biol Chem 261: 4337-4345, 1986.

Ikeya E, Nagase M, Honda N. Intraglomerular distribution of fibronectin in primary glomerular diseases. Clin Nephrol 24: 53-59, 1985.

Isaka Y, Fujiwara Y, Veda N, Kaneda Y, Kamada T, Imai E. Glomerulosclerosis induced by in vivo transfection of transforming growth factor ß or platelet derived growth factor gene in the rat kidney. J Clin Invest 92: 2597-2601, 1993.

Isaka Y, Brees DK, Ikegaya K, Kaneda Y, Imai E, Noble NA, Border WA. Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. Nature Medicine 4: 418-423, 1996.

Jaffer F, Saunder C, Shultz P, Throckmorton D, Weinshell E, Abboud HE. Regulation of mesangial cell growth by polypeptide mitogens. Am J Pathol 135: 261-269, 1989.

Johnson RJ, Ida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gowan AM. Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis: αsmooth muscle actin is a marker of mesangial cell proliferation. J Clin Invest 87: 947-858, 1991.

Johnson RJ, Raines EW, Floege J, Yoshimura A, Pritzl P, Alpers C, Ross R. Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. J Exp Med 175: 1413-1416, 1992.

Johnson RJ. The glomerular response to injury: Progression or resolution? Kidney Int 45: 1769-1782, 1994.

Johnson RJ, Lombardi D, Eng E, Gordon K, Alpers CE, Pritzl P, Floege J, Young B, Pippin J, Couser WG, Gabbiani G. Modulation of experimental mesangial proliferative nephritis by interferon λ. Kidney Int 47: 62-69, 1995.

Jones CL, Buch S, Post M, M°Culloch L, Liu E, Eddy AA. Pathogenesis of interstitial fibrosis in chronic purine aminonucleoside nephrosis. Kidney Int 40: 1020-1031, 1991.

Jones DB. Inflammation and repair of the glomerulus. Am J Pathol 134: 933-945, 1953.

Jorgensen F. The ultrastructure of the normal human glomerulus. Ejnar Munksgaard, Copenhagen, 1966.

Kagami S, Border WA, Ruoslahti E, Noble NA. Co-ordinated expression of ß1 intergrins and TGFß induced matrix proteins in glomerulonephritis. Lab Invest 69: 68-76, 1993.

Kaizuka M, Yamabe H, Osawa H, Inuma H, Tamura N, Tsunoda J, Fujita K, Shirato K, Onodera K. Thrombin stimulates tissue inhibitor of metalloproteinase-1 and type I collagen in human mesangial cells. J Am Soc Nephrol 6: 898, 1995 (abs).

Kaname S, Vehida S, Ogata E, Kurokawa K. Autocrine secretion of transforming growth factor ß in cultured rat mesangial cells. Kidney Int 42: 1319-1327, 1992.

Kanwar YS, Farquar MG. Presence of heparan sulfate in the glomerular basement membrane. Proc Natl Acad Sci 76: 1303-1397, 1979a.

Kanwar YS, Farquar MG. Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. Proc Natl Acad Sci 76: 4493-4497, 1979b.

Kanwar YS, Linker A, Farquar MG. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. J Cell Biol 86: 688-693, 1980.

Kanwar YS, Jakubowski ML, Rosenweig LJ. Distribution of sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. Eur J Cell Biol 31: 290-296, 1983a.

Kanwar YS, Rosenweig LJ, Jakubowski ML. Distribution of de novo synthesised sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. Lab Invest 49: 216-225, 1983b.

Kasiske BL, O'Donnell MP, Cleary MP, Keane WF. Treatment of hyperlipidaemia reduces glomerular injury in obese Zucker rats. Kidney Int 33: 667-672, 1988a.

Kasiske BL, O'Donnell MP, Garvis WJ, Keane WF. Pharmacologic treatment of hyperlipidaemia reduces glomerular injury in the $^{5}/_{6}$ nephrectomy model of chronic renal failure. Circ Res 62: 367-374, 1988b.

Kasiske BL, O'Donnell MP, Schmitz PG, Kim Y, Keane WF. Renal injury of diet induced hypercholesterolaemia in rats. Kidney Int 37: 880-891, 1990.

Katsura M, Ito A, Hirakawa S, Mori Y. Human recombinant interleukin 1α increases biosynthesis of collagenase and hyaluronic acid in cultured human chorionic cells. FEBS Lett 244: 315-318, 1989.

Kawasaki K, Yaoita E, Yamamoto T, Tamatani T, Miyasaka M, Kihara I. Antibodies against intracellular adhesion molecule-1 and lymphocyte injury in rat experimental crescentic glomerulonephritis. J Immunol 150: 1074-1083, 1993.

Keane WF, Kasiske BL, O'Donnell MP, Kim Y. The role of altered lipid metabolism in the progression of renal disease. Experimental evidence. Am J Kidney Dis 17: 38-42, 1991.

Keane WF, Kasiske BL, O'Donnell MP, Kim Y. Oxidised low density lipoprotein increases mesangial cell expression of type IV collagen [α(IV)] mRNA. (abs) J Am Soc Nephrol 3: 635, 1992.

Kefalides NA. Current status of chemistry and structure of basement membranes. In: Biology and chemistry of basement membranes. Kefalides NA (ed). Academic Press, New York, 215, 1978.

Kelly JD, Haldeman BA, Grant FJ, Murray MJ, Seifert RA, Bowen-Pope DF, Cooper JA, Kazlauskas A. Platelet-derived growth factor (PDGF) stimulates PDGF receptor subunit dimerisation and intersubunit trans-phosphorylation. J Biol Chem 266: 8987-8992, 1991.

Kerr PG, Nikilic-Paterson DJ, Lan HY, Rainone S, Tesch G, Atkins RC: Deoxyspergualin suppresses local macrophage proliferation in renal allograft rejection. Transplantation 58: 596-601, 1994.

Kitamura M, Suto TS, Yokoo T, Fine LG. The mesangial cell-derived suppressor of cytokine synthesis is transforming growth factor ß. J Am Soc Nephrol 6: 770, 1995 (abs).

Klahr S, Heifets M, Purkerson ML. The influence of anticoagulation on the progression of experimental renal disease. In: The progressive nature of renal disease. Mitch, Brenner, Stein (eds), Churchill Livingstone, New York, 1986.

Klahr S, Schreiner GF, Ichikawa I. Progression in renal disease. N Engl J Med 318: 1657-1666, 1988.

Knauss TC, Mene P, Ricanati SA, Kester M, Dubyak GR, Emancipator SN, Sedor JR. Immune complex activation of rat glomerular mesangial cells: dependence on the Fc region of the antibody. Am J Physiol 257 (Renal Fluid Electrolyte Physiol 26): F478-F485, 1989.

Kobayashi M, Koyama A, Narita M, Shigematsu H. Intraglomerular monocytes in human glomerulonephritis. Nephron 59: 580-585, 1991.

Koch R. Die aetiologie der tuberculose. Berl Klin Wschr 19: 221, 1882 (Translated by Dr and Mrs Max Pinner for the National Tuberculosis Association: The aetiology of tuberculosis. Baltimore:Waverly Press Inc. 1-48, 1932)

Kohan DE, Schreiner GF. Interleukin 1 modulation of renal epithelial glucose and amino acid transport. Am J Physiol 254 (Renal Fluid and Electrolyte Physiol 6) F879-F886, 1988.

Kohno M, Horio T, Yokokawa K, Yasunari K, Kurihara N, Takeda T. Endothelin modulates the mitogenic effect of PDGF on glomerular cells. Am J Physiol 266 (Renal Fluid Electrolyte Physiol 35): F894-F900, 1994.

Kreisberg J, Venkatachalam, Troyer D. Contractile properties of cultured glomerular mesangial cells. Am J Physiol 249 (Renal Fluid Electrolyte Physiol 18) F457-F463, 1985.

Kriegler M, Perez C, De Fay K, Albert I, Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein. Ramifications for the complex physiology of TNF. Cell 53: 45-53, 1988.

Kuncio GS, Neilson EG, Haverty T. Mechanism of tubulointerstitial fibrosis. Kidney Int 3(: 550-556, 1991.

Kurkinen M, Condon MR, Blumberg B, Barlow DP, Quinones S, Saus J, Pihlajaniemi T. Extensive homology between the carboxy-terminal peptides of mouse α1(IV) and α2(IV) collagen. J Biol Chem 262: 8496-8499, 1987.

Kusner DJ, Luebbers EL, Nowinski RJ, Konieczkowski M, King CH, Sedor JR. Cytokine and LPS induced synthesis of interleukin 8 from human mesangial cells. Kidney Int 39: 1240-1248, 1991.

Lacave R, Rondeau E, Ochi S, Delarve F, Scheuning WD, Sraer JD. Characterisation of a plasminogen activator and its inhibitor in human mesangial cells. Kidney Int 35: 806-811, 1989.

Laemmli UK. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature 227: 680-685, 1970.

Laiho M, Sakesela P, Andreasen A, Keski-Oja J. Enhanced production and extra cellular depositon of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. J Cell Biol 103: 2403-2410, 1986.

Laiho M, Weis FMB, Boyd FT, Ignotz RA, Massague J. Responsiveness to transforming growth factor ß restored by complementation between cells defective in TGFß receptor I and II. J Biol Chem 266: 9108-9112, 1991.

Lan HY, Paterson DJ, Atkins RC. Initiation and evolution of interstitial leukocyte infiltration in experimental glomerulonephritis. Kidney Int 40: 425-433, 1991.

Lan HY, Nikolic-Paterson DJ, Atkins RC. Trafficking of inflammatary macrophages from the kidney to draining lymph nodes during experimental glomerulonephritis. Clin Exp Immunol 92: 336-341, 1993a.

Lan HY, Nikolic-Paterson DJ, Zarama M, Vannice JL, Atkins RC. Suppression of experimental crescentic glomerulonephritis by the interleukin-1 receptor antagonist. Kidney Iny 43: 479-485, 1993b.

Lan HY, Nikolic-Paterson DJ, Mu W, Atkins RC. Local macrophage proliferation in the progression of glomerular and tubular injury in rat anti-GBM glomerulonephritis. Kidney Int 48: 753-760, 1995.

Largen PJ, Tam FWK, Rees AJ, Cattell V. Rat mesangial cells have a selective role in macrophage recruitment and activation. Exp Nephrol 3: 34-39, 1995.

La Rocca RV, Stein CA, Myers CE. Suramin - Prototype of a new generation of anti-tumour compounds. Cancer Cells 2: 106-115, 1990.

LaRochelle WJ, Giese N, Robbins KC, Aaronsen SA. Variant PDGF ligands and receptors-structure-function-relationships. NIPS 6: 56-63, 1991.

Latta H. The glomerular capillary wall. J Ultrastruct Res 32: 526-544, 1970.

Latta H. Ultrastructure of the glomerulus and juxta glomerular apparatus. In: Handbook of Physiology. Renal Physiology. Washington DC: Am Physiol Soc sect 8 1: 1-29, 1973.

Lavelle KJ, Durland BD, Yum MN. The effect of anti-macrophage antiserum. J Lab Clin Med 98: 195-205, 1981.

Lefkowith JB, Schreiner GF. Essential fatty acid deficiency depletes rat glomeruli of resident macrophages and inhibits angiotensin II induced eicosanoid synthesis. J Clin Invest

80: 947-956, 1987.

Leonard EJ, Yoshimura T. Human monocyte chemoattractant protein-1 (MCP-1). Immunology Today 11: 97-101, 1990.

Lianos EA, Zanglis A. Mesangial cell synthesis of platelet activating factor in response to defined inflammatory mediators. Kidney Int 33: 273, 1988 (abs).

Lianos EA. Lipid inflammatory mediators in glomerulonephritis. J Lab Clin Med 113: 535-536, 1989.

Liberek T, Topley N, Jorres A, Coles GA, Gahl GM, Williams JD. Peritoneal dialysis fluid inhibition of phagocyte function: Effects of osmolality and glucose concentration. J Am Soc Nephrol 3: 1508-1515, 1993.

Linder EA, Miettinen A, Tornroth T. Fibronectin as a marker for the glomerular mesangium in immunohistology of kidney biopsies. Lab Invest 42: 70-75, 1980.

Lopez JF, Palkovits M, Arato M, Mansour A, Akil H, Watson SJ. Localisation and quantification of pro-opiomelanocortin mRNA and glucocorticoid receptor mRNA in pituitaries of suicide victims. Neuroendocrinology 56: 491-501, 1992.

Lovett DH, Ryan JL, Sterzel RB. Stimulation of rat mesangial cell proliferation by macrophage interleukin 1. J Immunol 131: 2830-2836, 1983a.

Lovett DH, Sterzel RB, Kashgarian M, Ryan JL. Neutral proteinase activity produced in vitro by cells of the glomerular mesangium. Kidney Int 23: 342-349, 1983b

Lovett DH, Sterzel RB. Cell culture approaches to the analysis of glomerular inflammation. Kidney Int 30 246-254, 1986.

Lovett DH, Szamel M, Ryan JL, Sterzel RB, Gemsa D, Resch K. Interleukin 1 and the glomerular mesangium.I. Purification and characterisation of a mesangial cell-derived autogrowth factor. J Immunol 136: 3700-3705, 1986a.

Lovett DH, Resch K, Gemsa D. Interleukin 1 and the glomerular mesangium. II. Monokine stimulation of mesangial cell prostanoid secretion. Am J Pathol 129: 543-551, 1987.

Lovett DH, Haensch GM, Goppelt M, Resch K, Gemsa D. Activation of glomerular mesangial cells by the terminal membrane attack complex of complement. J Immunol 138: 2473-2473, 1987.

Lovett DH, Larson A. Cell cycle-dependent interleukin 1 gene expression by cultured glomerular mesangial cells. J Clin Invest 82: 115-124, 1988.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951.

Lyons RM, Gentry LE, Purchio AF, Moses HL.
Mechanism of activation of latent recombinant transforming growth factor ß1 by plasmin. J Cell Biol 110: 1361-1367, 1990.

Mackay CR, Imhof BA. Cell adhesion in the immune system. Immunol Today 14: 99-102, 1993.

MacKay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE. Transforming growth factor ß. Murine glomerular receptors and responses of isolated glomerular cells. J Clin Invest 83: 1160-1167, 1989.

MacKay K, Robbins AR, Bruce MD, Danielpour D. Identification of disulphide-linked transforming growth factor ß specific binding proteins in rat glomeruli. J Biol Chem 265: 9351-9356, 1990.

Madri JA, Roll FJ, Furthmayr H, Foidart JM. Ultrastructural localisation of fibronectin and laminin in the basement membranes of the murine kidney. J Cell Biol 86: 682-687, 1980.

Mahieu PR, Foidart JB, Dubois CH, Dechenne, Deheneffe J. Tissue culture of normal rat glomeruli. Contractile activity of the cultured mesangial cells. Invest Cell Pathol 3: 121-128, 1980.

Markovic-Lipkovski J, Muller C, Risher T, Bohle A, Muller GA. Association of glomerular and interstitial mononuclear leucocytes with different forms of glomerulonephritis. Nephrol Dial Transplant 5: 10-17, 1990.

Marti HP, Lee L, Kashgarian M, Lovett DH. Transforming growth factor $ß_1$ stimulates glomerular mesangial cell synthesis of the 72kD type IV collagenase. Am J Pathol 144: 82-94, 1994.

Martin J, Lovett DH, Gemsa D, Sterzel RB, Davies M. Enhancement of glomerular mesangial cell neutral proteinase secretion by macrophages: role of interleukin 1.
J Immunol 137: 527-529, 1986.

Martin J, Knowlden J, Davies M, Williams J. Identification and independent regulation of human mesangial cell metalloproteinases. Kidney Int 46: 877-885, 1994.

Martin GR, Timpl R. Laminin and other basement membrane components. Annu Rev Cell Biol 3: 57-85, 1987.

Martin GR, Timpl, Muller K, Kuhn K. The genetically distinct collagens. Trends Biochem Sci 10:285-287, 1985.

Massague J. The transforming growth factor ß family. Annu Rev Cell Biol 6: 597-641, 1990.

Matrisian LM, Leroy P, Ruhlmann C, Gesnel MC, Breathnach R. Isolation of the oncogene and epidermal growth factor induced transin gene: complex control in rat fibroblasts. Mol Cell Biol 6: 1679-1686, 1986.

Matrisian LM. Metalloproteinases and ther inhibitors in matrix remodelling. Trend Genet 6: 121-125, 1990.

Matsui TM, Heidaran T, Miki T, Toru M, Popescu N, LaRochelle W, Kraus M, Pierce J, Aaronsen SA. Isolation of a novel receptor cDNA establishes the existance of two PDGF receptor genes. Science 243: 800-803, 1989.

Matsumo K, Hatano M. Production of interleukin 1 in glomerular cultures from rats with nephrotoxic serum nephritis. Clin Exp Immunol 75: 123-128, 1989.

Matsumo K, Hatano M. Soluble immune complexes stimulate production of interleukin 1 by cultured rat glomerular mesangial cells. Am J Nephrol 11: 138-143, 1991.

Matsumoto K, Osakabe K, Katayama H, Hatano M. Impaired cell mediated immunity in focal segmental glomerulosclerosis by supressor cells. Nephron 32: 263-264, 1982.

Matsumoto K, Osakabe K, Katayama H, Yoshizawa N, Nagura Y, Harada M, Fujita T, Chi H, Hatano M. Impaired cell-mediated immunity in focal segmental glomerulosclerosis. Nephron 34: 220-223, 1983.

Mattana J, Singhal PC. Macrophage supernatants have both stimulatory and suppressive effects on mesangial cell proliferation. J Cell Physiol 154: 289-293, 1993.

Mattana J, Singhal PC. Macrophage Fc receptor activity modulates mesangial cell proliferation and matrix synthesis. Am J Physiol 266 (Renal Fluid Electrolyte Physiol,35): F568-F575, 1994.

McCaffrey TA, Falcone DJ, Brayton CF, Agarwal LA, Welt FGP, Weksler BB. Transforming growth factor ß activity is potentiated by heparin via dissociation of the transforming growth factor ß/α2-macroglogulin inactive complex. J Cell Biol 109: 441-448, 1989.

M°Donald JA. Extracellular matrix assembly. Annu Rev Cell Biol 4: 183-207, 1988.

M°Keown-Longo P. Fibronectin cell surface interactions. Rev Infect Dis 9: 5322-5334, 1987.

Melcion C, Lachman L, Killen PD, Morel-Maroger L, Striker GE. Mesangial cells, effect of monocyte products on proliferation and matrix synthesis. Transplant Proc 14: 559-564, 1982.

Mene P, Abboud HE, Dubiak GR, S Carpa A, Dunn MJ. Effects of PDGF on inositol phosphates, Ca^{2+} and contraction of mesangial cells. Am J Physiol 253: F458-F463, 1987.

Mercola M, Wang C, Kelly J, Brownlee C, Jackson-Grusby L, Stiles C, Bowen-Pope DF. Selective expression of PDGF A and its receptor during early mouse embryogenesis. Devl Biol 138: 114-122, 1990.

Merril CR, Goldman D, Sedman SA, Ebert MH. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1437-1438, 1981.

Meudlers Q, He CJ, Adida C, Peraldi MN, Schleung WD, Sraer JD, Rondeau E. Tumour necrosis factor α increases anti-fibrinolytic activity of cultured human mesangial cells. Kidney Int 42: 327-334, 1992.

Meyer TW, Anderson S, Rennke HG, Brenner WM. Converting enzyme inhibitor therapy limits glomerular injury in rats with renal insufficiency. Am J Med 79: suppl 3C, 31-36, 1985.

Middaugh CR, Mach H, Burke CJ, Volkin DB, Dabora JM, Tsai PK, Bruner MW, Ryan JA, Marfia KE. Nature of the interaction of growth factors with suramin. Biochem 31: 9016-9024, 1992.

Millan F, Denhez F, Kondaiah P, Ackhurst R. The embryonic gene expression patterns of TGFß1, 2 and 3 suggest differential developmental function in vivo. Development 111: 131-144, 1991.

Mizel SB, Mizel D. Purification to apparent homogeneity of murine interleukin 1. J Immunol 126: 834-837, 1981.

Moorhead JF, Chan MK, El-Nahas M, Varghese Z. Lipid nephrotoxicity in chronic progressive glomerular and tubulo-interstitial disease. Lancet 2: 1309-1312, 1982.

Moorhead JF, Wheeler DH, Varghese Z. Permissive role of lipids in progressive renal disease. Am J Med 87: 12N-20N, 1989.

Moorhead JF. Lipids and progressive kidney disease. Kidney Int 39 (suppl 3) S35-S40, 1991.

Morioka T, Narita I, Shimizu F, Oite T. Production by cultured human monocytes of mesangial cell proliferative factor(s) differing from Interleukin 1 and interleukin 6. Clin Exp Immunol 83: 182-186, 1991.

Mosher DF. Fibronectin. In: Biology of extracellular matrix. RD Mecham (ed), San Diego, Academic Press. 1989.

Mosquero JA. Increase production of fibronectin by glomerular cultures from rats with nephrotoxic nephritis. Lab Invest 68: 406-412, 1993.

Mulligan MS, Johnson KJ, Todd RF, Issekutz TB, Miyasaka M, Tamatani T, Smith CW, Anderson DC, Ward PA. Requirements for leukocyte adhesion molecules in nephrotoxic nephritis. J Clin Invest 91: 577-587, 1993.

Nakajima M, De Chavigny A, Johnson CE, Hamada JI, Stein CA, Nicholson GL. Suramin. A potent inhibitor of melanoma, heparanase and invasion. J Biol Chem 266: 9661-9666, 1991.

Nakamura T, Miller D, Ruoslahti E, Border WA. Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor ß. Kidney Int 41: 1213-1221, 1992.

Narita I, Morioka T, Arakawa M, Shimizu F, Oite T. Monocytes secrete factors regulating glycosaminoglycan synthesis in mesangial cells in vitro. Clin Exp Immunol 83: 497-504, 1991.

Nathan CF. Secretory products of macrophages. J Clin Invest 79: 319-326, 1987.

Neilson EG. Pathogenesis and therapy of interstitial nephritis. Kidney Int 35: 1257-1270, 1989.

Niiro H, Otsuka T, Kuga S, Nemoto Y, Abe M, Hara N, Nakano T, Ogo T, Niho Y. IL-10 inhibits prostaglandin E_2 production by lipopolysaccharide-stimulated monocytes. Int Immunol 6: 661-664, 1994.

Nikolic-Paterson DJ, Lan HY, Hill PA, Vannice JL, Atkins RC. Suppression of experimental glomerulonephritis by the interleukin-1 receptor antagonist: Inhibition of intercellular adhesion molecule-1 expression. J Am Soc Nephrol 4: 1695-1700, 1994.

Nikolic-Paterson DJ, Main IW, Tesch GH, Lan HY, Atkins RC. Interleukin-1 in renal fibrosis. Kidney Int 49: suppl 54, S88-S90, 1996

Nishikawa H, Guo Y-J, Miyasaka M, Tamatani T, Collins AB, Sy MS, M°Clusky RT, Andres G. Antibodies to intercellular adhesion molecule-1/lymphocyte function-associated antigen-1 prevent crescent formation in rat autoimmune glomerulonephritis. J Exp Med 177: 667-677, 1993.

Norum KR, Gjone E, Glomset JA. Familial lecithin:cholesterol acyltransferase deficiency, including fish eye disease. In: The metabolic bases of inherited disease. Scriver CR, Beaudet AL, Sly WS, Valle D (eds). New York, M°Graw-Hill, 1181-1194, 1989.

O'Donnell MP, Kasiske BL, Katz SA, Schmitz PG, Keane WF. Lovastatin but not enalapril reduces glomerular injury in Dahl sensitive rats. Hypertension 20: 651-658, 1992.

Oikawa S, Suzuki N, Sakuma E, Saito T, Namai K, Kotake H, Fujii Y, Toyota T. Abnormal lipoprotein and apolipoprotein pattern in lipoprotein glomerulopathy. Am J Kidney Dis 18: 553-558, 1991.

Okuda S, Oti Y, Tsuruda H, Onoyama K, Fujimi S, Fujishima M. Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. Kidney Int 29: 502-510, 1986.

Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor ß and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. J Clin Invest 86: 453-462, 1990.

Old RJ. Tumour necrosis factor (TNF). Science 30: 630-632, 1985.

Olivetti G, Anversa P, Rigamonti W, Vitali-Mazza L, Loud AV. Morphometry of the renal corpuscle during normal post-natal growth and compensatory hypertrophy. J Cell Biol 75: 573-580, 1977.

Olsen J, Heptinstall R. Non immunologic mechanism of glomerular injury. In: Pathology Reviews, Rubin, Damianov (eds), Humana Press, Clifton 155-168, 1989.

Olson JL, Hostetter TH, Rennke HG, Venkatachalam MA, Brenner BM. Altered glomerular permselectivity and progressive sclerosis following extreme ablation of renal mass. Kidney Int 22: 112-126, 1982.

Ooi YM, Weiss MA, Hsu A, Ooi BS. Mechanisms of supression of mouse mesangial cell proliferation by macrophage supernatants. J Immunol 130: 1790-1795, 1983.

Oomura A, Nakamura J, Arakawa M, Ooshima A, Isemura M. Alterations in the extracellular matrix components in human glomerular disease. Virchow Arch [A] 415: 151-159, 1989.

Pabst R, Sterzel RB. Cell renewal of glomerular cell types in normal rats. An autoradiographic analysis. Kidney Int 24: 626-631, 1983.

Pan CG, Bresnahan BA, Albrightson C, Lianos EA. Mesangial cell immune injury: synthesis, origin and role of TNF. J Am Soc Nephrol 2: 542a, 1993 (abs).

Parry DAD. The molecular and fibrillar structure of collagen and its relationship to the mechanical properties of connective tissue. Biophys Chem 29: 195-209, 1988.

Penttinen RP, Kobayashi J, Bornstein P. Transforming growth factor ß increases mRNA for matrix protein both in the presence and in the absence of changes in mRNA stability. Proc Natl Acad Sci USA 85: 1105-1108, 1988.

Purkerson ML, Tollesfsen DM, Klahr S, Schreiner GF. N-desulphated/acetylated heparin ameliorates the progression of renal disease in rats with subtotal renal ablation. J Clin Invest 81: 69-74, 1988.

Petterson EA, Colvin RB. Cold-insoluble globulin (fibronectin, LETS protein) in normal and diseased humam glomeruli: Papain sensitive attachment to normal glomeruli and deposition in crescents. Clin Immunol Immunopathol 11: 425-436, 1978.

Phillips AO, Steadman R, Topley N, Williams JD. Elevated D-glucose concentrations modulate TGFß1 synthesis by human cultured renal proximal tubular cells. The permissive role of platelet-derived growth factor. Am J Pathol 147: 362-374, 1995.

Radeke HH, Meier B, Topley N, Floege J, Habermehl GG, Resch K. Interleukin 1ß and tumour necrosis factor α induce oxygen radical production in mesangial cells. Kidney Int 37: 767-775, 1990.

Raugi GJ, Lovett DH. Thrombospondin secretion by cultured mesangial cells. Am J Pathol 129: 364-372, 1987.

Rayner HC, Ward L, Walls J. Cholesterol feeding following unilateral nephrectomy in the rat leads to glomerular hypertrophy. Nephron 57: 453-459, 1991.

Ren KY, Brentjens J Chen YK, Brodkin M, Noble B. Glomerular macrophage proliferation in experimental immune nephritis. Clin Immunol Immunopathol 60: 384-398, 1991.

Rennard SI, Berg R, Martin GR, Foidart JM, Gehron-Robey P. Enzyme-linked Immunoassay (ELISA) for connective tissue components. Analytical Biochem 104: 205-214, 1980.

Risdon RA, Sloper JC, de Wardener HE. Relationship between renal function and histologic changes found in renal-biopsy specimenns from patients with persistant glomerulonephritis. Lancet 2: 363-366, 1968.

Roberts AB, Frolik CA, Anzano MA, Assoian RK, Sporn MB. In: Methods in Molecular and Cell Biology. Barnes D, Sato G, Sirbasku D (eds), Liss, New York, 1983.

Roberts AB, Flanders KC, Kondaiah P, Thompson NL, Obberghen-Schilling E, Wakefield LM, Rossi P, Crombrugghe BD, Heine U, Sporn MB. Transforming growth factor ß: Biochemistry and roles in embryogenesis, tissue repair and remodelling and carcinogenesis. Rec Prog Horm Res 44: 157-197, 1988.

Roberts AB, Heine UI, Flanders KC, Sporn MB. TGFß: Major role in regulation of extracellular matrix. In: Ann NY Acad Sci "Structure, molecular biology and pathology of collagen" 580: 225-232, 1990a.

Roberts AB, Sporn MB. The transforming growth factor betas. In: Handbook of experimental pharmacology. Peptide growth factors and their receptors. Sporn MB, Roberts AB (eds), Berlin, Springer-Verlag, 419-472, 1990b.

Rodewald R, Karnovsky MJ. Structure of the glomerular slit diaphragm in the rat and mouse. J Cell Biol 60: 423-433, 1974.

Rogers KA, Hoover RL, Castellot JJ, Robinson JM, Karnovsky MJ. Dietary cholesterol-induced changes in macrophage characteristics. Am J Pathol 125: 284-291, 1986.

Rohde H, Wick G, Timpl R. Immunochemical characterisation of the basement membrane glycoprotein laminin. Eur J Biochem 102: 195-201, 1979.

Rosenzweig LJ, Kanwar YS. Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to ^{125}I-bovine serum albumin. Lab Invest 47: 177-184, 1982.

Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell 46: 155-169, 1986.

Ross R. Platelet-derived growth factor.Lancet 1: 1179-1182, 1989.

Rossi P, Karsenty G, Roberts AB, Roche NS, Sporn MB, de Crombrugghe B. A nuclear factor 1 binding site mediates the transcriptional activation of a type 1 collagen promoter by transforming growth factor ß. Cell 52: 405-414, 1988.

Rovin BH, Lefkowith JB, Lange LG, Schreiner GF. Glomerular production of a lipid monocyte chemotactic factor. J Am Soc Nephrol 1: 536, 1990a (abs).

Rovin BH, Lefkowith JB, Schreiner GF. Mechanisms underlying the anti-inflammatory effects of essential fatty acid deficiency in experimental glomerulonephritis: Inhibited release of a monocyte chemoattractant by glomeruli. J Immunol 145: 1238-1245, 1990b.

Rovin BH, Yoshimura T, Tan L. Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. J Immunol 148: 2148-2153, 1992.

Rovin BH, Tan LC. LDL stimulates mesangial fibronectin production and chemoattractant expression. Kidney Int 43: 218-225, 1993.

Rovin BH, Rumancik M, Tan L, Dickerson J. Glomerular expression of monocyte chemoattractant protein-1 in experimental and human glomerulonephritis. Lab Invest 71: 536-542, 1994.

Ruoslahti E, Hayman EG, Pierscbacher MD. Extracellular matrices and cell adhesion. Arteriosclerosis 5: 581-594, 1985.

Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and intergrins. Science 238: 491-497, 1987.

Ruoslahti E. Structure and biology of proteoglycans. Annu Rev Cell Biol 4: 229-255, 1988a.

Ruoslahti E. Fibronectin and its receptors. Annu Rev Biochem 57: 375-413, 1988b.

Saito T, Sato H, Oikawa J, Kudo K, Kurihara I, Nakayama K, Abe K, Yoshinaga K, Sakaguchi H. Lipoprotein glomerulopathy. Report of a normolipidemic case and review of the literature. Am J Nephrol 13: 64-68, 1993.

Sakai T, Kritz W. The structural relationship between mesangial cells and basement membrane of the renal glomerulus. Anat Embryol 176: 373-386, 1987.

Salo T, Lyons JG, Rahemtulla F, Birkendal-Hansen H, Larjava H. Transforming growth factor ß1 upregulation type IV collagenase expression in cultured human keratinocytes. J Biol Chem 266: 11436-11441, 1991.

Satriano JA, Hera K, Shan Z, Stanley ER, Mori T, Schlondorf D. Regulation of monocyte chemoattractant protein-1 and macrophage colony stimulating factor by IFN, tumour necrosis factor α, IgG aggregates and cAMP in mouse mesangial cells. J Immunol 150: 1971-1978, 1993.

Savill J, Smith J, Ren Y, Sarraf CE, Abbot F, Rees AJ. Glomerular macrophages and mesangial cells ingest neutrophils undergoing apoptosis. Kidney Int 42: 924-936, 1992.

Scheinman JI, Foidart JM, Gehron-Robey P, Fish AJ, Michael AF. The immunohistology of glomerular antigens.IV. Laminin, a defined noncollagen basement membrane glycoprotein. Clin Immunol Immunopathol 15: 175-189, 1980a.

Scheinman JI, Foidart JM, Michael AF. The immunohistology of glomerular antigens. V. The collagenous antigens of the glomerulus. Lab Invest 43: 373-381, 1980b.

Schlondorf D. The glomerular mesangial cell: An expanding role for a specialised pericyte. FASEB J 16: 272-282, 1987.

Schonholzer KW, Waldron M, Magil AB. Intraglomerular foam cells and human focal glomerulosclerosis. Nephron 62: 130-136, 1992.

Schreiner GF, Cotran RS, Pardo V, Unanue ER. A mononuclear cell compartment in experimental immunological glomerulonephritis. J Exp Med 147: 369-384, 1978.

Schreiner GF, Kiely JM, Cotran RS, Unanue ER. Characterisation of resident glomerular cells in the rat expressing Ia determinants and manifesting restricted interactions with lymphocytes. J Clin Invest 68: 920-931, 1981.

Schreiner GF, Cotran RS. Localisation of an Ia-bearing glomerular cell in the mesangium. J Cell Biol. 94: 483-488, 1982.

Schreiner GF, Cotran RS, Unanue ER. Modulation of Ia and leukocyte common antigen expression in rat glomeruli during the course of glomerulonephritis and aminonucleoside nephrosis. Lab Invest 51: 524-533, 1984a.

Schreiner GF, Unanue ER. Origin of the rat mesangial phagocyte and its expression of the leukocyte common antigen. Lab Invest 51: 515-523, 1984b.

Schreiner GF, Flye W, Brunt E, Korber K, Lefkowith J. Essential fatty acid depletion of renal allograft and prevention of rejection. Science 240: 1032-1033, 1988.

Schreiner GF, Rovin BH, Lefkowith JB. The anti-inflammatory effects of essential fatty acid deficiency in experimental glomerulonephritis: The modulation of macrophage migration and eicosanoid metabolism. J Immunol 143: 3192-3199, 1989.

Schreiner GF. Pathways leading from glomerular injury to glomerulosclerosis. Contrib Nephrol 86: 1-18, 1990.

Schreiner GF. The role of the macrophage in glomerular injury. Semin Nephrol 11: 268-275, 1991.

Schreiner GF, Kees-Folts D, Delmez JA. Characterisation of a macrophage specific lipid chemotactic factor in the urine and peritoneal dialysate of nephrotic patients with declining

renal function. J Am Soc Nephrol 3: 419, 1992 (abs).

Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO. Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell 35: 421-431, 1983.

Seifert RA, Hart CE, Phillips PE, Forstrom J, Ross R, Murray MJ, Bowen-Pope DF. Two differerent subunits associate to create isoform-specific platelet-derived growth factor receptors. J Biol Chem 264: 8771-8778, 1989.

Shankland SJ, Scholey JW, Ly H, Thai K. Expression of transforming growth factor ß during diabetic renal hypertrophy. Kidney Int 46: 430-442, 1994.

Shingu M, Nagai Y, Isayama T, Naono T, Nobumaga M, Nagai Y. The effects of cytokines on tissue metalloproteinase inhibitors (TIMP) and collagenase production by human chondrocytes and TIMP production by synivial cells and endothelial cells. Clin Exp Immunol 94: 145-149, 1993.

Shultz PJ, DiCorleto PE, Silver BJ, Abboud HE. Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. Am J Physiol 255 (Renal Fluid Electrolyte Physiol 24): F674-F684, 1988.

Silver BJ, Jaffer FE, Abboud HE. Platelet-derived growth factor (PDGF) synthesis in mesangial cells: induction by multiple peptide mitogens. Proc Natl Acad Sci USA 86: 1056-1060, 1989.

Simonsen MS, Dunn MJ. Leukotriene C_4 and D_4 contract rat glomerular mesangial cells. Kidney Int 30: 524-531, 1986.

Simonsen MS, Culp LA, Dunn MJ. Rat mesangial cell matrix interactions in culture. Exp Cell Res 184: 484-498, 1989a.

Simonsen MS, Wann S, Mene P, Dubyak GR, Kester M, Nakazoto Y, Sedor JR, Dunn MJ. Endothelin stimulates phospholipase C, Na^+/H^+ exchange, *c-fos* expression and mitogenesis in rat mesangial cells. J Clin Invest 83: 708-712, 1989b.

Simpson LO. Glomerular permeabilty: an alternative to the pore theory. Lancet 2:251-252, 1981.

Singhal PC, Mattana J, Garg P, Arya M, Shan Z, Gibbons N, Franki N. Morphine-induced macrophage activity modulates mesangial cell proliferation and matrix synthesis. Kidney Int 49: 94-102, 1996.

Smith RA, Baglioni C. The active form of tumour necrosis factor is a trimer. J Biol Chem 262: 6951-6954, 1987.

Soma Y, Grotendorst GR. TGFß stimulates primary human skin fibroblast DNA synthesis via an autocrine production of PDGF-related peptides. J Cell Physiol 140: 246-253, 1989.

Spigelman Z, Dowers A, Kennedy S, DiSorbo D, O'Brien M, Barr R, McCaffrey R. Antiproliferative effects of suramin on lymphoid cells. Cancer Res 47: 4694-4698, 1987.

Springer TA. Adhesion receptors of the immune system. Nature 346: 425-434, 1990.

Sraer J, Foidart J, Chansel D, Mahieu P, Kouzneyzova B, Ardaillou R. Prostaglandin synthesis by mesangial cells and epithelial glomerular cultured cells. FEBS Lett 104: 420-424, 1979.

Stahl RAK, Thaiss F, Disser M, Helmchen U, Hora K, Schlondorf D. Increased expression of monocyte chemoattractant protein-1 in anti-thymocyte antibody-induced glomerulonephritis. Kidney Int 44: 1036-1047, 1993.

Stenman S, Vaheri A. Distribution of a major connective tissue protein, fibronectin in normal human tissues. J Exp Med 147: 1054-1064, 1978.

Sterzel RB, Lovett DH, Foellmer HG, Perfetto M, Biemesderfer D, Kashgarian M. Mesangial hillocks. Nodular foci of exaggerated growth of cells and matrix in prolonged culture. Am J Pathol 125: 130-140, 1986.

Striker LJ, Killen PD, Chi E, Striker GE. The composition of glomerulosclerosis. Studies in focal sclerosis, crescentic glomerulonephritis. Lab Invest 51: 181-192, 1984.

Striker GE, Striker LJ. Glomerular cell culture. Lab Invest 53: 122-131, 1985.

Striker LJ, Doi T, Elliot S, Striker GE. The contribution of glomerular mesangial cells to progressive glomerulosclerosis. Semin Nephrol 9: 318-328, 1989.

Striker LJ, Peten EP, Elliot SJ, Doi T, Striker GE. Mesangial cell turnover: Effect of heparin and peptide growth factors. Lab Invest 64: 446-456, 1991.

Suzuki S, Ebihara I, Tomino Y, Koide H. Transcriptional activation of matrix genes by transforming growth factor ß in mesangial cells. Exp Nephrol 1: 229-237, 1993.

Tam FWK, Karkar AM, Smith J, Yoshimura T, Steinkasserer A, Kurple R, Langer K, Rees AJ. Differential expression of macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 in experimental glomerulonephritis. Kidney Int 49: 715-721, 1996.

Tamatani T, Kotani M, Miyasaki M. Characterisation of the rat leukocyte intergrin CD11/CD18, by the use of LFA-1 subunit specific monoclonal antibodies. Eur J Immunol 21: 627-633, 1991.

Tang WW, Feng L, Xia Y, Wilson CB. Extracellular matrix accumulation in immune-mediated tubulointerstitial injury. Kidney Int 45: 1077-1084, 1994.

Timpl R. Recent advances in the biochemistry of the baement membrane. Kidney Int 30: 293-298, 1986.

Tipping PG, Lowe MG, Holdsworth SR. Glomerular macrophages express augmented procoagulent activity in experimental fibrin related glomerulonephritis in rabbits. J Clin Invest 82: 1253-1259, 1988.

Tipping PG, Lowe MG, Holdsworth SR. Glomerular interleukin-1 production is dependent on macrophage infiltration in anti-GBM glomerulonephritis. Kidney Int 39: 103-110, 1991a.

Tipping PG, Leong TW, Holdsworth SR. Tumour necrosis factor production by glomerular macrophages in anti-glomerular basement membrane glomerulonephritis in rabbits. Lab Invest 65: 272-279, 1991b.

Tolins JP, Stone BG, Raij L. Interaction of Hyperlipidaemia and hypertension in initiation of glomerular injury. Kidney Int 41: 1254-1261, 1992.

Toole BP. Glycosaminoglycans in morphogenesis. In: Cell Biology and Extracellular Matrix, Hay ED (ed). New York, Plenum 259-294, 1981.

Topley N, Floege J, Wessel K, Hass R Radeke HH, Kaever V, Resch K. Prostaglandin E_2 production is synergistically increased in cultured human glomerular mesangial cells by combinations of IL-1 and tumour necrosis factor-α. J Immunol 143: 1989-1995, 1989.

Torbohm I, Berger B, Schonemark M, von Kempis J, Rother K, Hansch GM. Modulation of collagen type IV synthesis in human glomerular epithelial cells by interleukin 1. Clin Exp Immunol 75: 427-431, 1989.

Town B, Wills ED, Wilson EJ, Wormall A. Studies on suramin. The action of thr drug on enzymes and other proteins. General considerations. Biochem J 47: 149-158, 1950.

Van Furth R (editor). Mononuclear phagocytes: Functional Aspects. The Hague, The Netherlands: Nijhoff 1980.

Van Furth R. Phagocytic cells: development and distribution of mononuclear phagocytes in normal steady state and inflammation. In: Inflammation: Basic principles and clinical correlations. Galin JI, Goldstein IM, Synderman R (eds). NewYork, Raven Press Ltd. 281-295, 1988.

Van Goor H, Fidler V, Weening JJ, Grond J. Determination of focal and segmental glomerulosclerosis in the rat after renal ablation. Evidence for involvement of macrophages and lipids. Lab Invest 64: 754-765, 1991.

Van Goor H, van der Horst ML, Fidler V, Grond J. Glomerular macrophage modulation affects mesangial expansion in the rat after renal ablation. Lab Invest 66: 564-571, 1992.

Varani J, Mitra RS, McClenic BJ, Fligiel SEG, Inman DR, Dixit VM, Nickoloff BJ. Modulation of fibronectin production in normal human melanocytes and malignamt melanoma cells by interferon γ and tumour necrosis factor α. Am J Pathol 134: 827-836, 1989.

Viedt C, Burger A, Hansch GM. Fibonectin synthesis in tubular epithelial cells: up-regulation of the EDA splice variant by transforming growth factor ß. Kidney Int 48: 1810-1817, 1995.

Wade TP, Kasid A, Stein CA, LaRocca RV, Sargent ER, Gomella LG, Myers CE, Lineham WM. Suramin interference with transforming growth factor ß inhibition of human renal cell carcinoma in culture. J Surgical Research 53: 195-198, 1992.

Wagner C, Braunger M, Beer M, Rother K, Hansch GM. Induction of matrix protein synthesis in human glomerular mesangial cells by the terminal complement complex. Exp Nephrol 2: 51-56, 1994.

Wakefield LM, Smith DM, Broz S, Jackson M, Levinson AD, Sporn MB. Recombinant TGFß1 is synthesised as a two component latent complex that shows some structural features with the native platelet latent TGFß1 complex. Growth factors 1: 203-218, 1989.

Waterhouse P, Khokha R, Denhardt DT. Modulation of translation by 5' leader sequence of the mRNA encoding murine tissue inhibitor of metalloproteinases. J Biol Chem 265: 5585-5589, 1990.

Wheeler DC, Chana RS, Topley N, Peterson MM, Davies M, Williams JD. Oxidation of low density lipoprotein by mesangial cells may promote glomerular injury. Kidney Int 45: 1628-1636, 1994.

Weening JJ, Beukers JJB, Grond J, Elema JD. Genetic factors in focal segmental glomerulosclerosis. Kidney Int 29: 789-798, 1986.

Weiss MA, Ooi BS, Enguall, Ruoslahti E. Immunofluorescence localisation of fibronectin in the human kidney. Lab Invest 41: 340-347, 1979.

Weissgarten J, Berman S, Averbukh Z, Cohn M, Golik A, Cohen N, Modai D. Renal mononuclear cells secrete a factor triggering mesangial cell proliferation. NDT 8: 118-121, 1993.

Werb Z, Chin JR. Biosynthetic radiolabelling of cellular and secreted proteins of mononuclear phagocytes. In: Methods for studying mononuclear phagocytes. Adams DO, Edelson PJ, koren H (eds). New York: Academic 861-872, 1981.

Wiggins RC, Njoku N, Sedor JR. Tissue factor production by cultured rat mesangial cells. Stimulation by TNFα and lipopolysaccharide. Kidney Int 37: 1281-1285, 1990.

Wight TN, Raugi GJ, Mumby SM, Bornstein P. Light microscopic immunolocation of thrombospondin in human tissues. J Histochem Cytochem 33: 295-302, 1985.

Yamada KM, Olden K. Fibronectin, adhesive glycoprotein of cell surface and blood. Nature 327: 179-84, 1978.

Yamada KM. Fibronectin and cell surface interactions. In: Cell Biology of Extracellular Matrix. Hay ED (ed), New York, Plenum Press 111-146, 1991.

Yoshida Y, Fogo A, Shiraga H, Glick AD, Ichikawa I. Serial micropuncture analysis of single nephron function in subtotal renal ablation. Kidney Int 33: 855-867, 1988.

Yoshida Y, Kawamura T, Ikoma M, Fogo A, Ichikawa I. Effects of anti-hypertensive drugs on glomerular morphology. Kidney Int 36: 626-635, 1989a.

Yoshida Y, Fogo A, Ichikawa I. Glomerular haemodynamic changes versus hypertrophy in experimental glomerulosclerosis. Kidney Int 35: 654-660, 1989b.

Yoshioka K, Takemura T, Tohda M. Glomerular localisation of type III collagen in human kidney disease. Kidney Int. 35: 1203-1211, 1989.

Yoshioka K, Takemura T, Akano N, Matsubara K, Ooshima A, Maki S. Distribution of type I collagen in human kidney disease in comparison with type III collagen. J Pathol 162: 141-148, 1990.

Yoshimura T, Takeya M, Takahashi K. Molecular cloning of rat monocyte chemoattractant protein-1 (MCP-1) and its expression in rat spleen cells and tumour cell lines. Biochem Biophys Res Commun 174: 504-509, 1991.

Zardi L, Carnemolla B, Siri A, Peterson TE, Paolella G, Sebastio G, Baralle FE. Transformed human cells produce a new fibronectin isoform by preferential splicing of a previously unobserved exon. EMBO J 6: 2337-2342, 1987.

Zhang G, Moorhead PJ, El Nahas AM. Myofibroblasts and the progression of experimental glomerulonephritis. Exp Nephrol 3: 308-318, 1995.

Zimmermann KW. Uber den bau des glomerulus der saugerniere: weitare mitteilunger. Z Mikrosk Anat Forsch 32: 176-278, 1933.

Zhong WW, Burke PA, Hard AT, Walsh MJ, Hughes LA, Forse RA. Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. Arch Surg 128: 158-163, 1993.

Zhu L, Herrera GA, Murphy-Ullrich JE, Huang Z-Q, Sanders PW. Pathogenesis of glomerulosclerosis in light chain deposition disease. Role for transforming growth factor ß. Am J Pathol 147: 375-385, 1995.

Zoja C, Wang JM, Bettoni S, Sironi M, Renzi D, Chiaffarino F, Abboud HE, Van Damme J, Mantovani A, Remuzzi G, Rambaldi A. Interleukin 1ß and tumour necrosis factor α induce gene expression and producyion of leukocyte chemotactic factors, colony-stimulating factors and interleukin 6 in human mesangial cells. Am J Pathol 138: 991-1003, 1991.