GLOMERULAR SELF-DEFENCE: A ROLE OF MESANGIAL CELL-DERIVED TRANSFORMING GROWTH FACTOR-β

by

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ABSTRACT

Communication between resident glomerular cells and infiltrating macrophages via paracrine mediators plays a crucial role in the pathogenesis of glomerular disease. However, the precise manner of *in vivo* cross-talk between macrophages and glomerular cells remains unclear. The aim of the present studies is to elucidate the role of mesangial cells in the regulation of macrophage function. Mesangial cells were found to secrete factors that impair function of macrophages, and transforming growth factor-β1 (TGF-β1) was identified as one of the mesangial cell-derived molecules involved in this process. Mesangial cell-derived TGF-β1 reduced macrophage adhesiveness and caused consequent deactivation. Mesangial cell-derived TGF-β1 also inhibited production of proinflammatory cytokines by activated macrophages. It was hypothesised that, in certain inflammatory situations, mesangial cell-derived TGF-β1 may function as a “defender” against macrophage-mediated glomerular injury. To examine this possibility, a technique for *in vivo* macrophage transfer was developed. Lipopolysaccharide-stimulated reporter macrophages were transferred into normal rat glomeruli or nephritic glomeruli producing active TGF-β1. In the normal glomeruli, expression of activation markers (gelatinase B, inducible nitric oxide synthase, stromelysin) were induced in resident cells after the transfer of activated macrophages. In contrast, this induction was substantially repressed in the regenerating glomeruli that produced active TGF-β1. These results point to an intrinsic potential of mesangial cell-derived TGF-β1 to suppress the macrophage-initiated glomerular cell activation. TGF-β1 may be regarded as a possible, endogenous “defender” that attenuates the action of infiltrating macrophages in the glomerulus.
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LIST OF ABBREVIATIONS

ADP  Adenosine diphosphate
BMP  Bone morphogenetic protein
BSA  Bovine serum albumin
CIAA Chloroform-isoamylalcohol
CR   Complement receptor
DEPC Diethyl pyrocarbonate
DME-F12 Dulbecco’s modified Eagle’s medium/Ham’s F12
ECM  Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
EGTA Ethylene glycol tetraacetic acid
FCS  Foetal calf serum
FITC Fluorescein isothiocyanate
G418 Geneticin
GBM  Glomerular basement membrane
GM-CSF Granulocyte/monocyte colony stimulating factor
HSP  Heat shock protein
ICAM Intercellular adhesion molecule
Ig   Immunoglobulin
IL   Interleukin
IL-1ra IL-1 receptor antagonist protein
iNOS Inducible nitric oxide synthase
lacZ β-galactosidase gene
LAP  Latency associated peptide
LFA  Lymphocyte function-associated antigen
LPS  Lipopolysaccharide
LTBP Latent TGF-β-binding protein
| **MΦ**  | Macrophage                                      |
| **Mad** | Mothers against decapentaplegic                |
| **MC**  | Mesangial cell                                 |
| **MCP** | Macrophage chemoattractant protein             |
| **MMP** | Matrix metalloproteinase                        |
| **MOPS**| 3-[N-Morpholino]propanesulfonic acid          |
| **neo** | Neomycin resistance gene                       |
| **NO**  | Nitric oxide                                   |
| **PAF** | Platelet activating factor                     |
| **PAI** | Plasminogen activator inhibitor                |
| **PBS** | Phosphate buffered saline                     |
| **PDGF**| Platelet-derived growth factor                 |
| **RGD** | Arg-Gly-Asp sequence                           |
| **RI, RII, RIII, RIV, RV, RVI** | TGF-β receptor Type I, II, III, IV, V, VI    |
| **RNase**| Ribonuclease                                   |
| **SDS** | Sodium dodecyl sulphate                        |
| **SOD** | Superoxide dismutase                           |
| **SSC** | Saline sodium citrate                          |
| **sTNFR** | Soluble TNF receptor                          |
| **TGF-β**| Transforming Growth Factor-β                   |
| **TIE** | TGF-β inhibitory element                       |
| **TIMP**| Tissue inhibitor of metalloproteinase          |
| **TNF** | Tumour necrosis factor                         |
| **VCAM**| Vascular cell adhesion molecule                |
| **VLA** | Very late antigen                              |
| **X-gal** | 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside |
CHAPTER ONE

INTRODUCTION
The cytokine, transforming growth factor-β (TGF-β) has been the focus of extensive investigation over the past decade [reviewed in Bitzer et al. 1998]. It is now well-recognised that TGF-β is a pleiotropic and complex cytokine. A wide range of functions, many of which represent opposing activities, have been attributed to TGF-β. This molecule plays a role in the maintenance of organ functions, embryogenesis, normal development, tissue repair, immune-regulation and inflammation [Miller et al. 1989; Massague, 1990]. On the other hand, overexpression of TGF-β is involved in certain pathological processes including fibrogenesis [Border and Noble, 1993]. In the kidney, a number of reports have demonstrated association between elevated levels of TGF-β and renal diseases [Border and Noble, 1993]. During the course of glomerulonephritis, for example, overexpression of TGF-β has been shown to be an important stimulator of extracellular matrix (ECM) synthesis that causes excessive matrix deposition [reviewed in Border and Noble, 1994b]. Based on these data, TGF-β has been regarded as a “blackguard” molecule that contributes to glomerulosclerosis. However, this molecule may have the potential to be anti-inflammatory by suppression of glomerular cell mitosis and cytokine responses, and inactivation of infiltrating cells [Kitamura and Sütö, 1997a]. In particular, TGF-β is one of the most potent deactivators of macrophages which play a pivotal role in the generation of various glomerular diseases. We hypothesised that TGF-β may act as a “defender” against macrophage-mediated glomerular injury.

Using a technique for in vivo macrophage transfer, the present study aimed to explore whether and how endogenous TGF-β affects pathogenic actions of macrophages in the glomerulus.
1.2. PATHOGENESIS OF GLOMERULONEPHRITIS

The major clinical symptoms of glomerulonephritis are direct or indirect results of structural and functional alterations in the kidney. These changes include; i) increased permeability of the glomerular capillary wall, leading to extravasation of plasma proteins into Bowman’s space and hence into the urine, which is termed as proteinuria, ii) loss of filtration surface area, which leads to decreased glomerular filtration rate, and subsequently to oliguria or anuria, iii) disturbances of sodium chloride and water excretion, leading to fluid retention, which may result in oedema and hypertension, iv) damage to basement membranes, leading to accumulation of cellular elements of the blood in the lumen of nephrons, which manifest themselves as increased excretion of casts, erythrocytes, leukocytes and epithelial cells.

Light microscopic evaluation of the glomerular morphology shows enlarged, hypercellular glomeruli. The capillary lumina are occluded by proliferating mesangial and epithelial cells. Infiltration of the mesangium by polymorphonuclear leukocytes and monocytes/macrophages can also be observed. In some cases, accumulation of epithelial cells and/or monocytes/macrophages in Bowman’s space leading to the formation of crescents can also be seen. The most characteristic electron microscopic finding is the presence of electron-dense deposits by the epithelial side of the GBM. These “humps” may also be found subendothelially and in the mesangium. In the diffuse form of glomerulonephritidies, these morphologic changes may involve the majority of the glomeruli. In contrast, only some of the glomeruli are affected in the focal-segmental form of the disease.
The factors responsible for structural and functional alterations of the glomerulus and hence for the development of glomerulonephritis have been investigated extensively. A growing body of evidence has suggested that immune mechanisms play central roles in the pathogenesis of glomerulonephritis.

Using sophisticated immunological methods to detect several types of antibodies, antigens and complement factors, the aetiologies of immune-mediated glomerulonephritides have been well established. Most forms of glomerulonephritides are characterised by deposition of antibody-antigen complexes in the glomerulus. Based upon the origin of antigens, two major humoral mechanisms have been identified in the pathogenesis of immunologic glomerular injury.

In the first instance, antibodies are produced against antigens present within the kidneys. The best known intrarenal antigens are intraglomerular structures, including the glomerular basement membrane (GBM) and glomerular cell membranes.

The interaction between glomerular antigens and antibodies results in immune deposit formation followed by the activation of the complement system, recruitment of leukocytes/monocytes and release of lysosomal enzymes/nephritogenic mediators, which subsequently leads to glomerular injury. The antibody reaction with the GBM antigen results in a linear IgG deposition in the glomeruli.

In the second humoral mechanism, soluble circulating antigens react with antibodies and form immune complexes. Due to the increased permeability of the capillaries, these complexes can be deposited in the glomerulus, the GBM or the mesangium. The antigens responsible for eliciting an immune reaction are of extraglomerular origin, and can be endogenous (circulating tumour antigens, native DNA, thyreoglobulin etc.) and/or exogenous (bacterial or viral antigens, toxic agents including antibiotics etc.).
Accumulated immune complexes induce activation of mediator systems involving the complement system, resident glomerular cells and their bioactive products, coagulation proteins, platelets, leukocytes, monocytes/macrophages and leukocyte/macrophage-derived substances.

Infiltration of inflammatory cells, proliferation of resident glomerular cells and deposition of immune complexes can be observed in the majority of glomerulonephritides. Recent reports have also described a mechanism leading to glomerular injury in the absence of any inflammatory hypercellular lesions [Couser, 1993], termed as non-inflammatory glomerular injury. This process includes injury induced by antibodies alone or by the C5b-C9 membrane attack complex of complement. Antibodies produced against glomerular epithelial cells induce alterations in cell function including reduced adhesiveness accompanied by increased glomerular permeability and subsequent proteinuria [Couser, 1993]. A role for the C5b-C9 membrane attack complex in the pathogenesis of non-inflammatory glomerular injury has been suggested in several forms of glomerulonephritis including Heymann nephritis [Kerjaschki and Farquhar, 1982].

After deposition of immune complexes in the glomerulus, several interrelated mediation pathways are activated resulting in tissue injury. The involvement of the most important mediator systems in the pathogenesis of immunologic glomerular injury is outlined below.
1.2.1. **MEDIATORS OF IMMUNOLOGIC GLOMERULAR INJURY**

1.2.1.1. **Role of the complement system**

Following antibody-antigen interactions, the complement system may be activated through C3. This activation may occur by the classical or the alternative pathway. During complement activation, the soluble components of the complement system are activated in a specific order leading to the release of several biologically active factors and to the assembly of the common terminal C5b-C9 membrane attack complex.

The classical pathway is activated by immune complexes. C1 binds to the Fc region of complexed immunoglobulins and activates C2 and C4 which are cleaved and form the C3 convertase. C3 is then activated and cleaved into C3a and C3b. C3a is an anaphylatoxin, which may play a role in the suppression of several immune responses. C3b can also bind to CR3 receptors on the surface of macrophages. This interaction leads to the activation of macrophages. C3b then induces the cleavage of C5 into C5a and C5b, and facilitates the assembly of the membrane attack complex. C5b also serves as an anaphylatoxin and a chemoattractant for leukocytes. The assembly of the membrane attack complex results in the formation of membrane lesions and cell death.

The alternative pathway is activated by fungal or bacterial substances, IgA or IgE. Continually produced C3b binds to Factor B making it susceptible to cleavage by Factor D, leading to the formation of the C3 convertase. Further activation of C3 results in the acceleration of the alternative pathway. Regulation of this process is maintained by the inhibitory Factors H and I.
1.2.1.2. Role of polymorphonuclear leukocytes

Complement activation leads to the release of several biologically active products, including anaphylatoxins, macrophage stimulators, chemotaxins for leukocytes and vasoactive agents. These factors lead to the recruitment of polymorphonuclear leukocytes into the glomerulus. Neutrophil adherence occurs through a number of receptors including those for Fc, C3b, as well as lymphocyte function-associated antigen (LFA)-1 and CR3 [Johnson et al. 1988]. Activated leukocytes release proteases including collagenases and elastases, stimulators of macrophage chemotaxis, reactive oxygen intermediates and clot promoting factors. These materials can subsequently destroy glomerular tissue especially the GBM.

1.2.1.3. Role of platelets

The role of platelets in glomerular injury and thrombogenesis has long been established [Cameron, 1984]. Platelet aggregation is induced by several inflammatory mediators including platelet activating factor (PAF) derived from polymorphonuclear leukocytes, monocytes and mesangial cells, TXA$_2$, and thrombin [Hirata et al. 1993]. Besides the crucial role of platelets in thrombogenesis, platelets contain various growth factors including platelet-derived growth factor (PDGF), TGF-β, insulin-like growth factor-1 and platelet-derived endothelial growth factor [Johnson et al. 1992]. Furthermore, activated platelets can also release eicosanoids, reactive oxygen intermediates, vasoactive peptides, complement, proinflammatory cytokines and proteolytic enzymes [Cameron, 1984]. These mediators may lead to thrombus
formation, glomerular cell proliferation, leukocyte accumulation and complement activation, which subsequently cause further glomerular injury [Cameron, 1984; Gesualdo et al. 1991].

1.2.1.4. Role of resident glomerular cells

Resident glomerular cells, especially mesangial cells, produce a number of potentially injurious mediators and mediate glomerular inflammatory processes. The mesangium can respond to cytokines released by infiltrating leukocytes and monocytes/macrophages. Among the numerous proinflammatory cytokines, interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) have been shown to play central roles in various inflammatory processes [Dinarello, 1991]. IL-1 and TNF-α are produced by a wide variety of cells including infiltrating macrophages [Dinarello, 1991] and resident endothelial and mesangial cells [Lovett et al. 1983a; Miossec et al. 1986; Baud et al. 1992]. Blocking IL-1 and TNF-α action in the kidney resulted in the attenuation of experimental glomerulonephritides, suggesting that these cytokines play a crucial role in the pathogenesis of several glomerular diseases [Sedor et al. 1992; Sedor, 1992]. Similarly to TNF-α, IL-1 released by infiltrating cells and resident cells has the capacity to enhance expression of adhesion molecules and stimulate production of inflammatory mediators including eicosanoids, cytokines, chemokines, growth factors, metalloproteinases and reactive oxygen/nitrogen species by macrophages [Sedor, 1992; Shah, 1995; Sarén et al. 1996; Yokoo and Kitamura, 1996b]. Furthermore, it has been shown that activated mesangial cells have the ability to produce macrophage chemoattractant protein-1 (MCP-1) and colony stimulating factors [Budde et al. 1989; Mori et al. 1990; Zoja et al. 1991]. This implies that
glomerular cells participate in the recruitment and accumulation of macrophages under
certain inflammatory situations.

1.2.1.5. Role of monocytes/macrophages in glomerular injury

Accumulation of mononuclear cells in the glomerulus is a characteristic feature of
glomerulonephritis [Main et al. 1992]. The major population of infiltrating cells is the
monocyte/macrophage lineage [Hooke et al. 1987]. Depletion of these cells by
irradiation, antibodies or pharmacological agents prevents glomerular hypercellularity
and matrix expansion and reduces the degree of proteinuria in several models of
glomerulonephritis [Schreiner et al. 1978; Lavelle et al. 1981; Holdsworth and Neale,
1984; van Rooijen et al. 1990; van Diemen-Steenvoorde et al. 1991]. This line of
evidence suggests a crucial role for infiltrating macrophages in glomerular injury.

The mechanisms responsible for the recruitment of macrophages into the glomerulus
have not been fully elucidated, but it is likely that leukocyte adhesion molecules and
chemotactic factors play crucial roles in this process. Macrophages express β2
integrins (LFA-1, CR3) and β1 integrins (very late antigen (VLA)-4, L-selectin) which
play a role in their adhesion onto endothelial cells [reviewed in Cattell, 1994]. The
major leukocyte binding molecules on the surface of endothelial cells, which appear to
be important in macrophage trafficking, are intercellular adhesion molecule (ICAM)-1
and vascular cell adhesion molecule (VCAM)-1. ICAM-1 is constitutively expressed
on endothelial cells in many organs including the kidney [Bishop and Hall, 1989],
whereas VCAM-1 expression is induced in inflammatory situation [Rice et al. 1991].
The factors responsible for this induction are thought to be IL-1, TNF-α and
interferon \( \gamma \) released by activated macrophages and other resident cells [Main et al. 1992].

A number of other molecules have also been implicated in the recruitment of macrophages into the glomerulus. MCP-1 has been shown to play an important role in macrophage recruitment [Yoshimura and Leonard, 1992]. Inducible expression of MCP-1 by proinflammatory cytokines is observed in a variety of cell types, including glomerular mesangial cells [Zoja et al. 1991]. Largen et al. showed that immunoadsorption with anti-MCP-1 antibody inhibited chemotactic activity of supernatants from mesangial cells \textit{in vitro} [Largen et al. 1995]. Recent reports have documented upregulated MCP-1 mRNA in several forms of glomerulonephritides \textit{in vivo} [Tam et al. 1996; Sekiguchi et al. 1997; Wenzel et al. 1997]. Administration of an anti-MCP-1 antibody resulted in the attenuation of glomerulonephritis [Wada et al. 1996; Fujinaka et al. 1997]. It is likely that other chemotactic factors, including complement factors C3b and C5b, PDGF, eicosanoids and PAF, may also contribute to macrophage localisation at inflammatory sites, although their roles have not been fully explored [Cattell, 1994].

Upon accumulation and activation at inflammatory sites, macrophages perform a number of tasks, including phagocytosis, antigen presentation and production of inflammatory mediators [Johnston, 1988]. Macrophages secrete proteolytic enzymes including metalloproteinases and elastases which play a role in the degradation of the extracellular matrix (ECM) [Baricos and Shah, 1991]. Activated macrophages also abundantly produce reactive oxygen/nitrogen species and proinflammatory cytokines, including IL-1, TNF-\( \alpha \), IL-6 and PDGF [Main et al. 1992]. These cytokines stimulate intrinsic glomerular cells to mitogenesis and production of inflammatory mediators including cytokines, chemokines, neutral proteases, bioactive lipids, coagulation products and reactive oxygen/nitrogen species [Pfeilschifter and Schwartzzenbach,
1990; Zoja et al. 1991; Sedor et al. 1992]. These mediators subsequently induce cell proliferation, leukocyte influx and destruction of glomerular structure, thus contributing to the progression of glomerular disease.

These results suggest that infiltrating macrophages serve as important effector cells in the generation of glomerulonephritidies. Therefore, endogenous mechanisms counteracting pathogenic actions of activated macrophages are essential to the prevention of and recovery from glomerular injury. Regulation of macrophage function by macrophage-deactivating cytokines is described below.

1.3. REGULATION OF MACROPHAGE FUNCTION BY MACROPHAGE-DEACTIVATING FACTORS

During resolution of glomerulonephritis, activated macrophages should be deactivated and cleared from the glomerulus. Several mediators including IL-1 receptor antagonist (IL-1ra), soluble TNF-α receptor (sTNFR), decorin, osteonectin, inhibitors of complement factors, prostacyclins, lipoxins, nitric oxide (NO), and tissue inhibitor of metalloproteinases (TIMPs), have been shown to block macrophage functions via inhibiting specific macrophage-derived proinflammatory cytokines (IL-1, TNF-α), polypeptide growth factors (TGF-β, PDGF), bioactive lipids, and proteases [reviewed in Dubois et al. 1990; Johnson et al. 1992; Main et al. 1992; Tang et al. 1994].

Macrophage-deactivating factors are produced by various cells including endothelial and epithelial cells as well as infiltrating leukocytes [Szuro-Sudol and Nathan, 1982; Fiorentino et al. 1991]. Recently, it has been shown that glomerular cells, especially mesangial cells, also produce macrophage deactivators in vitro [Fouqueray et al.
1995]. *In vivo*, expression of certain macrophage-deactivating factors including IL-4, IL-10, IL-13 and TGF-β is upregulated in glomeruli subjected to various glomerulonephritidies [Bogdan et al. 1992; Ballardie et al. 1994; Sütö et al. 1996a]. In the glomerulus, macrophage deactivators released from resident cells may play a role in the prevention of or recovery from macrophage-mediated glomerular injury. The putative function of macrophage deactivators in glomerular disease is summarised below.

1.3.1. **IL-4**

The major source of the macrophage deactivator cytokine IL-4 is type 2 T helper cells but resident glomerular cells including mesangial cells and podocytes have also been implicated in the production of this cytokine [Furusu et al. 1997]. Increased expression of IL-4 mRNA has been reported in certain types of glomerulonephritis including IgA nephropathy and lupus nephritis [Ballardie et al. 1994; Furusu et al. 1997]. IL-4 is a potent macrophage deactivator. IL-4 inhibits macrophage production of IL-1 and reactive oxygen/nitrogen species and stimulates expression of IL-1ra [Kluth and Rees, 1996]. Administration of IL-4 has been shown to attenuate crescent formation, macrophage infiltration and proteinuria in experimental anti-GBM nephritis [Kitching et al. 1997].
1.3.2. **IL-6**

*In vitro*, glomerular epithelial cells and mesangial cells have the ability to produce IL-6 in response to proinflammatory stimuli including LPS, IL-1β or TNF-α [Sterzel et al. 1993; Boswell et al. 1994]. Enhanced mesangial expression of IL-6 has been reported in mesangial proliferative glomerulonephritis [Takemura et al. 1994]. The macrophage inhibitory function of IL-6 during glomerular inflammation is further supported by Karkar and coworkers, who showed that pretreatment with IL-6 attenuated leukocyte infiltration, expression of cytokines and proteinuria in nephrotoxic serum nephritis [Karkar et al. 1997]. IL-6 may deactivate macrophages by several mechanisms including suppression of IL-1β, TNF-α and MCP-1 synthesis, inhibition of reactive oxygen/nitrogen intermediates or stimulation of IL-1ra and sTNFR expression [Kluth and Rees, 1996].

1.3.3. **IL-10**

The cells responsible for the production of IL-10 are mainly infiltrating macrophages, but activated mesangial cells can also produce IL-10 [Fouqueray et al. 1995]. IL-10 has the ability to inhibit IL-1 and TNF-α synthesis and adherence of macrophages [Fiorentino et al. 1991]. Similarly to IL-4 and IL-6, IL-10 also inhibits production of reactive oxygen/nitrogen intermediates [Moore et al. 1993]. Furthermore, IL-10 stimulates synthesis of IL-1ra, which has anti-inflammatory properties by antagonising IL-1 [Moore et al. 1993]. IL-10 expression is upregulated in the nephritic glomerulus [Kluth and Rees, 1996]. Similarly to IL-4, administration of IL-10 attenuates crescent
formation and leukocyte accumulation in anti-GBM glomerulonephritis [Tipping et al. 1997].

1.3.4. **IL-13**

IL-13 shares many biologic responses with IL-4. IL-13 is capable of inhibiting monocyte secretion of IL-1, TNF-α, and granulocyte/monocyte colony stimulating factor (GM-CSF). IL-13 also inhibits production of NO by monocytes/macrophages [Kluth and Rees, 1996]. IL-13 is synthesised by type 2 T helper cells [Kluth and Rees, 1996]. Enhanced renal expression of IL-13 has been shown in anti-GBM glomerulonephritis [Kluth and Rees, 1996]. Currently, it is not established whether resident glomerular cells can produce IL-13.

1.3.5. **LEUKAEMIA INHIBITORY FACTOR (LIF)**

LIF is produced by various cell types [Hartner et al. 1994]. In vitro, mesangial cells also have the capacity to produce LIF [Hartner et al. 1994]. This production is upregulated in response to LPS, IL-1β or TNF-α [Kluth and Rees, 1996]. Increased expression of LIF has been detected in anti-GBM nephritis [Tang et al. 1996]. A role for LIF in the prevention of glomerular inflammation has been suggested by Tang et al., who showed that pretreatment with LIF leads to suppression of macrophage accumulation, inhibition of cytokine synthesis and attenuation of proteinuria [Tang et al. 1996].
1.3.6. **PROSTACYCLINS**

It has been shown that glomerular endothelial, epithelial and mesangial cells have the ability to produce prostacyclins PGE$_2$ and PGI$_2$ [Jim et al. 1982; Kreisberg et al. 1982]. Expression of these prostacyclins is upregulated by several inflammatory mediators including IL-1β, TNF-α, IL-6, reactive oxygen metabolites, nitric oxide and PDGF [Rahman et al. 1987]. *In vivo*, enhanced expression of PGE$_2$ is observed in a number of glomerular inflammatory processes including anti-GBM glomerulonephritis [Rahman et al. 1987]. Systemic administration of PGE$_2$ has been shown to inhibit glomerular leukocyte infiltration and prevent proteinuria in immune-complex glomerulonephritis [McLeish et al. 1985], suggesting that prostacyclins may play beneficial roles in leukocyte-mediated glomerular injury.

1.3.7. **LIPOXINS**

Lipoxins are generated from leukotriens by lipoxin synthases present in a wide range of cells including mesangial cells [Garrick et al. 1989]. Lipoxins inhibit the proinflammatory actions of leukotriens produced in glomerular inflammatory processes. Lipoxins A$_4$ and B$_4$ have been shown to suppress LTB$_4$-mediated leukocyte recruitment [Lee et al. 1989]. Enhanced production of lipoxin A$_4$ is observed in certain glomerular diseases including anti-GBM nephritis [Nassar and Badr, 1995]. In glomerular inflammation, lipoxins produced by resident cells may inhibit accumulation of leukocytes and monocytes/macrophages through suppression of their chemotaxis, adhesion and transmigration [Serhan et al. 1995].
1.3.8. **NITRIC OXIDE (NO)**

NO is a potent effector molecule which participates in a number of physiological and pathological processes. In the glomerulus, NO is constitutively produced by glomerular endothelial cells [Raij and Baylis, 1995], however, stimulated mesangial cells also have the ability to synthesise this molecule [Cattell and Cook, 1993]. NO may protect the glomerulus from injury by inhibiting mesangial cell proliferation, thrombosis and cytokine expression as well as scavenging of superoxide [Garg and Hassid, 1989; Shultz and Raij, 1992; Kubes et al. 1993; Florquin et al. 1994]. NO also functions as an inhibitor of leukocyte superoxide production [Wanikiat et al. 1997]. NO has been shown to inhibit leukocyte adhesion through the downregulation of adhesion receptors on the surface of endothelial cells [de Caterina et al. 1995]. These results suggest that NO may play an important role in the regulation of macrophage function in glomerular inflammatory processes.

1.3.9. **TGF-β**

One of the best characterised deactivators of macrophages is TGF-β. Several studies have shown expression of TGF-β in certain glomerular diseases [Border and Noble, 1994b]. TGF-β is known to have suppressive effects on macrophage production of injurious mediators including inflammatory cytokines, reactive oxygen/nitrogen species and NO [Tsunawaki et al. 1988; Ding et al. 1990]. The macrophage-deactivating potential of TGF-β is explored in detail in this thesis. Our current knowledge on the TGF-β superfamily is summarised in the following sections.
1.4. THE TGF-β SUPERFAMILY

TGF-βs belong to a superfamily of at least 25 structurally related molecules which were first identified as regulators of morphogenesis and differentiation [reviewed in Laiho and Keski-Oja, 1992; Kingsley, 1994]. All family members are synthesised and secreted as inactive pro-peptides by a wide range of cells. After secretion, the latent molecule is proteolytically cleaved, forming the mature peptide of 110-140 amino acids. Other sub-groups of the family include activins and inhibins, bone morphogenetic proteins, Müllerian inhibitory substances, decapentaplegic-C gene products and growth differentiation factors 1 and 3.

There are three mammalian isoforms, TGF-β1, -β2 and -β3. Among these, TGF-β1 is most abundantly produced by mammalian cells. Two additional TGF-β isoforms have been described: TGF-β4 and TGF-β5 are present in chicken and frog, respectively. The homology amongst the five isoforms ranges from 60 to 80 % [Massague, 1990]. The TGF-β isoforms are well conserved between species.

1.4.1. STRUCTURE

TGF-β1 was first described as a soluble factor present in the conditioned medium of murine sarcoma virus [de Larco and Todaro, 1978]. Later it was also purified from platelets, bone, kidney, lung and placenta [Massague, 1990]. Mature TGF-βs are homodimeric polypeptides with a molecular weight of 25 kDa [Massague, 1990]. These are synthesised and secreted as inactive pro-peptides of 390 amino acids,
consisting of a 5' signalling sequence, a pro-domain called latency associated peptide (LAP) and a 3' mature protein domain. The latent TGF-β may form complexes with latent TGF-β binding proteins (LTBP) of varying sizes prior to secretion [Wakefield et al. 1988] (Figure 1.1.).

\[ \text{LTBP} \]
\[ \text{LAP} \]
\[ \text{TGF-β} \]
\[ \text{LAP} \]

**Figure 1.1.: The high molecular weight latent TGF-β complex.**

The complex comprises the latent TGF-β binding protein (LTBP), the latency associated peptide (LAP) and the mature homodymeric peptide (TGF-β).

The role of LAP in regulating TGF-β activity has not been fully characterised. It has been suggested that LAP plays a crucial role in the secretion of the latent complex [Sha et al. 1989]. Since LTBP does not associate with mature TGF-β1, its role is not entirely clear [Kanzaki et al. 1990]. However, amino acid sequence analysis revealed that LTBP contains epidermal growth factor-like domains, an Arg-Gly-Asp (RGD) sequence, which may mediate interactions with integrins [Ruoslahti and
Pierschbacher, 1987]. Thus LTBP may play a role in binding the latent TGF-β complex to matrix proteins.

1.4.2. \textit{ACTIVATION}

TGF-β isoforms are secreted as latent complexes and subsequently activated extracellularly by proteolytic cleavage. The \textit{in vivo} process of this activation has not been fully elucidated, but evidence suggests possible contribution of plasmin, a serine protease, to the cleavage of latent TGF-β at the cell surface [Lyons et al. 1988]. TGF-β may regulate its own activity by increasing the expression of plasminogen activator inhibitor (PAI-1). TGF-β may also down-regulate urokinase directly [Nunes et al. 1995].

1.4.3. \textit{TGF-β RECEPTORS}

The biological actions of TGF-βs are mediated through binding to specific cell membrane receptors. Expression of TGF-β receptors is ubiquitous and almost all cells respond to TGF-β in a cell type-specific manner. Three different TGF-β receptors have been identified; i.e., type I (RI) (53 kDa), type II (RII) (70-80 kDa) and type III (RIII) (250-350 kDa) [Massague, 1996]. RIII is a proteoglycan, named betaglycan and is not required for signalling [Andres et al. 1989]. Betaglycan may be found in a soluble as well as a membrane-bound form [Andres et al. 1989]. The membrane-bound form presents TGF-β to RII whereas the soluble form binds TGF-β and inhibits its binding to membrane receptors [Lopez-Casillas et al. 1994]. Both type I and type II receptors are required for TGF-β signalling.
TGF-β initially binds to RII, which is a constitutively active kinase. The TGF-β-RII complex then recruits RI, which becomes phosphorylated by RII. RI and RII form a heteromeric complex and generate signals via serine threonine kinases [Massague, 1996]. In addition to these three receptors, several other cell surface proteins have been shown to bind TGF-β. Types IV, V, and VI receptors have been isolated from pituitary tumour cells, bovine liver and a wide range of other cells [Cheifetz et al. 1988; O’Grady et al. 1991; Segarini et al. 1992]. However, the functional significance of these receptors is still unclear. Currently, the downstream signal transduction pathways responsible for propagating TGF-β’s effects are not fully defined. Some studies have indicated the involvement of tumour suppressor proteins, mitogen-activated protein kinase pathways and the Mad family of molecules [Alexandrow and Moses, 1995; Yamaguchi et al. 1995; Massague, 1996].

1.4.4. BIOLOGICAL ACTIVITY

TGF-β is a multifunctional cytokine whose biological actions depend on cell types. The current dogma is that TGF-β is generally an inhibitor of cell growth and a stimulator of ECM formation. The diversity of its actions on mammalian cells, coupled with almost universal expression of TGF-β receptors, makes this cytokine an important regulator of pathophysiologic processes, i.e., control of cell proliferation, adhesion, migration and phenotypic change. TGF-β has been shown to have dichotomous effects on regulating organogenesis/embryogenesis, tissue repair, inflammation, fibrosis and oncogenesis [Massague, 1990]. It is suggested that the biological activities of TGF-β isoforms are similar, but differences in efficacy and potency may exist depending on cell type. In certain pathophysiological situations, each isoform may have distinct biological effects [Ohta et al. 1987; Shah et al. 1995].
1.5. EXPRESSION OF TGF-β IN GLOMERULAR CELLS

Expression of TGF-β is observed in the normal glomeruli [MacKay et al. 1990; Okuda et al. 1990; Ando et al. 1995; Yamamoto et al. 1996]. The physiological meaning of this observation is currently unclear. Since TGF-β generally functions as a differentiation factor [Massague 1990], the basal levels of TGF-β detected in the normal glomerulus could contribute to the maintenance of normal glomerular microenvironment.

In the glomerulus, increased expression of TGF-β has been closely correlated with various pathological conditions. The induction of TGF-β has been reported in various glomerular diseases including anti-Thy 1 glomerulonephritis, anti-GBM nephritis, Habu-venom glomerulonephritis, puromycin-induced nephrosis, diabetic glomerulopathy and nephropathy associated with ureteral obstruction, as well as in human diseases such as IgA nephropathy, focal/segmental glomerulosclerosis, crescentic glomerulonephritis, lupus nephritis, diabetic nephropathy and human immunodeficiency virus nephropathy [Border and Noble, 1994b; Yamamoto et al. 1996].

Although the cell type responsible for TGF-β production in the glomerulus has not been fully elucidated, several reports suggest that mesangial cells may be the predominant source [Okuda et al. 1990; Yamamoto et al. 1994]. In vitro, mesangial cells have the ability to secrete a substantial amount of latent TGF-β1 and to convert it to the mature form [Kaname et al. 1992; Kitamura et al. 1996; Sütö et al. 1996a]. It has also been shown that cultured mesangial cells synthesise plasminogen activator [Lacave et al. 1989; Sappino et al. 1991] that participates in the in vivo activation of
TGF-β [Lyons et al. 1988]. These data suggest that mesangial cells play a role in both production and activation of TGF-β1 in the affected glomeruli. Other potential sources of TGF-β may include platelets and infiltrating cells, especially macrophages [Assoian et al. 1983; Ding et al. 1994; Lianos et al. 1994]. The contribution of resident endothelial or epithelial cells to the production of glomerular TGF-β in pathological conditions is still poorly defined.

1.6. PROFIBROTIC ACTION OF TGF-β IN THE GLOMERULUS

Excessive deposition of ECM is a hallmark of tissue fibrosis. A number of studies have suggested a significant role for TGF-β in fibrogenesis of various organs including the kidney, liver, lung, brain, joint and skin [Border and Noble, 1994a].

In glomerular disease, TGF-β has been regarded as a “blackguard” that contributes to glomerulosclerosis [Border and Ruoslahti, 1992; Border and Noble, 1993; Border and Noble, 1994a; Border and Noble, 1994b]. Generally, TGF-β induces deposition of ECM via stimulating matrix production, decreasing matrix-degrading activity and facilitating matrix assembly and integrin expression [Roberts and Sporn, 1990; Kitamura et al. 1992; Kagami et al. 1993].

In cultured glomerular mesangial and epithelial cells, TGF-β1 induces production of collagens, fibronectin and proteoglycans [Bruijn et al. 1994]. TGF-β upregulates production of PAI-1 in isolated glomeruli and thereby suppresses the activity of plasminogen activator, which is involved in the activation of matrix-degrading metalloproteinases [Tomooka et al. 1992]. These data suggest that TGF-β may be an important factor in the generation of glomerulosclerosis (Figure 1.2.).
In glomerular diseases, upregulation of local TGF-β is closely correlated with the degree of mesangial matrix expansion [Border and Ruoslahti, 1992; Border and Noble, 1993; Border and Noble, 1994a; Border and Noble, 1994b]. There is a growing body of evidence which supports the crucial role of TGF-β1 in the accumulation of ECM in vivo. Isaka et al. reported that introduction of a TGF-β1 cDNA into the normal glomerulus induced ECM deposition [Isaka et al. 1993]. Border and coworkers demonstrated that systemic inhibition of TGF-β activity by anti-TGF-β antiserum or decorin (the natural inhibitor of TGF-β) attenuated accumulation of ECM in an acute model of anti-Thy 1 glomerulonephritis [Border et al. 1990; Border et al. 1992; Isaka et al. 1996a; Isaka et al. 1996b]. Similarly, Akagi et al. showed that inhibition of TGF-β1 expression by antisense oligodeoxynucleotides suppressed ECM accumulation in the same experimental disease [Akagi et al. 1996]. These data strongly suggest that excessive action of TGF-β1 contributes to the transient expansion of ECM in certain glomerulonephritidies.

On the other hand, it still remains unclear whether sustained, local expression of TGF-β leads to irreversible glomerulosclerosis. To address this issue, Sanderson et al. generated transgenic mice that express TGF-β1 under the control of an albumin promoter [Sanderson et al. 1995]. These mice abundantly produced biologically active TGF-β1 in the liver, exhibited high levels of circulating, mature TGF-β1 and developed progressive glomerulosclerosis. Histological analyses of the glomerulus revealed substantial deposition of immunoglobulins. The glomerulosclerosis observed in this model may represent immune-complex-mediated glomerular injury. Nevertheless, the findings mentioned above suggest a possible role for TGF-β in the generation of irreversible glomerulosclerosis.
1.7. **ANTI-INFLAMMATORY ACTION OF TGF-β IN THE GLOMERULUS**

1.7.1. **GENERAL ANTI-INFLAMMATORY PROPERTIES**

Several studies have described the beneficial effects of TGF-β on certain pathologic situations. For example, TGF-β enhances wound healing and bone formation [Mundy, 1991; Sporn and Roberts, 1993]. This molecule acts as an autocrine tumour suppressor [Coombs et al. 1993; Markowitz et al. 1995] and may inhibit the pathogenesis of atherosclerosis [Grainger et al. 1994; Grainger et al. 1995]. Other beneficial properties of TGF-β include protection of tissue cells from reperfusion injury [Lefer et al. 1993] and/or suppressing actions of certain cytokines and reactive oxygen/nitrogen species [Lefer, 1991; Perrella et al. 1996].

Lymphocytes and monocytes/macrophages possess high affinity TGF-β receptors. It has been shown that TGF-β causes significant suppression of immune functions and inflammatory processes. This molecule, for example, inhibits proliferation of T cells, B cells, thymocytes and natural killer cells [Rook et al. 1986; Kehrl et al. 1986a]. It inhibits certain functions of lymphocytes including inhibition of immunoglobulin production by B lymphocytes [Kehrl et al. 1991] and cytokine production by thymocytes/T lymphocytes [Ristow, 1986; Kehrl et al. 1986b]. TGF-β inhibits neutrophil and T cell adhesion to the endothelium [Gamble and Vadas, 1988] and deactivates macrophages [Tsunawaki et al. 1988]. This molecule also antagonises immunomodulatory effects of inflammatory cytokines IL-1, IL-2, IL-3, colony stimulating factors and interferons α and γ [Wahl et al. 1989].
In several inflammatory diseases, TGF-β exerts anti-inflammatory effects. Systemic administration of TGF-β attenuates the activity of experimental autoimmune diseases including collagen arthritis, allergic encephalomyelitis, insulitis, Sjögren syndrome and systemic lupus erythematosus [Brandes et al. 1991; Kuruvilla et al. 1991; Raz et al. 1993; Pankewycz et al. 1994; Dang et al. 1995].

The crucial role of constitutively expressed, endogenous TGF-β1 was raised by two groups which showed that targeted disruption of the mouse TGF-β1 gene resulted in a multifocal inflammatory disease [Shull et al. 1992; Kulkarni et al. 1993]. TGF-β thus plays an important role in the maintenance of normal immune and organ functions.

**Figure 1.2:** Profibrotic and anti-inflammatory actions of TGF-β.

Profibrotic actions of TGF-β include stimulation of extracellular matrix (ECM) production, inhibition of matrix degradation and enhancement of matrix assembly. TGF-β exerts its anti-
inflammatory actions via inhibiting mitogenesis and cytokine responses of glomerular cells and suppressing several functions of infiltrating leukocytes.

1.7.2. INHIBITION OF GLOMERULAR CELL PROLIFERATION

TGF-β is a potent inhibitor of mitogenesis in the glomerulus. Externally added TGF-β inhibits proliferation of cultured mesangial, epithelial and endothelial cells [Jaffer et al. 1989; Nakamura et al. 1992; Choi and Ballermann, 1995]. Similarly, TGF-β1 inhibits the proliferative response of isolated normal glomeruli [Kitamura et al. 1995]. A possible mechanism responsible for the antiproliferative action of TGF-β is the intervention in cell-cycle machinery [Derynck, 1994; Alexandrow and Moses, 1995; Shoecklmann et al. 1996].

During the course of glomerulonephritis, mitogenic mediators released by infiltrating cells play crucial roles in stimulating resident glomerular cells to proliferate. For example, Lovett et al. have reported that macrophage-derived IL-1 stimulates mesangial cell mitogenesis in vitro [Lovett et al. 1983b]. It has been shown that TGF-β inhibits production of cytokines by leukocytes [Bogdan et al. 1992]. TGF-β may exert its antiproliferative effect on glomerular cells, in part, via inhibition of the release of mitogenic cytokines by activated local leukocytes.

Despite the accumulating data in vitro, the effect of TGF-β on glomerular cell proliferation in vivo is still controversial. Isaka et al. reported that transfection of a TGF-β1 gene into the normal rat glomerulus induced modest mitogenesis of resident cells [Isaka et al. 1993]. Using antisense oligonucleotides, the same group recently showed that suppression of TGF-81 expression in anti-Thy 1 glomerulonephritis did
not affect cell proliferation [Akagi et al. 1996]. Using the similar experimental model and an *ex vivo* gene transfer approach, Kitamura et al. demonstrated that introduction of a mutated gene coding for the active form of TGF-β1 suppressed mitogenesis of glomerular cells [Kitamura et al. 1995].

1.7.3. *SUPPRESSION OF CYTOKINE RESPONSES OF THE GLOMERULUS*

In the kidney, IL-1 and TNF-α are involved in the pathogenesis of several glomerular diseases [Sedor et al. 1992; Sedor, 1992]. These cytokines induce mesangial cell proliferation, leukocyte influx and production of injurious mediators which may lead to the generation of glomerular injury [Pfeilschifter and Schwartzenbach, 1990; Zoja et al. 1991; Sedor et al. 1992]. TGF-β has the ability to counteract the effects of proinflammatory cytokines [Dubois et al. 1990; Dinarello, 1991; Redini et al. 1993; van Beuningen et al. 1993]. It has been shown that mesangial cells and/or glomeruli treated with TGF-β1 exhibited blunted responses to IL-1β and TNF-α [Pfeilschifter and Vosbeck, 1991; Kitamura et al. 1995; Danoff and Jiang, 1996; Vervoolderdonk et al. 1996]. In some particular pathological situations, TGF-β could serve as a safeguard of the glomerulus by inhibiting the actions of these proinflammatory cytokines. Preliminary evidence by Nakagawa et al. showed that administration of the TGF-β inhibitor decorin into rats subjected to anti-GBM nephritis led to exacerbation of the disease; i.e., enhanced glomerular cellularity, accelerated crescent formation and increased proteinuria [Nakagawa et al. 1994]. Expression of TGF-β is observed in this experimental model. Since IL-1 plays a crucial role in the generation of anti-GBM nephritis [Lan et al. 1993a; Tang et al. 1994], TGF-β might exhibit its beneficial effects by attenuating the actions of IL-1.
1.7.4. **TGF-β AND MACROPHAGE FUNCTION**

Infiltration of monocytes/macrophages is a typical feature of glomerulonephritis [Main et al. 1992]. These infiltrating cells are mainly monocytes/macrophages [Hooke et al. 1987].

Activated macrophages secrete various inflammatory mediators including cytokines, proteinases and reactive oxygen/nitrogen species [Nathan, 1987], and contribute to glomerular damage [Baricos and Shah, 1991; Shah, 1991; Sedor, 1992;]. Using glomerular cells in culture, several studies have reported the role of macrophages in glomerular cell activation. For example, conditioned media from activated macrophages induce mesangial cell mitogenesis and matrix production [Lovett et al. 1983b; Zoja et al. 1991].

TGF-β is known to be a powerful macrophage deactivator [Nathan, 1991]. At picomolar concentrations, for example, TGF-β suppresses generation of hydrogen peroxide by macrophages [Tsunawaki et al. 1988]. TGF-β may act as a potential defender against macrophage-mediated glomerular injury.

1.7.4.1. **Attenuation of macrophage adhesiveness**

Adhesiveness of macrophages is an important factor which determines retention of macrophages at inflammatory sites. Adhesion also serves as a priming stimulus for functional alteration in monocytes/macrophages; e.g., differentiation, migration,
phagocytosis, oxidative burst and gene expression [Springer and Anderson, 1986; Haskill et al. 1988; Newman and Tucci, 1990; Shaw et al. 1990; Azevedo and de Souza, 1992; Xing et al. 1992]. Based on these, adhesion of macrophages within the glomerulus may be a crucial event which regulates the activity of local macrophages.

Macrophages express a variety of adhesion receptors for cellular and matrix ligands, including integrins and scavenger receptors. Monocytes/macrophages utilise integrins for adhesion to ECM within the microenvironment [Rosen and Gordon, 1987; Hynes, 1992]. Macrophages also use scavenger receptors and complement receptor 3 (CR3; β2 integrins) to adhere to various biological and artificial substrata including tissue culture plastic [Fraser et al. 1993; Hogasen et al. 1995; Hughes et al. 1995]. It has been reported that TGF-β1 downregulates expression of scavenger receptors and CR3 in macrophages [Bottalico et al. 1991; Hogasen et al. 1995].

1.7.4.2. Suppression of macrophage cytokine synthesis

Activated macrophages are capable of producing a variety of inflammatory cytokines including IL-1β, IL-6 and TNF-α. These macrophage-derived factors cause mitogenesis, production of ECM and secretion of inflammatory mediators by resident glomerular cells [Melcion et al. 1982; Lovett et al. 1983b], thus contribute to glomerular injury. Expression of IL-1, IL-6 and TNF-α is detectable in several types of glomerular diseases [Tang et al. 1994] where macrophages play a crucial role. Previous reports have shown that TGF-β1 inhibits production of interferon-γ, TNF-α, IL-1 and lymphotoxin by peripheral blood mononuclear cells and peritoneal macrophages [Espevik et al. 1987; Bogdan et al. 1992].
1.7.4.3. Suppression of macrophage production of other inflammatory mediators

1.7.4.3.1. Reactive oxygen intermediates

Reactive oxygen intermediates (superoxide anion, hydrogen peroxide and the hydroxyl radical) have been recognised as crucial mediators in the pathogenesis of glomerular injury [Shah, 1991]. Oxygen radical metabolites generated by infiltrating cells and/or resident glomerular cells can induce cell dysfunction and death, leading to progression of disease. In certain glomerulopathies, reactive oxygen species have been shown to play a role in the degradation of GBM leading to proteinuria [Shah, 1991]. Shah et al. showed that oxidants induce glomerular expression of chemoattractants of monocytes, reduce the activity of ADPase that inhibits thrombus formation and, thereby, contribute to the pathogenesis of proliferative glomerulonephritis [Shah, 1991].

Reactive oxygen species from neutrophils and monocytes/macrophages have been demonstrated to play a pathogenic role during the course of glomerular inflammation [Shah, 1991]. In response to various stimuli, these cells exhibit respiratory burst which leads to increased generation of reactive oxygen metabolites. Enhanced generation of reactive oxygen metabolites has been reported in macrophages isolated from glomeruli subjected to anti-GBM nephritis and anti-Thy 1 glomerulonephritis [Boyce et al. 1989; Oberle et al. 1992].

It is known that TGF-β1 and TGF-β2 strongly inhibit the respiratory burst of macrophages at picomolar concentrations [Tsunawaki et al. 1988]. In both experimental diseases, TGF-β is upregulated in the glomerulus [Border and Noble,
TGF-β might, therefore, be a protective molecule by inhibiting macrophage functions in macrophage-dependent glomerular injury.

1.7.4.3.2. **Nitric oxide**

Recent studies have implicated a crucial role for NO as an important regulator of a wide variety of physiological functions in mammalian organs, including the kidney [Cattell and Cook, 1993]. NO is generated from L-arginine by NO synthase (NOS). In the glomerulus, expression of constitutive NOS is observed [Cattell and Cook, 1993]. The basal production of NO may play a role in the regulation of glomerular haemodynamics [Raij and Baylis, 1995]. In contrast, expression of the inducible form of nitric oxide synthase (iNOS) is observed in various forms of glomerulonephritis, including anti-GBM nephritis, Heymann nephritis, anti-Thy 1 nephritis and in situ immune complex glomerulonephritis [Cattell and Cook, 1993]. The severity of certain glomerular diseases is correlated with increased iNOS expression in the glomerulus. The crucial role for NO has been demonstrated in autoimmune glomerulonephritis and anti-Thy 1 nephritis using NOS inhibitors [Weinberg et al. 1994; Narita et al. 1995]. In normal and nephritic glomeruli, infiltrating monocytes/macrophages have been identified as the major sources of elevated NO [Cattell and Cook, 1993]. However, resident mesangial cells also have the ability to express iNOS in response to proinflammatory cytokines including IL-1β and TNF-α [Cattell and Cook, 1993].

Expression of iNOS by activated macrophages is inhibited by several mediators including IL-4, IL-10 and TGF-βs [Oswald et al. 1992]. It has been shown that TGF-β1, -β2 and -β3 suppress generation of NO by activated macrophages [Ding et al.
Currently, the precise mechanism involved in the regulation of glomerular iNOS expression in normal and pathological conditions has not been fully elucidated.

1.7.4.3.3. **Metalloproteinases**

Members of the family of matrix metalloproteinases (also called collagenases or gelatinases) are key enzymes in matrix degradation. Glomerular expression of metalloproteinases including gelatinase A, gelatinase B and stromelysin is enhanced in certain glomerular inflammatory processes such as membranoproliferative glomerulonephritis and diabetic nephropathy [Baricos and Shah, 1991; Lovett et al. 1992; Maynard et al. 1994].

Gelatinase B synthesised by infiltrating leukocytes and resident glomerular cells degrades type IV and V collagens that are abundantly present in the glomerular ECM [Couchman et al. 1994]. Tightly controlled expression of this enzyme is linked to embryogenesis and wound healing, and its uncontrolled expression is associated with certain pathologies such as tumour invasion/metastasis and inflammation [Liotta et al. 1980; Unemori et al. 1991; Sarén et al. 1996]. Excessive glomerular production of gelatinase B leads to structural alteration of the mesangial matrix and GBM [Baricos and Shah, 1991; Couchman et al. 1994]. Currently, however, the mechanisms involved in the regulation of gelatinase B expression in normal and diseased glomeruli have not been explored.

Stromelysin (transin) is a multipotent enzyme which degrades basement membrane collagens as well as proteoglycans and matrix glycoproteins [Reynolds, 1996]. Similarly to gelatinase B, stromelysin plays a role in organ development and wound healing [Matrisian et al. 1986]. Enhanced mesangial expression of stromelysin has
been reported in proliferative glomerulonephritis [Marti et al. 1992]. Overexpression of stromelysin induces an aberrant breakdown of the glomerular matrix and thereby contributes to its structural and functional alteration [Baricos and Shah, 1991]. Mesangial cells have the ability to produce stromelysin abundantly in response to the macrophage-derived proinflammatory cytokine IL-1β [Kitamura et al. 1994a]. When stromelysin was overexpressed via genetic manipulation, mesangial cells exhibited an altered phenotype: enhanced mitogenesis and migration [Kitamura et al. 1994b; Kitamura, 1998].

The expression of metalloproteinases is regulated by cytokines (particularly IL-1), growth factors and hormones [Reynolds, 1996]. Generally, TGF-β downregulates synthesis of metalloproteinases including gelatinase B and stromelysin [Roberts and Sporn, 1990].
CHAPTER TWO

MATERIALS AND METHODS
2.1. MATERIALS

2.1.1. REAGENTS AND CULTURE MEDIA

Chemicals of analytical or tissue culture grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified. Solvents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture dishes were purchased from Greiner (Dursley, Gloucestershire, UK). Other tissue culture plasticware was purchased from Becton Dickinson (Lincoln Park, NJ, USA).

Dulbecco’s modified Eagle’s medium/F-12 (DME-F12), foetal calf serum (FCS) and phosphate bufferd saline (PBS) were purchased from GIBCO BRL (Gaithersburg, MD, USA). Human TGF-β1 was obtained from Genzyme (Cambridge, MA, USA). For blocking studies, an anti-TGF-β1 neutralising antibody was obtained from Promega (Southampton, Hampshire, UK). Radioactive isotope-labelled materials were purchased from Amersham International Ltd (Little Chalfont, Buckinghamshire, UK). Laminin, collagen type IV or basement membrane Matrigel™ were purchased from Collaborative Biomedical Products / Becton Dickinson Labware (Bedford, MA, USA). For immunoblot analyses a Vectastain ABC Kit and a peroxidase substrate kit AEC were obtained from Vector Laboratories (Peterborough, Cambridgeshire, UK).
2.1.2. **CELLS**

The macrophage cell line J774.2 and the endothelial cell line ECV304 were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). The macrophage cell line NR8383, the fibroblastic cell lines NRK49F, NIH3T3 and the epithelial cell lines LLCPK1, MDCK and CCL64 from the American Type Culture Collection (Rockville, MD, USA).

Mesangial cell clones SM43 and FM14 were established from isolated glomeruli of a male Sprague-Dawley and Fisher rat by a limiting dilutional method and identified as being of mesangial cell phenotype by their morphological features and positive staining for desmin, α-smooth muscle actin and Thy 1 [Kitamura et al. 1994c]. Antibodies used were: rabbit antidesmin antiserum (1:20 dilution), mouse anti-mesangial cell monoclonal antibody 1-22-3 [Kawachi et al. 1992b] (1:20 dilution), mouse anti-α-smooth muscle actin monoclonal antibody (1:200 dilution), goat anti-rabbit immunoglobulins fluorescein isothiocyanate (FITC) (1:32 dilution), and goat anti-mouse immunoglobulins FITC (1:50 dilution) [Kitamura et al. 1994c].

SM43 mesangial cells stably transfected with a rat IL-10 cDNA [Feng et al. 1993], a human IL-13 cDNA [Minty et al. 1993], and a human IL-1 receptor antagonist cDNA [Yokoo and Kitamura, 1996a] were established using a modified calcium phosphate co-precipitation method [Kitamura et al. 1994b]. FM14 mesangial cells transfected with a porcine TGF-β cDNA [Samuel et al. 1992] were also established.
All cells were maintained in DME-F12, supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 10 % FCS. DME-F12 containing 1 % FCS (1%FCS/DME-F12) was generally used as the basal assay medium.

For the preparation of isolated glomeruli, male Sprague-Dawley rats (3 - 5 months old) were sacrificed and glomeruli were isolated from their kidneys by the conventional sieving method. Purified glomeruli were collected and used for primary culture. DME-F12 and 15 % FCS was used as culture medium [Kitamura et al. 1991].

2.1.3. CONDITIONED MEDIA

2.1.3.1. Mesangial cell-conditioned medium

To prepare conditioned media, confluent cultures of each cell line grown in the presence of 10 % FCS in 100 mm culture plates were washed twice with PBS and incubated in 4 ml of 1%FCS/DME-F12 at 37 °C for 48 hours. The conditioned media were then passed through a 0.2 μm filter (Nalgene, Rochester, NY) and stored at -80 °C until use. The culture medium conditioned by SM43 was used as the standard mesangial cell-conditioned medium. DME-F12 containing 1 % FCS was also incubated in the absence of cells for 48 hours, filtered, and used as control medium for
assays. In a certain experiment, conditioned medium prepared from serum-depleted, inactivated mesangial cells was utilised. For this purpose, confluent SM43 cells were precultured in 0.5%FCS/DME-F12 for 72 hours and then incubated in 4 ml of 1%FCS/DME-F12 for 48 hours.

2.1.3.2. Macrophage-conditioned medium

Macrophage-conditioned media were prepared as follows. NR8383 cells were seeded in 6-well plates at a density of 5 x 10^5 cells/well and cultured in 2 ml 1% FCS/DME-F12. In certain experiments, cells were exposed to 1:1 diluted mesangial cell-conditioned medium during this pre-incubation. After 6 hours, cultures were stimulated with or without 1 µg/ml of LPS (Escherichia coli 0111:B4; Sigma) and incubated for an additional 18 hours. Media were then replaced with 2 ml of fresh DME-F12 containing 1 % FCS, and cells were further incubated for 24 hours. The media were collected, filtered and used for experiments.

2.1.3.3. Medium conditioned by isolated glomeruli

Conditioned media were prepared from isolated normal and nephritic glomeruli. To create the latter, anti-Thy 1 glomerulonephritis was induced by the monoclonal antibody 1-22-3 [Kawachi et al. 1992b] in adult male Sprague-Dawley rats (body weight 250-350 g; 4 rats), as described previously [Kitamura et al. 1994c; Kitamura et al. 1995]. In this nephritis model, glomerular expression of TGF-β1 is up-regulated.
from day 4 and sustained at least until day 14 [Okuda et al. 1990]. Media conditioned by normal or nephritic (day 5, 7 and 14) glomeruli were prepared by incubating isolated glomeruli (1 x 10^4) in 1 ml of 0.5% FCS/DME-F12 for 24 hours. Conditioned media diluted at 1:4 (20%) were used for assessment of TGF-β bioactivity.

2.2. METHODS

2.2.1. EFFECT OF MESANGIAL CELL-DERIVED TGF-β ON MACROPHAGE ADHESIVENESS

2.2.1.1. Experimental design

Adhesiveness of macrophages is an important factor that determines retention and activity of macrophages at inflammatory sites. The aim of this study was to investigate the role of mesangial cell-derived factors in macrophage adhesiveness. Mouse and rat macrophages were seeded on different substrata, exposed to mesangial cell-conditioned medium, and their adhesiveness was examined. To determine the specificity of the effect of mesangial cell-derived medium, media conditioned by other cell lines were also tested. Amongst various putative mediators, a role for mesangial cell-derived TGF-β1 was especially investigated. We examined production of active TGF-β1 by cultured mesangial cells. The effect of exogenous TGF-β1 on macrophage
adhesiveness was tested using serial concentrations of human TGF-β1. To identify TGF-β1 as one of the mesangial cell-derived molecules involved in this process, an anti-TGF-β1 neutralising antibody was used for blocking studies.

To examine whether altered macrophage adhesiveness is associated with altered macrophage function, mitogenic activity and cytokine expression were examined in adherent and non-adherent macrophages exposed to mesangial cell-conditioned medium.

2.2.1.2. Assessment of macrophage adhesiveness

Murine J774.2 macrophages (5x10^4 cells/well) were suspended in 2 - 10 times diluted mesangial cell-conditioned medium or control medium and plated in 24-well tissue culture plates. Mesangial cell-conditioned medium diluted at 1:1 with 1%FCS/DME-F12 was generally used for studies. The morphology of macrophages was assessed by light microscopic analysis. The number of floating cells was determined at 30 minutes and 24 hours after seeding. Viability of the cells was assessed by trypan blue exclusion. Reversibility of macrophage adhesiveness was examined by replating detached cells in fresh control medium. The same study was also performed using NR8383 rat macrophages.

To investigate the effect of mesangial cell-conditioned medium on the adhesion of macrophages onto extracellular matrices, tissue culture plates were coated with laminin, collagen type IV or basement membrane Matrigel™. The effect of mesangial cell-conditioned medium was tested as described above.
To examine the effect of TGF-β1 on macrophage adhesiveness, human TGF-β1 (0 - 10 ng/ml) was added to cultures of macrophages. To investigate the involvement of TGF-β1 as an active component in mesangial cell-conditioned medium, conditioned medium was prepared in the absence of FCS. The conditioned medium was pre-incubated with or without 25 μg/ml of an anti-TGF-β1 neutralising antibody for 10 minutes at room temperature, diluted at 1:9 with 1% FCS/DME-F12, and retested. The specificity of the anti-TGF-β1 neutralising antibody was confirmed by the observation that the CCL64 cell growth-inhibitory effect of TGF-β1 was abrogated by the antibody, but not by non-immune rabbit IgG (data not shown).

Heat-inactivated conditioned medium was utilised to examine involvement of thermolabile factors as active entities in mesangial cell-conditioned medium. Mesangial cell-conditioned medium or control medium diluted at 1:9 was heated at 80 °C for 10 minutes, and the efficacy was retested. Generally, heat treatment inactivates the majority of peptide factors with the exception of certain molecules including TGF-β1 [Kitamura et al. 1996].

2.2.1.3. Assessment of macrophage mitogenesis

J774.2 macrophages suspended in mesangial cell-conditioned medium diluted at 1:1 were seeded in 24-well plates at a density of 2.5 x 10^4 cells/well. After incubation for 48 hours, the cells were pulsed with 1 μCi of [3H]-thymidine per well for 3 hours. Adherent and non-adherent macrophages were then harvested separately, and the cell numbers were counted using a haemocytometer. Incorporation of radioactivity was measured by liquid scintillation counting, and values expressed as [3H]-thymidine incorporation per cell.
2.2.1.4. Assessment of cytokine expression in adherent and non-adherent macrophages

J774.2 macrophages were cultured for 24 hours in the presence of mesangial cell-conditioned medium (1:1 dilution) or control medium and stimulated by LPS (1 µg/ml) for an additional 24 hours. Adherent and non-adherent cells were separately harvested from identical culture plates, and Northern blot analysis was performed on the expression of IL-1β and IL-6 [Kitamura et al. 1994b]. For hybridisation, a mouse IL-1β cDNA [Gray et al. 1986] and a mouse IL-6 cDNA [van Snick et al. 1988] were used.

2.2.1.5. Assessment of TGF-β1 production by mesangial cells

2.2.1.5.1. Northern blot analysis

Expression of TGF-β1 mRNA in mesangial cells was evaluated by Northern blot analysis. Total RNA was extracted by a single-step method [Chomczynski and Sacchi, 1987]. In brief, confluent cultures of mesangial cells were washed with ice-cold PBS three times. Cells were lysed in 500 µl of lysis buffer (4 M guanidinium thyocyanate, 25 mM sodium citrate, 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol) with shearing by a 23 G needle. Sequentially, 50 µl of 2 M sodium acetate, 500 µl of water-saturated phenol and 100 µl of chloroform-isoamylalcohol mixture were added and mixed, followed by centrifugation at 4 °C for 20 minutes. The resultant aqueous phase was
transferred into a new tube, and re-centrifuged for 10 minutes. RNA was precipitated by 600 µl of isopropanol. After centrifugation, the pellet was dissolved in Tris-EDTA (pH 7.4). 10 µg of each sample was electrophoresed on 1.2 % agarose gel. RNA was transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using 1 M ammonium acetate.

For hybridisation, a porcine TGF-β1 cDNA [Samuel et al. 1992] was labelled with $^{32}$P-dCTP using a random priming method [Maniatis et al. 1989]. The membranes were hybridised with the probe overnight at 65 °C, then washed under non-stringent conditions (4X SSC, 0.5 % SDS, 50 °C) and exposed to Kodak XAR films at - 80 °C. Densitometric analysis was performed using a computerised program, NIH Image. Values were normalised relative to the level of β-actin mRNA which was used as a loading control.

2.2.1.5.2. Western blot analysis

Two milliliters of control media or mesangial cell-conditioned media containing 0.5 % FCS was precipitated with the same volume of 10 % trichloroacetic acid at 4 °C. After centrifugation, each pellet was dissolved in 10 µl of 0.5 N sodium hydroxide, electrophoresed in a 10 % polyacrylamide gel, and transferred electrophoretically onto a nitrocellulose membrane [Kitamura et al. 1995]. Immunoblot analysis was performed using an anti-human TGF-β1 antibody (1:500 dilution), a Vectastain ABC Kit and a peroxidase substrate kit AEC following the protocols provided by the manufacturers.
2.2.1.5.3  Bioassay

The growth inhibition assay of the mink lung epithelial cell line, CCL64 [Cone et al. 1988], was used to determine the biological activity of TGF-β in mesangial cell-conditioned medium [Kitamura et al. 1995]. CCL64 cells were seeded in 24-well plates at a density of $5 \times 10^3$ cells/well. After incubation for 24 hours in the presence of 10 % FCS, cells were further incubated in 1 % FCS/DME-F12 for 24 hours and then exposed to serially diluted mesangial cell-conditioned medium. After 48 hours, $[^3]$H]-thymidine (1 μCi/well) was added, incubated for 3 hours, and incorporation of radioactivity was measured by liquid scintillation counting (Tri-Carb® Liquid Scintillation Analyzer, Canberra Packard, Bangbourne, Berkshire, UK). As standards, activated human TGF-β1 (0 - 5 ng/ml) was used.

2.2.2.  EFFECT OF MESANGIAL CELL-DERIVED TGF-β ON MACROPHAGE CYTOKINE SYNTHESIS

2.2.2.1.  Experimental design

The aim of this study was to investigate the effect of mesangial cell-derived factors on cytokine production by macrophages. First, induction of IL-1β, IL-6 and TNF-α was examined in macrophages stimulated by LPS. To assess whether macrophages secrete the active form of cytokines, the biological activity of IL-1 was evaluated in macrophage-conditioned media.
To investigate the effect of mesangial cell-derived medium on the induction of cytokines in macrophages, macrophages were treated with serial dilutions of mesangial cell-derived medium and stimulated with LPS. The effect of conditioned media derived from other cell lines as well as isolated normal and nephritic glomeruli was also examined.

Mesangial cells produce biologically active TGF-β1. To examine the effect of mesangial cell-derived TGF-β1 on macrophage cytokine expression, macrophages were pretreated with several concentrations of TGF-β1 and then stimulated. Expression of cytokines was examined by Northern blot analysis. To identify TGF-β1 as the macrophage deactivator in mesangial cell-derived medium, a blocking experiment with a neutralising anti-TGF-β1 antibody was performed.

2.2.2.2. Northern blot analysis of macrophage cytokine synthesis

J774.2 macrophages (5 x 10^5 cells/well) were cultured in 6-well culture plates for 24 hours, and then stimulated with LPS (1 μg/ml). After 3, 6, 9, 12 and 24 hours, cells were harvested for Northern blot analysis of IL-1β, IL-6 and TNF-α.

To examine the effect of mesangial cell-derived factors on the cytokine expression, macrophages were pretreated with diluted (2 - 40 times) mesangial cell-conditioned media for 6 hours and then stimulated with LPS for 18 hours. In certain experiments, macrophages were stimulated with LPS for 6 hours and then treated with diluted (1:1) mesangial cell-conditioned media for 18 hours. The effect of mesangial cell-conditioned media pretreated with heat (80 °C, 10 minutes) was also tested.
To examine the effect of TGF-β1 on macrophage cytokine synthesis, human TGF-β1 (0 - 10 ng/ml) was added to cultures of macrophages. To identify TGF-β1 as an active component in mesangial cell-conditioned medium, mesangial cell-conditioned medium pre-incubated with an anti-TGF-β1 neutralising antibody (5 µg/ml) was used.

Northern blot analysis was performed, as described earlier. For the hybridisation, a mouse IL-1β cDNA [Gray et al. 1986], a mouse IL-6 cDNA [van Snick et al. 1988], and a human TNF-α cDNA [Pennica et al. 1984] were used.

2.2.2.3. Northern blot analysis of macrophage deactivator expression in mesangial cells

SM43 mesangial cells were cultured in 6-well plates in the presence of 10% FCS/DME-F12 and subjected to Northern blot analyses of macrophage deactivators IL-10, IL-13 and TGF-β1. As positive controls, mesangial cells stably transfected with IL-10, IL-13 and TGF-β1 were used.

2.2.2.4. Assessment of TGF-β1 production by mesangial cells

To determine that active TGF-β is present in mesangial cell-derived medium, Western blot analysis and bioassay were performed as described earlier.
2.2.2.5. Bioassay of IL-1

To evaluate the biological activity of IL-1 secreted by activated macrophages, gelatinase B expression in mesangial cells was used as an indicator. Control mesangial cells and mesangial cells expressing IL-1ra were exposed to medium conditioned by activated macrophages for 18 hours. Expression of gelatinase B was examined by Northern analysis. For hybridisation, a mouse gelatinase B cDNA [Tanaka et al. 1993] was used.

2.2.3. CROSS-TALK BETWEEN MACROPHAGES AND MESANGIAL CELLS IN CULTURE

2.2.3.1. Experimental design

The aim of this investigation was to examine cross-talk between macrophages and mesangial cells in more detail. For this purpose, we utilised co-culture of mesangial cells and macrophages. Using gelatinase B and iNOS as indicator molecules, this study aimed to explore cross-talk between these cells via paracrine regulators.

NR8383 macrophages, mesangial cells and macrophages co-cultured with mesangial cells were stimulated with LPS, and production of gelatinase B and iNOS was separately examined in macrophages and mesangial cells.
To identify the macrophage-derived stimulator for mesangial gelatinase B production, expression of IL-1β was examined in unstimulated and LPS-stimulated macrophages by Northern blot analysis. To confirm the role of macrophage-derived IL-1β as the stimulator, mesangial cells overexpressing IL-1ra were treated with macrophage-conditioned media, and gelatinase B production was examined by gelatin zymography.

To investigate mesangial cell-derived modifiers of macrophage gelatinase B and iNOS production, macrophages were treated with mesangial cell-derived media, stimulated with LPS and subjected to Northern and zymographic analyses.

To identify the macrophage inhibitor produced by mesangial cells, expression of TGF-β1 was examined. The effect of externally added TGF-β1 on gelatinase B and iNOS expression in activated macrophages was also investigated. For blocking experiments, an anti-TGF-β1 neutralising antibody was used.

2.2.3.2. Gelatin zymography

Production of gelatinase B by mesangial cells was examined by gelatin zymography. Mesangial cells suspended in 10 % FCS/DME-F12 were seeded in 24-well plates at a density of 1 x 10^5 cells/well. After 24 hours, cells were washed and pre-incubated in 1 % FCS/DME-F12 with or without 1 μg/ml of LPS or 10 ng/ml of IL-1β (human recombinant; Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan) for 24 hours. Media were then replaced with fresh 1% FCS/DME-F12 (0.3 ml/well), incubated for an additional 24 hours and collected for analysis. To examine the effect of macrophage-conditioned media, mesangial cells (SM43 cells and SM43 cells expressing IL-1ra)
were exposed to conditioned media diluted at 1:1 with 1% FCS/DME-F12 during the pre-incubation period.

To investigate the effect of mesangial cell-conditioned medium and TGF-β1 on macrophage production of gelatinase B, NR8383 macrophages suspended in 1% FCS/DME-F12 were seeded in 24-well tissue culture plates at a density of 5 x 10^4 cells/well and exposed to; i) 2 - 10 times diluted (10 - 50 %) mesangial cell-conditioned medium, ii) diluted (1:9) conditioned medium pretreated with an anti-TGF-β1 neutralising antibody (30 μg/ml; Promega, Southampton, UK), or iii) 0.05 - 10 ng/ml of TGF-β1 (Genzyme, Cambridge, MA). After 6 hours, cells were stimulated with LPS (1 μg/ml), incubated for an additional 18 hours, and media were collected for zymographic analyses.

Production of gelatinase B by co-culture of mesangial cells and macrophages was examined as follows. Mesangial cells suspended in 10% FCS/DME-F12 were seeded in 6-well plates at a density of 1 x 10^6 cells/well. After 24 hours, media were replaced with 1% FCS/DME-F12 (2 ml/well) and further incubated for 24 hours. Macrophages were then seeded on plastic or on mesangial cells at a density of 5 x 10^5 cells per well. After 24 hours, media were replaced with fresh 1% FCS/DME-F12 (4 ml/well) containing 1 μg/ml of LPS. After a 24 hour incubation period, media were collected and subjected to gelatin zymography.

Zymographic analysis of gelatinase B was performed as follows [Yokoo and Kitamura, 1996b; Kitamura et al. 1995]. Aliquots of conditioned media (5 μl) were mixed with 5 μl of 2X sample buffer (4 % SDS, 0.005 % bromophenol blue, and 20 % glycerol in 0.5 M Tris-HCl; pH 6.8) and applied to 10 % acrylamide gels containing 0.1 % gelatin. After electrophoresis, SDS was removed from the gels by incubation in 2.5 % Triton X-100 at room temperature for 30 minutes. The gels were
then incubated overnight at 37 °C in a developing buffer containing 0.2 M NaCl, 5 mM CaCl₂, and 0.02 % Brij35 in 50 mM Tris-HCl (pH 7.6). Gels were stained in a solution containing 30 % methanol, 10 % glacial acetic acid and 0.5 % Coomassie G250 and destained in the same solution without dye. To quantify gelatinolytic activity, transparent bands on gels were inverted to positive images and subjected to densitometric analyses using Image Master ID (Pharmacia Biotech, Uppsala, Sweden). All the zymographic data are presented as positive images.

2.2.3.3. **Northern blot analysis**

Expression of gelatinase B, IL-1β and iNOS in macrophages was investigated by Northern blot analysis. NR8383 macrophages suspended in 10% FCS/DMEM-F12 were seeded in 6-well plates at a density of 1 x 10⁶ cells/well and stimulated with or without LPS (1 µg/ml). In some experiments, macrophages were pretreated with 1:1 diluted mesangial cell-conditioned medium or 10 ng/ml of TGF-β1 for 6 hours and then stimulated by LPS. After 18 hours, cells were harvested and subjected to RNA extraction. To examine the expression of TGF-β1, gelatinase B and iNOS in mesangial cells, confluent cultures were incubated with or without 10 ng/ml of IL-1β for 24 hours.

The effect of “bystander” mesangial cells on macrophage gelatinase B and iNOS expression was investigated as follows. NR8383 macrophages were seeded on plastic or on confluent mesangial cells at a density of 5 x 10⁵ cells/well (6-well plates). After 6 hours, cultures were stimulated by LPS for 18 hours. Macrophages were then separated from mesangial cells and subjected to Northern analysis. In certain
experiments, expression of iNOS mRNA in mesangial cells co-cultured with macrophages was also determined.

Northern blot analysis was performed, as described earlier. For hybridisation, a mouse IL-1β cDNA [Gray et al. 1986], a mouse gelatinase B cDNA [Tanaka et al. 1993], a porcine TGF-β1 cDNA [Samuel et al. 1992] and a mouse iNOS cDNA [a gift from Dr. S. Moncada] were labelled with $^{32}$P-dCTP using a random priming method. Values were normalised relative to the levels of β-actin and GAPDH mRNAs that were used as loading controls.

2.2.3.4. Assessment of TGF-β1 production by mesangial cells

To determine that active TGF-β is present in mesangial cell-derived medium, Western blot analysis and bioassay were performed as described earlier.

2.2.4. CROSS-TALK BETWEEN MACROPHAGES AND MESANGIAL CELLS IN THE GLOMERULUS

2.2.4.1. Experimental design

The aim of this study was to investigate the cross-talk between resident glomerular cells and macrophages in the glomerular microenvironment.
To examine whether activated macrophages can affect functions of resident cells, activated reporter macrophages were transferred into normal rat glomeruli via renal artery injection. After the cell transfer, glomeruli were isolated and incubated ex vivo for 24 hours. Induction of gelatinase B, iNOS and stromelysin in resident glomerular cells was examined by Northern blot analysis.

The role of endogenous TGF-β1 in the macrophage-mediated glomerular cell activation was investigated using nephritic glomeruli producing active TGF-β1. Activated reporter macrophages were transferred into normal rat kidneys or kidneys subjected to anti-Thy 1 glomerulonephritis. After the cell transfer, glomeruli were isolated, incubated ex vivo for 24 hours, and Northern blot analysis was performed on the expression of gelatinase B, iNOS and stromelysin.

2.2.4.2. Creation of reporter macrophages

In the presence of 10 µg/ml polybrene, NR8383 macrophages were exposed to 1:1 diluted conditioned medium from a helper-free packaging cell line ΩE/BAG [Kitamura et al. 1994c] that produces a replication-incompetent BAG virus. This retroviral vector introduces a bacterial β-galactosidase gene (lacZ) and a neomycin phosphotransferase gene (neo) under the control of a Moloney murine leukemia virus long terminal repeat and a simian virus 40 promoter, respectively [Price et al. 1987]. Stable infectants were selected in the presence of the neomycin analogue G418 (500 µg/ml), and the reporter macrophage BAGMAC<sup>NR</sup> was established (Figure 2.1.). Expression of β-
galactosidase was examined by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay [Kitamura, 1996].

Figure 2.1.: Establishment of reporter macrophages.

Reporter macrophage BAGMAC\textsubscript{NR} was established by transducing NR8383 alveolar macrophages with the replication-defective BAG retrovirus. This retrovirus introduces a bacterial β-galactosidase gene \textit{(lacZ)} and a neomycin phosphotransferase gene \textit{(neo)}. Stable infectants were selected in the presence of the neomycin analogue G418, and BAGMAC\textsubscript{NR} cells were established.

2.2.4.3. Transfer of reporter macrophages into the glomerulus

Via the renal artery, BAGMAC\textsubscript{NR} cells (1 x 10^6) previously treated with or without 1 μg/ml LPS for 14 - 24 hours were delivered into normal rat glomeruli or glomeruli in
the regeneration phase of acute anti-Thy 1 glomerulonephritis (day 7) (male Sprague-Dawley rats, 250-350 g) [Kitamura et al. 1994c]. Anti-Thy 1 glomerulonephritis was induced by intravenous injection with the monoclonal antibody 1-22-3 [Kawachi et al. 1992b]. In this experimental model, TGF-β1 is upregulated in mesangial cells during the regeneration of the glomeruli, i.e. from day 4 until day 14 [Okuda et al. 1990]. Immediately after the cell injection, both kidneys were removed and processed for glomerular isolation by the conventional sieving method [Kitamura et al. 1991]. One third (0.5 - 1 x 10⁴) of the glomeruli isolated from each kidney were immediately frozen at - 80 °C (Figure 2.2.).

**Figure 2.2.**: *Transfer of reporter macrophages into the glomerulus.*

BAGMAC^{NR} macrophages stimulated with LPS were transferred into normal rat glomeruli or glomeruli subjected to acute anti-Thy 1 glomerulonephritis (day 7). After the cell transfer, glomeruli were isolated and used for X-gal assay and Northern blot analysis.
2.2.4.4. X-gal assay

To evaluate cell transfer efficiency, isolated glomeruli were subjected to X-gal assay [Kitamura et al. 1994c; Kitamura, 1996]. Glomeruli were fixed in 0.5 % glutaraldehyde, 2 mM MgCl$_2$, and 1.25 mM EGTA in PBS at 4 °C overnight and incubated at 37 °C for 1 hour in a reaction buffer containing 1 mg/ml X-gal (Sigma), 20 mM K$_3$Fe(CN)$_6$, 20 mM K$_4$Fe(CN)$_6$•3H$_2$O, 2 mM MgCl$_2$, 0.01 % sodium deoxycholate and 0.02 % NP-40 in PBS. More than 100 glomeruli were randomly selected, and the percentages of X-gal-positive glomeruli were determined by light microscopy.

2.2.4.5. Neomycin subtraction method

Two thirds of the glomeruli isolated from normal kidneys or kidneys subjected to anti-Thy 1 glomerulonephritis were incubated *ex vivo* for 24 hours in 1% FCS/DME-F12 with or without G418 (200 µg/ml) and stored at -80 °C. Unmodified glomerular cells are susceptible to neomycin whereas BAGMAC$^{NR}$ cells transduced with *neo* are resistant to this drug. Using gelatinase B as an indicator, we showed that 200 µg/ml of neomycin reduced glomerular gelatinase B production to 21 % of the level of untreated glomeruli. In contrast, the production of gelatinase B by activated BAGMAC$^{NR}$ cells
was not affected by 200 μg/ml of G418. Similarly, Northern blot analyses revealed that this concentration of G418 abrogated expression of gelatinase B by isolated glomeruli whereas that of BAGMAC\textsuperscript{NR} cells was unaffected [Kitamura and Sütö, 1997b]. Using this difference, it is possible to subtract the contribution of resident cells from the total responses of BAGMAC\textsuperscript{NR}-containing glomeruli (Figure 2.3.).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure23.png}
\caption{Neomycin subtraction method.}
\end{figure}

BAGMAC\textsuperscript{NR} cells transduced with \textit{neo} are resistant to G418. In contrast, exposure of resident cells to G418 leads to cell death. Using this difference, it is possible to subtract the contribution of resident cells from the total responses of BAGMAC\textsuperscript{NR}-containing chimeric glomeruli.
2.2.4.6. Northern blot analysis

After the transfer of reporter BAGMAC<sup>NR</sup> macrophages into normal rat kidneys or kidneys subjected to anti-Thy 1 glomerulonephritis, glomeruli were isolated. One third of the glomeruli from each kidney was immediately stored at -80 °C. The remaining glomeruli were incubated *ex vivo* for 24 hours in the presence or absence of the neomycin analogue G418 (200 µg/ml) and then stored at -80 °C. Total RNA was extracted from isolated glomeruli and Northern blot analysis was performed as described earlier. For hybridisation, a rat stromelysin-1 cDNA [Matrisian et al. 1985], a mouse gelatinase B cDNA [Tanaka et al. 1993], and a mouse iNOS cDNA [a gift from Dr. S. Moncada] were labelled with <sup>32</sup>P-dCTP using a random priming method. Values were normalised relative to the levels of β-actin and GAPDH mRNAs that were used as loading controls.

2.2.4.7. Assessment of TGF-β activity

Conditioned media derived from normal and nephritic glomeruli (day 7) were diluted at 1:4, and tested for TGF-β activity using the growth inhibition assay of CCL64 cells, as described earlier [Kitamura et al. 1995].
2.2.5. STATISTICAL ANALYSIS

Assays were performed in quadruplicate. Data are expressed as means ± SE. Statistical analyses were performed using the non-parametric Mann-Whitney U test. \( P \) values less then 0.05 were considered to be significantly different.
CHAPTER THREE

RESULTS
3.1. IMPAIRMENT OF MACROPHAGE ADHESIVENESS BY MESANGIAL CELL-DERIVED TGF-β

3.1.1. EFFECT OF MESANGIAL CELL-DERIVED MEDIUM ON MACROPHAGE ADHESIVENESS

In inflammatory processes, adhesion of macrophages to substrata triggers several functional alterations in macrophages, subsequently leading to their activation. Activated macrophages secrete various mediators, which may contribute to further injury and destruction of glomerular structures. The purpose of this study was to evaluate the effect of mesangial cell-derived media on macrophage adhesiveness. Murine J774.2 macrophages were suspended in serial dilutions of mesangial cell-derived medium and plated on plastic substrata. The number of floating cells was determined at 30 minutes and 24 hours after seeding. After 30 minutes, over 99% of macrophages adhered, and mesangial cell-derived medium did not affect this initial adhesion (not shown). However, within 24 hours, mesangial cell-derived medium significantly induced detachment of macrophages in a concentration-dependent manner (Figure 3.1A.). The number of detached cells in mesangial cell-derived medium diluted at 1:1 was approximately 10 times (7 - 13 times) higher than that in control medium. To establish that the effect was not specific to J774.2 cells, the same study was conducted with NR8383 rat macrophages. Mesangial cell-derived medium affected adhesion of the rat macrophages in a similar fashion; i.e., 1:1 diluted conditioned medium generated 19 ± 1 fold (mean ± SE) increase in the number of detached NR8383 cells (data not shown). Subsequent studies were therefore confined to J774.2 cells.
In the glomerulus, mesangial cells are surrounded by ECM composed of collagens, glycoproteins and proteoglycans. Therefore, the effect of mesangial cell-derived media on macrophage adhesiveness to different ECM components was examined. A similar inhibitory effect of mesangial cell-derived medium on adhesiveness was observed in macrophages seeded on Matrigel, laminin, or collagen type IV (Figure 3.1B.). Interestingly, mesangial cell-derived medium was more effective in macrophages seeded on uncoated plastic than those on ECM-coated plates. All further experiments were, therefore, conducted using uncoated plastic as described below. Throughout the studies, more than 98% of detached macrophages were viable as determined by trypan blue exclusion.

To investigate whether the reduced adhesiveness was reversible, detached macrophages pretreated with mesangial cell-derived medium were suspended in control medium and replated into 24-well plates. As shown in Figure 3.1C., re-seeded macrophages exhibited significant impairment of initial adhesion at 30 minutes, but the majority of the cells regained adhesiveness within 24 hours. No significant difference in the number of non-adherent cells was detected at 24 hours compared to mesangial cell-derived medium-untreated macrophages.

We tested whether the activity of mesangial cell-derived medium is affected by activation states of mesangial cells. As demonstrated in Figure 3.1D., the conditioned medium from serum-depleted “inactive” cells also substantially reduced adhesiveness of macrophages. Compared to the standard mesangial cell-derived medium, however, the medium from inactivated cells showed modest but significant reduction in this activity (87 ± 2% vs. standard mesangial cell-derived medium (100%), mean ± SE, P<0.05).
Figure 3.1: Effect of mesangial cell conditioned medium (MC medium) on macrophage adhesiveness.

Confluent rat mesangial cell line SM43 cultured in a 100 mm culture plate was incubated in 4 ml of 1% FCS/DME-F12 at 37 °C for 48 hours. The conditioned medium was passed through a 0.2 μm filter and used as MC medium. DME-F12 containing 1% FCS was also incubated in the absence of cells for 48 hours, filtered, and used as control medium for assays.

A. Dose-dependent effect of MC medium. J774.2 macrophages were suspended in 2 - 10 times diluted MC medium or control medium and plated in 24-well tissue culture plates (5x10⁴ cells/well). After 24 hours, the number of detached cells was counted. Assays were performed in quadruplicate. Data are presented as means ± SE.

B. Effect of MC medium on macrophage adhesiveness to different matrix constituents. Tissue culture plates were coated with basement membrane Matrigel, laminin, or collagen type IV, and the effect of MC medium (1:1 dilution) was tested. Statistically significant differences (P<0.05) compared to the effect of control medium on each substrate (*) or to the effect of MC medium on uncoated plastic (†) are shown.
C. Reversibility of impaired adhesiveness of macrophages. 
Following the treatment with MC medium for 24 hours, detached macrophages were suspended in fresh control medium and replated into 24-well plates. As a control experiment, macrophages were pretreated with control medium for 24 hours, suspended in fresh control medium and replated. After 0.5, 2, and 24 hours, the number of floating cells was counted, and fold increases against the values of control were calculated. Asterisks indicate statistically significant differences (P<0.05) compared to controls.

D. Effect of MC medium derived from serum-depleted mesangial cells. 
Confluent SM43 cells were precultured in 0.5% FCS/DME-F12 for 72 hours and incubated in 4 ml of 1% FCS/DME-F12 for 48 hours. The prepared conditioned medium from inactivated mesangial cells (inactivated) was diluted at 1:1 and added to macrophages. Statistically significant differences (P<0.05) compared to the effect of control medium (control) (*) or standard MC medium (standard) (†) are shown.
3.1.2. **EFFECT OF CONDITIONED MEDIA FROM NON-MESANGIAL CELL LINES**

To examine that the factor responsible for the attenuation of macrophage adhesiveness is secreted by mesangial cells but not other cells, conditioned media derived from LLCPK1 epithelial cells, NRK49F fibroblasts, and ECV304 endothelial cells were tested for the effect on macrophage adhesiveness. J774.2 macrophages were suspended in 1:1 diluted conditioned media and seeded in 24-well culture plates. After 24 hours, the number of floating cells was evaluated. In contrast to mesangial cell-derived medium, none of the conditioned media from non-mesangial cell lines induced macrophage detachment (Figure 3.2.).

3.1.3. **PRODUCTION OF TGF-β1 BY MESANGIAL CELLS**

To identify the active component in mesangial cell-derived medium, we focused on TGF-β1 since; i) TGF-β is one of the most potent suppressors of macrophage function [Tsunawaki et al. 1988], ii) cultured mesangial cells have the ability to produce active TGF-β1 [Kaname et al. 1992; Kitamura et al. 1996], and iii) TGF-β1 may inhibit expression of adhesion receptors in macrophages [Bottalico et al. 1991; Hughes et al. 1995]. Expression of TGF-β1 mRNA in SM43 mesangial cells was examined by Northern blot analysis. Mesangial cells constitutively expressed a 2.5 kb transcript, consistent with the predicted size of TGF-β1 mRNA, as shown in Figure 3.3A. Immunoblot analysis on mesangial cell-derived medium detected a 25 kD band.
corresponding to the active form of TGF-β1 (Figure 3.3B.). By using the growth inhibition assay of the mink lung epithelial cell line CCL64, we confirmed that mesangial cell-derived medium contained biologically active TGF-β (Figure 3.3C.). The estimated concentration of active TGF-β1 was 5 ng/ml. In contrast, conditioned medium derived from NRK49F fibroblasts, LLCPK1 epithelial cells or ECV304 endothelial cells did not exert any inhibitory effect on the mitogenesis of CCL64 cells (data not shown).

Figure 3.2.: Effect of media conditioned by non-mesangial cell lines on macrophage adhesiveness.

The fibroblastic cell line NRK49F, the epithelial cell line LLCPK1, and the endothelial cell line ECV304 were cultured in 100 mm culture plates. The confluent cultures were incubated in 4 ml of 1% FCS/DME-F12 at 37 °C for 48 hours, and passed through a 0.2 µm filter. Adhesion assays were performed using 1:1 diluted conditioned media. Fold increases in the number of non-adherent cells compared to the value of control medium (control) are demonstrated. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference (P<0.05) compared to control. Assays were performed in quadruplicate.
**Figure 3.3.** Expression of transforming growth factor-β1 (TGF-β1) in mesangial cells under basal culture conditions.

A. Northern blot analysis. Total RNA was extracted from confluent SM43 mesangial cells, and Northern blot analysis was performed on the expression of TGF-β1. The position of 28S and 18S ribosomal RNAs is shown on the left.

B. Immunoblot analysis of TGF-β1 in MC medium. Control medium and MC medium were precipitated with 10% trichloroacetic acid and electrophoresed on a 10% polyacrylamide gel. Immunoblot analysis was performed using an anti-TGF-β1 antibody which recognises the active form of TGF-β1. The colour reaction was developed using an immunoperoxidase method. The position of molecular-weight markers (30 and 22 kD) is shown on the left.

C. Bioassay of TGF-β activity. TGF-β bioactivity was evaluated by a growth inhibition assay using the indicator cell line, CCL64. Cells were exposed to 2 - 16 times diluted mesangial cell conditioned media, pulsed with [³H]-thymidine, and incorporation of radioactivity was measured by liquid scintillation counting. As a standard, human TGF-β1 (0.1 - 5 ng/ml) was used.
To examine the effect of TGF-β1 on macrophage adhesiveness, several concentrations of TGF-β1 were added to cultures of macrophages. As shown in Figure 3.4A., TGF-β1 induced macrophage detachment in a dose-dependent manner. The effect was detected at concentrations more than 50 pg/ml and reached to maximum at 5 ng/ml. The number of detached cells induced by 5 ng/ml TGF-β1 was 10 times higher than that of control.

To elucidate the involvement of TGF-β1 as an active entity of mesangial cell-derived medium, diluted conditioned medium was pretreated with an anti-TGF-β1 neutralising antibody, and the efficacy was retested. Compared to untreated medium, the effect of mesangial cell-derived medium treated with the antibody was partially but significantly (35 ± 4 %, mean ± SE, P<0.05) abrogated (Figure 3.4B.). These data suggest that TGF-β1 is an active component involved in the regulation of macrophage adhesiveness by mesangial cell-derived medium.

To examine involvement of other soluble factors as active entities in mesangial cell-derived medium, diluted mesangial cell-derived medium was heated at 80 °C, and the efficacy was retested. Although the active form of TGF-β1 is known to be heat-stable, the heat treatment significantly reduced the total activity of mesangial cell-derived medium (67 ± 4 % vs. unheated mesangial cell-derived medium (100 %)) (Figure 3.4C.). It is noteworthy that the heat-inactivated mesangial cell-derived medium still retained the substantial ability to induce macrophage detachment compared to control medium.
**Figure 3.4.: Identification of TGF-β1 in mesangial cell-derived medium (MC medium) as an active component.**

A. Effect of TGF-β1 on macrophage adhesiveness. Macrophages were exposed to several concentrations of TGF-β1 (0 - 10 ng/ml). After 24 hours, the number of detached cells was evaluated.

B. Effect of an anti-TGF-β1 neutralising antibody on the activity of MC medium. MC medium was pre-incubated with or without 25 μg/ml of an anti-TGF-β1 neutralising antibody for 10 minutes at room temperature, diluted at 1:9 with 1% FCS/DME-F12, and its effect retested. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference (*P<0.05). Assays were performed in quadruplicate.

C. Effect of heat-treated MC medium on macrophage adherence. MC medium or control medium diluted at 1:9 was heated at 80 °C for 10 minutes, and the efficacy was retested. Statistically significant differences (*P<0.05) compared to the effect of untreated or heat-treated control medium (*) or untreated MC medium (†) are shown. Data are presented as means ± SE.
We examined whether the reduced adhesiveness of macrophages treated with mesangial cell-derived medium is associated with altered mitogenic activity. Macrophages were pretreated with mesangial cell-derived medium, and incorporation of $[^3]$H-thymidine was examined. Adherent and non-adherent macrophages were harvested separately, and incorporation of radioactivity per cell was evaluated. Compared to attached cells, detached macrophages showed a significant reduction in mitogenic activity (Figure 3.5A.). The mean value in non-adherent cells was $0.038 \pm 0.013$ cpm/cell vs. $0.265 \pm 0.021$ cpm/cell in adherent cells ($P<0.05$).

J774.2 macrophages stimulated by LPS express the proinflammatory cytokines IL-1β and IL-6 [Kitamura et al. 1996]. Using these molecules as indicators of activation, we examined whether altered adhesiveness affects the response of macrophages to stimuli. Macrophages were pretreated with diluted mesangial cell-derived medium or control medium and stimulated by LPS. Adherent and non-adherent cells were separately harvested from identical culture plates, and Northern blot analysis was performed. As demonstrated in Figure 3.5B., treatment with mesangial cell-derived medium markedly suppressed the expression of IL-1β and IL-6 in adherent macrophages, as reported previously [Kitamura et al. 1996]. Under the same culture conditions, detached macrophages exposed to mesangial cell-derived medium exhibited lower levels of cytokine expression than adherent cells. The normalised values of mRNA level in non-adherent cells were 36 % (IL-1β) and 37 % (IL-6) compared to adherent cells (100 %).
A. Mitogenic activity. Macrophages suspended in MC medium (1:1 dilution) were seeded in 24-well plates at a density of 2.5 x 10^4 cells/well. After incubation for 48 hours, the cells were pulsed with 1 μCi of [3H]-thymidine per well for 3 hours. Adherent and non-adherent macrophages were then harvested separately, and the cell numbers were determined. Incorporation of radioactivity was measured by liquid scintillation counting, and values expressed as [3H]-thymidine incorporation per cell. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference (P<0.05). Assays were performed in quadruplicate.

B. Cytokine expression. Macrophages were cultured for 24 hours in the presence or absence of MC medium (1:1 dilution) and stimulated by LPS (1 μg/ml) for an additional 24 hours. Adherent and non-adherent cells were separately harvested from identical culture plates, and Northern blot analysis was performed for evaluation of IL-1β and IL-6 mRNA. As a loading control, expression of β-actin mRNA is shown.

**Figure 3.5.** Activity of detached macrophages following the treatment with mesangial cell-derived medium (MC medium).

![Graph showing mitogenic activity with adherent and non-adherent macrophages]

![Graph showing cytokine expression with MC Medium, IL-1β, IL-6, and β-actin]
3.1.6. **SUMMARY**

1. Mesangial cell-derived medium significantly induced detachment of macrophages from substrata. The reduced adhesiveness of macrophages was reversible, and cell viability was unaffected by mesangial cell-derived medium.

2. The factor which reduced macrophage adhesiveness was uniquely secreted by mesangial cells. TGF-B1 was identified as an active component involved in the suppression of macrophage adhesiveness.

3. Once macrophages became non-adherent, they were deactivated in terms of their mitogenesis and cytokine productivity in response to stimuli.
3.2. INHIBITION OF MACROPHAGE CYTOKINE SYNTHESIS BY MESANGIAL CELL-DERIVED TGF-β

Activated macrophages are capable of producing a number of proinflammatory cytokines, which stimulate resident glomerular cells to mitogenesis and production of extracellular matrix proteins [Melcion et al. 1982; Lovett et al. 1983b]. On the other hand, several cell types have been shown to produce factors which suppress the function of macrophages [Szuro-Sudol and Nathan, 1982]. The aim of this study was to investigate the effect of mesangial cell-derived factors on the synthesis of proinflammatory cytokines by activated macrophages.

3.2.1. INDUCTION OF PROINFLAMMATORY CYTOKINES IN MACROPHAGES

J774.2 macrophages were stimulated with LPS and the kinetics of the expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α was examined. Compared to unstimulated control, LPS markedly induced expression of IL-1β and TNF-α within 3 hours, and IL-6 expression within 6 hours after stimulation. The expression of IL-1β and IL-6 gradually increased for up to 24 hours, whereas the expression of TNF-α reached a plateau at 6 hours and then declined thereafter (Figure 3.6A.).

To examine whether increased expression of cytokines is associated with protein synthesis, IL-1 bioactivity in macrophage conditioned media was tested. As an indicator, expression of gelatinase B in mesangial cells was used. Gelatinase B expression has been shown to be inducible in response to IL-1β [Yokoo and Kitamura,
Northern blot analysis revealed that compared to control media, conditioned media from LPS-stimulated macrophages induced expression of gelatinase B mRNA in mesangial cells. This stimulatory effect was abrogated in mesangial cells expressing IL-1ra, the specific inhibitor of IL-1 (Figure 3.6B.). These results showed that conditioned media derived from activated macrophages contain the active form of IL-1β.

3.2.2. DOSE-DEPENDENT SUPPRESSION OF MACROPHAGE CYTOKINE EXPRESSION BY MESANGIAL CELL-DERIVED MEDIUM

To investigate the effect of mesangial cell-derived conditioned medium on the expression of proinflammatory cytokines by activated macrophages, J774.2 cells were pretreated with serial dilutions of mesangial cell conditioned media for 6 hours, and then stimulated with LPS. Northern blot analysis revealed that mesangial cell-derived conditioned medium inhibited expression of IL-1β, IL-6 and TNF-α mRNA by activated macrophages in a dose-dependent manner (Figure 3.7A.). The normalised values of mRNA levels were 96, 90, 85, 45, 16 % (IL-1β), 96, 91, 43 % (IL-6), and 100, 100, 81, 47, 47 % (TNF-α) compared to non-treated cells (100 %).

To test whether mesangial cell conditioned medium could inhibit expression of cytokines in pre-activated macrophages, J774.2 cells were stimulated by LPS for 6 hours and then exposed to diluted mesangial cell-derived conditioned media. Northern blot analysis showed that mesangial cell conditioned medium dose-dependently downregulated expression of cytokines in pre-activated macrophages (Figure 3.7B.). Normalised mRNA levels were similar to those listed above.
Figure 3.6: Induction of IL-1β, IL-6 and TNF-α in LPS-stimulated macrophages.

A. Kinetics of macrophage cytokine expression in response to LPS. J774.2 macrophages were stimulated with LPS (1 µg/ml) for 3, 6, 9, 12, and 24 hours, and then harvested. Northern blot analyses on cytokine expression were performed. As control, unstimulated macrophages were used.

B. Biological activity of macrophage-derived IL-1.
Untransfected SM43 mesangial cells and mesangial cells constitutively expressing IL-1ra (SM43/IL-1ra) were exposed to diluted (1:1) conditioned media derived from unstimulated (-) and LPS-stimulated (+) macrophages. Expression of gelatinase B in mesangial cells was examined by Northern blot analysis. Expression of β-actin was used as loading control. The position of ribosomal RNAs is shown on the right.
Figure 3.7: *Dose-dependent inhibition of macrophage cytokine synthesis by mesangial cell-derived conditioned media (MC media).*

A. Treatment with MC media before LPS stimulation.
J774.2 macrophages were exposed to serial dilutions (0 - 50 %) of MC media for 6 hours and then stimulated with LPS (1 μg/ml) for 18 hours. Cells were then harvested and expression of IL-1β, IL-6 and TNF-α was examined.

B. Treatment with MC media after LPS stimulation.
Macrophages were stimulated with LPS for 6 hours and then exposed to diluted MC media for 18 hours. Cells were harvested and Northern blot analyses were performed.

C. Effect of MC media on the biological activity of IL-1 derived from activated macrophages. J774.2 macrophages were pretreated with diluted (1:1) control [MC media (-)] or mesangial cell-derived media [MC media (+)] for 6 hours and then stimulated with (+) or without (-) LPS for 18 hours. Media were then replaced with DME-F12 containing 10 % FCS, and macrophages were further incubated for 24 hours. The media were then collected and added to SM43 and SM43/IL-1ra mesangial cells. Mesangial expression of gelatinase B was examined by Northern blot analysis. As a loading control, expression of β-actin is shown.
To examine whether the mesangial cell-derived factor inhibits synthesis of the active forms of cytokines, conditioned media derived from activated macrophages pretreated with or without mesangial cell-derived media were exposed to untransfected or IL-1ra-transfected mesangial cells. Compared to media from unstimulated macrophages, conditioned media from activated macrophages induced gelatinase B expression in untransfected mesangial cells (100%). This induction was suppressed to 28% by the media from activated macrophages that were pretreated with mesangial cell-derived media. None of the test media induced gelatinase B expression in IL-1ra overexpressing mesangial cells (Figure 3.7C.). These results showed that the mesangial cell-derived factor suppressed both induction and synthesis of active IL-1.

3.2.3. EFFECT OF CONDITIONED MEDIA FROM NON-MESANGIAL CELL LINES

Conditioned media from fibroblastic, epithelial and endothelial cell lines were tested for the effect on the expression of cytokines by activated macrophages. J774.2 macrophages were exposed to conditioned media derived from NIH3T3 fibroblasts, NRK49F fibroblasts, LLCPK1 epithelial cells, MDCK epithelial cells, CCL64 epithelial cells, or ECV304 endothelial cells as well as from mesangial cells. Macrophages were incubated with the media for 6 hours, then stimulated by LPS for 18 hours. Northern blot analyses were performed on the expression of cytokines. In contrast to mesangial cell-derived medium, none of the conditioned media derived from other cell lines inhibited expression of IL-1β, IL-6 and TNF-α by activated macrophages (Figure 3.8.). These findings suggest that the inhibitor of macrophage cytokine synthesis is secreted by mesangial cells exclusively.
Figure 3.8.: Effect of conditioned media derived from endothelial, epithelial or fibroblastic cell lines on macrophage cytokine expression.

Macrophages were exposed to diluted (1:1) conditioned media from NIH3T3 fibroblasts, NRK49F fibroblasts, LLCPK1 epithelial cells, MDCK epithelial cells, CCL64 epithelial cells, ECV304 endothelial cells as well as SM43 and FM14 mesangial cells. Cells were then stimulated with LPS (1 μg/ml). Northern blot analysis was performed to examine expression of IL-1B, IL-6 and TNF-α by activated macrophages. Expression of β-actin is shown as loading controls.

3.2.4. EFFECT OF CONDITIONED MEDIA DERIVED FROM ISOLATED NORMAL AND NEPHRITIC GLOMERULI

We elucidated the effect of conditioned media from normal isolated glomeruli on macrophage cytokine expression. Macrophages were treated with diluted (1:4) conditioned media and then stimulated by LPS. Northern blot analyses revealed that,
compared to control, conditioned media from normal isolated glomeruli suppressed the expression of IL-1β, IL-6 and TNF-α by activated macrophages (Figure 3.9.).

To investigate the effect of media derived from inflamed glomeruli on macrophage cytokine expression, media derived from isolated glomeruli subjected to anti-Thy 1 glomerulonephritis were tested. Compared to normal glomeruli, nephritic glomeruli (day 5) exhibited enhanced suppressive effect on cytokine expression by activated macrophages. Normalised mRNA levels were 47% (IL-1β), 39% (IL-6), and 33% (TNF-α) compared to normal glomeruli (100%).

![Figure 3.9.](image)

**Figure 3.9.:** *Effect of conditioned media from isolated normal or nephritic glomeruli on macrophage cytokine expression.*

Macrophages were exposed to diluted (1:4) conditioned media derived from isolated normal glomeruli or glomeruli subjected to anti-Thy 1 glomerulonephritis (day 5 and 14) and then stimulated with LPS (1 μg/ml). Northern blot analysis was performed on the expression of IL-1β, IL-6 and TNF-α by activated macrophages. β-actin was used as loading control.
3.2.5.  IDENTIFICATION OF THE ACTIVE ENTITY IN
MESANGIAL CELL-DERIVED CONDITIONED MEDIA

3.2.5.1.  Expression of macrophage-deactivating cytokines by
mesangial cells

To elucidate the active entity in mesangial cell-derived media, expression of the best
characterised macrophage-deactivating cytokines was examined in mesangial cells.
Northern blot analysis revealed that mesangial cells constitutively expressed a 2.5 kb
transcript corresponding to the size of TGF-β1 mRNA (Figure 3.10.). No expression
of IL-10 or IL-13 mRNA was detected in mesangial cells by Northern analyses.

Immunoblot analysis on mesangial cell-derived media revealed a 25 kDa band,
indicative of the presence of active TGF-β1 in the media (Figure 3.3B.). Using the
growth inhibition assay of the mink lung epithelial cell line CCL64, we confirmed that
mesangial cells secrete active TGF-β1 into the media (not shown). The estimated
concentration of active TGF-β1 was 6.4 - 7.2 ng/ml.

3.2.5.2.  Effect of exogenous TGF-β on macrophage cytokine expression

J774.2 macrophages were exposed to several concentrations of TGF-β1 for 6 hours,
and then stimulated by LPS for an additional 18 hours. Northern blot analyses showed
that TGF-β1 suppressed expression of IL-1β, IL-6 and TNF-α by activated macrophages in a dose-dependent manner (Figure 3.11.).

Figure 3.10.: *Expression of macrophage-deactivating cytokines in mesangial cells.*

Northern blot analyses of IL-10, IL-13 and TGF-β1 in SM43 mesangial cells. As positive controls, stably transfected cell clones SM43/IL-10, SM43/IL-13, and FM14/TGFB1 were used. The position of ribosomal RNAs is shown on the left (+). Arrowheads denote the expressed exogenous transcripts. An arrow shows the endogenous TGF-β1 transcript.
Figure 3.11.: **Dose-dependent inhibition of macrophage cytokine synthesis by exogenous TGF-β1.**

J774.2 macrophages were pretreated with several concentrations of TGF-β1 (0 - 10 ng/ml) for 6 hours. Cells were then stimulated with LPS (1 μg/ml) for 18 hours and then harvested. Expression of IL-1β, IL-6 and TNF-α were examined by Northern blot analysis. β-actin was used as loading control.

### 3.2.5.3. Identification of TGF-β1 as the mesangial cell-derived suppressor of macrophage cytokine synthesis

TGF-β present in the media in inactive form can be activated by heat-treatment. Therefore, control media and conditioned media from mesangial cells were heated at 80 °C for 10 minutes and added to macrophages. After LPS stimulation, macrophage cytokine synthesis was examined by Northern blot analyses. Compared to unheated mesangial cell conditioned media (100 %), heated media further suppressed IL-1β (18
%, IL-6 (9 %) and TNF-α (26 %) expression by activated macrophages (Figure 3.12.), confirming the involvement of TGF-β in the process.

Diluted mesangial cell-derived conditioned medium was treated with an anti-TGF-β1 neutralising antibody, and the effect on macrophage cytokine expression was examined. The inhibition of TGF-β1 activity abrogated the suppressive effect of mesangial cell conditioned medium, confirming that the active entity is mesangial cell-derived TGF-β1 (Figure 3.12.). Normalised mRNA levels were 81 % (IL-1β), 75 % (IL-6) and 98 % (TNF-α) compared to control (100 %).

Figure 3.12.: Identification of the active entity in mesangial cell-derived conditioned media.

Mesangial cell-derived conditioned medium (CM) was pre-incubated with or without 5 μg/ml of an anti-TGF-β1 neutralising antibody for 10 minutes at room temperature, diluted at 1:4 with 1 % FCS, and its effect was retested. CM or control medium diluted at 1:4 was heated at 80 °C for 10 minutes, and the efficacy was retested. J774.2 macrophages were exposed for 6 hours to control medium (control), heated control medium, 1:4 diluted CM, heated CM, CM pretreated with the anti-TGF-β1 neutralising antibody (CM+anti-TGF-β), or human TGF-β1 (5 ng/ml) (TGF-β). After
LPS stimulation for 18 hours, cells were harvested and Northern blot analyses were performed on the expression of IL-1β, IL-6 and TNF-α by activated macrophages. Expression of β-actin was used as loading control.

3.2.6. SUMMARY

1. Mesangial cell-derived medium inhibited production of IL-1β, IL-6 and TNF-α by activated macrophages.

2. The factor which inhibited cytokine production by activated macrophages was uniquely secreted by mesangial cells as well as nephritic glomeruli where mesangial cells are activated.

3. TGF-β1 was identified as the major suppressor of macrophage cytokine production.
3.3. INHIBITION OF MACROPHAGE METALLOPROTEINASE PRODUCTION BY MESANGIAL CELL-DERIVED TGF-β

3.3.1. SYNERGISTIC INTERACTION BETWEEN MACROPHAGES AND MESANGIAL CELLS FOR GELATINASE B PRODUCTION

Mesangial cells and macrophages produce gelatinase B in response to stimuli [Okuda et al. 1990; Kitamura et al. 1995; Yokoo and Kitamura, 1996b]. Using this molecule as an indicator, interaction between mesangial cells and macrophages was investigated. Macrophages, mesangial cells, and macrophages co-cultured with mesangial cells were stimulated with LPS, and zymographic analysis was performed on the conditioned media (Figure 3.13.).

Figure 3.13.: Production of gelatinase B by NR8383 rat macrophages (MΦ), rat mesangial cells (MC), and their co-culture (MΦ+MC) in response to lipopolysaccharide (LPS).

Mesangial cells were seeded in 6-well plates at a density of 1 x 10^6 cells/well. After 24 hours, media were replaced with DME-F12 containing 1% FCS (2 ml/well) and further incubated for 24 hours. NR8383 macrophages were seeded in empty wells or wells
containing mesangial cells at a density of 5 x 10^5 cells/well. After 24 hours, media were replaced with fresh 1 % FCS/DME-F12 with (+) or without (-) 1 μg/ml of LPS. After a 24 hour incubation, media were collected and subjected to gelatin zymography. Position of gelatinase B and gelatinase A is indicated on the left.

Under basal culture conditions, NR8383 macrophages produced a low level of gelatinase B. When stimulated with LPS, the expression level was upregulated. Although LPS-stimulated mesangial cells did not exhibit upregulation of gelatinase B, co-culture with macrophages stimulated by LPS showed higher levels of gelatinase B expression (306 %) compared to macrophages alone (100 %).

3.3.2. IDENTIFICATION OF A MACROPHAGE-DERIVED STIMULATOR OF MESANGIAL GELATINASE B EXPRESSION

To examine whether activated macrophages secrete factors that induce mesangial expression of gelatinase B, mesangial cells were treated with culture medium conditioned by unstimulated or LPS-stimulated NR8383 macrophages. Zymographic analysis showed that the medium from stimulated macrophages substantially (5 fold) upregulated the mesangial production of gelatinase B compared to the medium from unstimulated macrophages that had only a modest effect (Figure 3.14.).
To identify the responsible molecules involved, we focused on the role of IL-1β, because: i) macrophages produce IL-1β abundantly in response to LPS [Kitamura et al. 1996; Sütő et al. 1996b], and ii) IL-1β is a prominent stimulator of gelatinase B in mesangial cells [Kitamura et al. 1995; Yokoo and Kitamura, 1996b]. Using Northern blot analysis, we first examined the expression of IL-1β in LPS-stimulated and unstimulated NR8383 cells. As shown in Figure 3.15A., NR8383 macrophages expressed a low level of basal IL-1β mRNA. After LPS stimulation, the expression was markedly upregulated. Externally added IL-1β substantially induced gelatinase B expression in mesangial cells at both mRNA (Figure 3.15B.) and protein levels (15 fold) (Figure 3.15C.). Furthermore, the mesangial production of gelatinase B in response to the macrophage-conditioned medium was suppressed by 75 % in mesangial cells that stably express IL-1ra (Figure 3.15D.).
A. Expression of IL-1β mRNA in un-stimulated and stimulated macrophages. NR8383 cells were stimulated with (+) or without (-) LPS for 18 hours, and Northern blot analysis was performed on IL-1β expression. Position of ribosomal RNAs (28S and 18S) is indicated on the right. Expression of β-actin is shown as a loading control.

B. Effect of IL-1β on gelatinase B mRNA expression in mesangial cells. Mesangial cells were stimulated with (+) or without (-) IL-1β (10 ng/ml) for 24 hours, and Northern blot analysis was performed on gelatinase B expression.

C. Effect of IL-1β on the production of gelatinase B by mesangial cells. Mesangial cells were stimulated with (+) or without (-) IL-1β for 24 hours. Media were then replaced with fresh 1% FCS/DME-F12, incubated for an additional 24 hours and collected for zymographic analysis.

D. Effect of the macrophage-conditioned medium on gelatinase B production by mesangial cells stably expressing human IL-1 receptor antagonist protein (IL-1ra). Untransfected mesangial cells [IL-1ra (-)] and IL-1ra-transfected cells [IL-1ra (+)] were exposed to control medium (1% FCS/DME-F12) or 1:1 diluted conditioned medium from activated macrophages. After 24 hours, media were replaced with fresh 1% FCS/DME-F12, incubated for an additional 24 hours and collected for zymographic analysis.
3.3.3. **IDENTIFICATION OF A MESANGIAL CELL-DERIVED INHIBITOR OF MACROPHAGE GELATINASE B EXPRESSION**

We sought mesangial cell-derived paracrine regulators for macrophage production of gelatinase B. NR8383 macrophages were exposed to serial dilutions of mesangial cell-derived medium, stimulated with or without LPS, and zymographic analysis was performed on the conditioned media. Unexpectedly, the mesangial cell-derived medium did not induce but inhibited the production of gelatinase B by activated macrophages in a dose-dependent manner (Figure 3.16A.). Basal expression of gelatinase B was also repressed by the mesangial cell-conditioned medium (data not shown). At concentrations higher than 25%, the inducible gelatinase B activity was abolished by the treatment with mesangial cell-derived medium. This inhibition of enzymatic activity was not due to induction of metalloproteinase inhibitors, since expression levels of gelatinase A were unaltered. Furthermore, Northern blot analysis revealed that activated macrophages in co-culture with mesangial cells exhibited a markedly depressed expression of gelatinase B mRNA compared to stimulated macrophages not in co-culture (Figure 3.16B.).

To identify the inhibitor involved in the mesangial cell-conditioned medium, we examined the role of TGF-β1, because; i) mesangial cells have the ability to generate the active form of TGF-β1 [Kitamura et al. 1996; Sütö et al. 1996b], and ii) TGF-β1 is known to be a prominent macrophage deactivator that inhibits production of several inflammatory mediators [Kitamura and Sütö, 1997a]. Consistent with our previous reports [Kitamura et al. 1996; Sütö et al. 1996b], Northern and western blot analyses revealed that SM43 mesangial cells expressed a 2.5 kb TGF-β1 transcript (Figure
3.17A.) and generated the 25 kDa mature TGF-β1 (Figure 3.3B.). By using the growth inhibition assay of the mink lung epithelial cell line CCL64, it was confirmed that mesangial cell-conditioned medium, utilised in this study, contained biologically active TGF-β. The estimated concentration of active TGF-β1 was approximately 5 ng/ml.

A. B.

Figure 3.16.: Effect of mesangial cell-derived factor(s) on gelatinase B expression in activated macrophages.

A. Effect of mesangial cell-derived medium (MC medium) on gelatinase B production by macrophages.
NR8383 macrophages suspended in 1% FCS/DME-F12 were seeded in 24-well tissue culture plates at a density of 5 x 10⁴ cells/well and exposed to 2 - 10 times diluted (10 - 50 %) mesangial cell-conditioned medium. After 6 hours, cells were stimulated with (+) or without (-) LPS and incubated for an additional 18 hours. Culture media were then collected and used for zymographic analysis.

B. Effect of “bystander” mesangial cells on the expression of gelatinase B mRNA in activated macrophages.
NR8383 cells were seeded in empty wells (MΦ) or wells containing confluent mesangial cells (co-cultured MΦ). After 6 hours, cultures were stimulated with (+) or without (-) LPS. After 18 hours, macrophages were separately harvested and used for Northern analysis.
To determine the identity of TGF-β1 as the mesangial cell-derived inhibitor, NR8383 macrophages were pretreated with or without TGF-β1, stimulated by LPS, and Northern analysis of gelatinase B was performed. As shown in Figure 3.17B., externally added TGF-β1 suppressed the macrophage expression of gelatinase B by 72%. This was confirmed by another assay, gelatin zymography. TGF-β1 ranging from 0.05 to 10 ng/ml inhibited LPS-induced gelatinase B production by macrophages in a dose-dependent manner (Figure 3.17C.). Furthermore, zymographic analysis revealed that the inhibitory activity of the mesangial cell-conditioned medium was obliterated (78%) by treatment with an anti-TGF-β1 neutralising antibody (Figure 3.17D.).

3.3.4. SUMMARY

1. Conditioned media from activated macrophages induced gelatinase B expression in mesangial cells. Macrophage-derived IL-1β was identified as the active component involved in the stimulation of mesangial gelatinase B production.

2. Mesangial cell-derived medium or co-culture with mesangial cells significantly inhibited production of gelatinase B by activated macrophages. TGF-β1 was identified as the mesangial cell-derived suppressor of macrophage gelatinase B production.
Figure 3.17.: Identification of a mesangial cell-derived inhibitor of macrophage gelatinase B production as transforming growth factor-β1 (TGF-β1).

A. Expression of TGF-β1 mRNA in mesangial cells. Position of ribosomal RNAs (28S and 18S) is shown on the right.

B. Effect of TGF-β1 on the expression of gelatinase B in LPS-stimulated macrophages. NR8383 macrophages were pretreated with or without TGF-β1 (10 ng/ml) for 6 hours and stimulated by LPS. After 18 hours, cells were subjected to Northern analysis.

C. Effect of TGF-β1 on macrophage production of gelatinase B. NR8383 macrophages were pretreated with 0.05 - 10 ng/ml TGF-β1 for 6 hours and stimulated with LPS. After 18 hours, media were collected for zymographic analysis.

D. Effect of an anti-TGF-β1 neutralising antibody on the inhibitory activity of mesangial cell-conditioned medium. Mesangial cell-derived medium (MC medium) was pre-incubated with or without 30 µg/ml of an anti-TGF-β1 neutralising antibody for 10 minutes at room temperature, diluted at 1:9 with 1% FCS, and its effect was retested. NR8383 macrophages were exposed for 6 hours to control medium (1% FCS/DME-F12), 1:9 diluted MC medium, or MC medium pretreated with the anti-TGF-β1 neutralising antibody and stimulated with (+) or without (-) LPS. After 18 hours, media were collected for zymographic analysis.
3.4. SUPPRESSION OF MACROPHAGE iNOS EXPRESSION BY MESANGIAL CELL-DERIVED TGF-β

3.4.1. INDUCTION OF iNOS EXPRESSION IN MESANGIAL CELLS BY ACTIVATED MACROPHAGES

Rat mesangial cells were cultured with or without NR8383 macrophages and stimulated with LPS. Expression of iNOS mRNA in mesangial cells was examined by Northern blot analysis. Under basal culture conditions, mesangial cells did not express iNOS mRNA. Unstimulated macrophages did not alter mesangial expression of iNOS. Expression of iNOS mRNA was not detectable in mesangial cells stimulated with LPS. In contrast, mesangial cells co-cultured with stimulated macrophages expressed substantial levels of iNOS mRNA. This result implied that paracrine stimulators from activated macrophages induced expression of iNOS in mesangial cells (Figure 3.18.).

Figure 3.18.: Stimulatory effect of activated macrophages on iNOS expression by mesangial cells.
Mesangial cells were seeded in 24-well tissue culture plates and grown to confluence. NR8383 cells were then suspended in 1 % FCS/DME-F12 and added to wells containing confluent mesangial cells (MΦ +). 1 % FCS/DME-F12 was added to control wells containing mesangial cells only (MΦ −). After 6 hours, cultures were stimulated with (+) or without (-) LPS. After 18 hours, mesangial cells were separately harvested and used for Northern analysis. Expression of GAPDH was used as loading control.

3.4.2. EFFECT OF MESANGIAL CELL-DERIVED FACTORS ON iNOS EXPRESSION BY ACTIVATED MACROPHAGES

To examine the effect of mesangial cell-derived paracrine regulators on the expression of iNOS by activated macrophages, rat NR8383 cells were cultured with or without rat mesangial cells for 24 hours and then stimulated with LPS for an additional 24 hours. Northern blot analyses revealed that expression of iNOS mRNA was not detectable in unstimulated macrophages. When stimulated by LPS, the expression was markedly induced. However, when macrophages co-cultured with mesangial cells were stimulated by LPS, the induction of iNOS was abolished (Figure 3.19.).

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Figure 3.19.: Suppression of iNOS expression in activated macrophages by mesangial cells.
NR8383 cells were seeded in empty wells (MC −) or wells containing confluent cultures of mesangial cells (MC +). LPS-stimulated (+) and unstimulated (-) macrophages were separately harvested and Northern blot analysis on the expression of iNOS was performed. As loading control, expression of GAPDH was used.

3.4.3. **IDENTIFICATION OF A MESANGIAL CELL-DERIVED INHIBITOR OF MACROPHAGE iNOS EXPRESSION**

To identify the inhibitor involved in the mesangial cell-conditioned medium, the role of TGF-β1 was examined. Consistent with our previous reports [Kitamura et al. 1996; Sütö et al. 1996b], our current results demonstrate that mesangial cells have the ability to generate the active form of TGF-β1 (Figure 3.3B.) and mesangial cell-conditioned medium, utilised in this study, contained biologically active TGF-β (Figure 3.3C.).

To determine the identity of TGF-β1 as the mesangial cell-derived inhibitor, NR8383 macrophages were pretreated with or without TGF-β1, stimulated by LPS, and Northern analysis on macrophage iNOS expression was performed. As shown in Figure 3.20., externally added TGF-β1 suppressed the macrophage expression of iNOS by 79%.

![Figure 3.20: Effect of TGF-β1 on the expression of iNOS in LPS-stimulated macrophages.](image-url)
NR8383 macrophages were exposed to TGF-β1 (10 ng/ml) for 6 hours and then stimulated by LPS. After 18 hours, cells were harvested and Northern blot analysis was performed.

3.4.4. SUMMARY

1. Activated macrophages induced iNOS expression in mesangial cells.

2. Bystander mesangial cells inhibited expression of iNOS in activated macrophages. TGF-β1 was identified as the major suppressor of macrophage iNOS expression.
3.5. COUNTERACTION BY RESIDENT CELLS AGAINST EFFECTOR FUNCTION OF MACROPHAGES IN THE GLOMERULUS: A ROLE OF TGF-β

Using cross-feeding and co-culture studies, we elucidated a paracrine regulatory mechanism between macrophages and mesangial cells. We found that; i) activated macrophages produce IL-1 that activates mesangial cells, and ii) this stimulatory effect is counteracted by activated mesangial cells via the macrophage deactivator TGF-β1 [Sütö et al. 1996b] (Figure 3.21.). We hypothesised that, in certain inflammatory situations, mesangial cell-derived TGF-β1 may function as a “defender” against macrophage-mediated glomerular injury. To examine this hypothesis, activated macrophages were transferred into normal and TGF-β1-producing nephritic glomeruli, and expression of activation markers (gelatinase B, iNOS and stromelysin) was examined in isolated glomeruli.

3.5.1. INDUCTION OF GLOMERULAR GELATINEASE B, iNOS AND STROMELYSIN EXPRESSION BY ACTIVATED MACROPHAGES

To investigate the role of activated macrophages in the glomerular expression of gelatinase B, iNOS and stromelysin, reporter macrophages expressing β-galactosidase were stimulated with LPS and transferred into normal left kidneys of rats via renal artery injection. Figure 3.22A. shows typical appearance of macrophages accumulated in glomeruli of injected left kidneys (top). About 73.2 ± 3.4 % (mean ± SE, n=10) of glomeruli isolated from the injected kidneys were positive for X-gal staining.
Figure 3.21: Paracrine regulatory loop between macrophages and mesangial cells.

Activated macrophages secrete IL-1β which stimulates mesangial cells. This effect is counteracted by mesangial cell-derived TGF-β1.

β-Galactosidase-positive cells were not detected in glomeruli from contralateral kidneys (Figure 3.22A, bottom). Using these isolated glomeruli, expression of gelatinase B, iNOS and stromelysin was examined by Northern blot analysis (Figure 3.22B.). Immediately after the cell transfer, expression of gelatinase B, iNOS and stromelysin transcripts was not detectable in either injected or non-injected glomeruli. After ex vivo incubation of these chimeric glomeruli for 24 hours, gelatinase B, iNOS and stromelysin mRNAs were dramatically upregulated in macrophage-transferred glomeruli when compared to unmodified glomeruli. This induction was abolished by
treatment with G418, showing that activated macrophages stimulated resident cells of normal glomeruli to upregulate these activation markers.

3.5.2. **SUPPRESSION OF GLOMERULAR GELATINEASE B, iNOS AND STROMEYSIN EXPRESSION BY ENDOGENOUS TGF-β1**

To examine whether the macrophage-mediated glomerular expression of gelatinase B, iNOS and stromelysin is inhibited by endogenous TGF-β1, an experimental model of anti-Thy 1 glomerulonephritis was used. In this model, TGF-β1 is upregulated in activated mesangial cells during the regeneration of the glomerulus [Okuda et al. 1990; Yamamoto et al. 1994]. BAGMACNR macrophages stimulated with LPS were transferred into normal rat glomeruli or glomeruli subjected to acute anti-Thy 1 glomerulonephritis (day 7). After the cell transfer, glomeruli were isolated and used for TGF-β bioassay, X-gal assay and Northern blot analysis. As demonstrated in Figure 3.23A, regenerating glomeruli produced substantial levels of biologically active TGF-β (as evidenced by inhibition of [3H]-thymidine incorporation by CCL64 cells), compared to normal glomeruli. X-gal assay on isolated glomeruli showed that there was no significant difference in percentages of X-gal-positive glomeruli between normal (73.2 ± 3.4 %, n=10) and nephritic conditions (71.0 ± 2.9 %, n=6). Under this experimental condition, expression of gelatinase B, iNOS and stromelysin was examined by Northern blot analysis. Immediately after the cell transfer, gelatinase B, iNOS and stromelysin transcripts were not detectable in normal and inflamed glomeruli. After ex vivo incubation of these chimeric glomeruli, gelatinase B, iNOS and stromelysin mRNAs were induced in macrophage-transferred, normal glomeruli. This induction was abolished by the treatment with G418. However, in the
regenerating nephritic glomeruli that express TGF-β1, the induction of gelatinase B, iNOS and stromelysin by activated macrophages was dramatically suppressed (15-21 % compared to normal glomeruli, 100 %) (Figure 3.23B.).

These results, together with our in vitro data, support the idea that, in certain pathologic situations, mesangial cell-derived TGF-β acts as a defending molecule against macrophage-mediated glomerular cell-activation.

3.5.3. SUMMARY

1. In vivo transfer of activated macrophages into normal rat glomeruli induced gelatinase B, iNOS and stromelysin expression in resident cells.

2. The macrophage-triggered expression of gelatinease B, iNOS and stromelysin was inhibited in the nephritic glomeruli where TGF-β1 was abundantly expressed in mesangial cells.
A. Detection of reporter macrophages in isolated glomeruli.

Reporter macrophage BAGMAC\textsuperscript{NR} cells (1 \times 10^6 cells), the NR8383 cells stably expressing a bacterial \(\beta\)-galactosidase gene and a neomycin resistance gene, were stimulated with LPS for 16 hours and transferred into normal rat kidneys via renal artery injection. Immediately after the cell transfer, glomeruli were isolated from both kidneys and subjected to 5-bromo-4-chloro-3-indolyl \(\beta\)-D-galactopyranoside (X-gal) assay to detect \(\beta\)-galactosidase activity. Top: injected side, bottom: non-injected side.
**B.** Expression of gelatinase B, iNOS and stromelysin in normal rat glomeruli after the delivery of stimulated macrophages.

BAGMAC\(^{NR}\) cells were stimulated with LPS and transferred into normal rat kidneys. Glomeruli were isolated from both kidneys, and one third (0.5 - 1 x 10^4) of them were frozen at -80 °C (d0). The remaining glomeruli were incubated for 24 hours in 1% FCS/DME-F12 with (+) or without (-) the neomycin analogue G418 (200 μg/ml) and stored at -80 °C (d1). Expression of gelatinase B, iNOS and stromelysin was examined by Northern blot analysis. \(\text{MO} (-)\): non-injected right kidney, \(\text{MO} (+)\): injected left kidney.

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A. Assessment of TGF-β bioactivity generated by normal and nephritic glomeruli.

Conditioned media were prepared from isolated normal (NG-CM) and inflamed rat glomeruli (IG-CM). To create the latter, anti-Thy 1 glomerulonephritis was induced by the monoclonal antibody 1-22-3. Media conditioned by normal or nephritic (day 7) glomeruli were prepared by incubating isolated glomeruli (1 x 10⁴) in 1 ml of 0.5 % FCS/DME-F12 for 24 hours. The media were then collected, diluted at 1:4 (20 %) and used for assessment of TGF-β activity using the mink lung epithelial cell line CCL64, as described in Methods. As standards, human TGF-β1 (0.1 - 5 ng/ml) was used (open bar). Assays were performed in quadruplicate, and data presented as means ± SE. An asterisk indicates a statistically significant difference (P<0.05) compared to control medium. control: 0.5 % FCS/DME-F12.

Figure 3.23.: Suppression of macrophage activation markers by TGF-β1-producing nephritic glomeruli.
B. Suppression of gelatinase B, iNOS and stromelysin induction in regenerating glomeruli after the delivery of LPS-stimulated macrophages.

BAGMAC\textsuperscript{NR} cells were stimulated with LPS for 16 hours and transferred into normal kidneys [\(\alpha\text{-Thy1}(-)\)] or nephritic kidneys subjected to anti-Thy 1 glomerulonephritis [\(\alpha\text{-Thy1}(+)\); day 7]. One third (0.5 - 1 \(\times\) 10\(^{5}\)) of isolated glomeruli were immediately frozen at -80 °C (d0). The remaining glomeruli were incubated for 24 hours in 1 % FCS/DME-F12 with (+) or without (-) the neomycin analogue G418 (200 \(\mu\text{g/ml}\)) and stored at -80 °C (d1). Expression of gelatinase B, iNOS and stromelysin was examined by Northern blot analysis. A representative result from three independent experiments is shown.
CHAPTER FOUR

DISCUSSION
The macrophage has been identified as the crucial modulator of glomerular injury [Main et al. 1992]. This is based upon the findings that: i) Infiltration of macrophages is a hallmark of various glomerular diseases [Monga et al. 1981; Hooke et al. 1987]. ii) In vitro, activated macrophages serve as effector cells via their secreted pathogenic mediators including cytokines, proteolytic enzymes and reactive oxygen/nitrogen species [Nathan, 1987]. iii) Macrophage infiltration is closely correlated with histopathological changes and proteinuria [Atkins et al. 1976; Magil et al. 1981; Monga et al. 1981]. iv) Depletion of macrophages attenuates glomerular damage and proteinuria in experimental glomerulonephritis [Hunsicker et al. 1979; Holdsworth et al. 1980; Lavelle et al. 1981; Holdsworth and Neale, 1984; van Diemen-Steenvoorde et al. 1991]. Communication between resident glomerular cells and infiltrating macrophages, therefore, plays a crucial role during the course of glomerulonephritis. However, currently, it is unclear how resident glomerular cells modulate the functions of infiltrating cells. To address this issue, the role of mesangial cells on macrophage functions including adhesion and production of pro-inflammatory mediators was examined in vitro. Furthermore, using an in vivo macrophage transfer technique, the interaction between activated macrophages and resident glomerular cells in the glomerulus was investigated.

We found that mesangial cell-derived factors reduce adhesiveness of macrophages and cause subsequent deactivation. We identified TGF-β1 as one of the mesangial cell-derived molecules involved in this process. We also demonstrated that mesangial cell-derived TGF-β1 inhibits synthesis of proinflammatory cytokines, metalloproteinases and iNOS by activated macrophages in vitro. Using a technique for in vivo macrophage transfer into the glomerulus, we also found that activated macrophages upregulate expression of gelatinase B, iNOS and stromelysin in resident cells, and that this induction is attenuated in nephritic glomeruli that produce active TGF-β1.
4.1. MESANGIAL CELL-DERIVED TGF-β1 REDUCES ADHESIVENESS OF MACROPHAGES AND CAUSES SUBSEQUENT DEACTIVATION

Adhesion of macrophages plays a crucial role in the initiation of inflammatory processes by influencing their number and function at inflammatory sites. Although accumulation of macrophages is one of the most typical pathological features in various forms of glomerulonephritis [Main et al. 1992], little is understood about the mechanisms involved in macrophage adhesiveness within the glomerular microenvironment. We showed that mesangial cells, but not other cells tested, release factors which induce macrophage detachment from various substrata. We identified that TGF-β1 is one of the factors involved in this activity.

The inhibitory effect of mesangial cell-derived medium on macrophage adhesiveness can be explained by several possible mechanisms. Certain factors secreted from mesangial cells could reduce adhesiveness via cytotoxicity. However, our data showed that the decreased adhesiveness was reversible and that cell viability and morphology was unaffected by mesangial cell-derived medium. Mesangial cell-derived factors might directly interfere with interactions between macrophages and substrata, but the fact that the initial adhesion of macrophages was not affected by the mesangial cell-derived medium, excluded this possibility. Mesangial cells may modulate macrophage adhesiveness by suppressing expression of certain adhesion receptors. Indeed, our data implied the requirement of new protein synthesis for the adhesive ability of macrophages since; i) macrophages pretreated with mesangial cell-derived medium showed reduced initial adhesion, ii) this impaired adhesiveness gradually recovered to the normal level following the withdrawal of mesangial cell-derived medium, and iii)
cycloheximide, a protein synthesis inhibitor, delayed recovery of impaired adhesiveness [T. Süto, preliminary results].

Macrophages use various receptors to adhere to the endothelium, ECM or other cells [Fraser et al. 1993; Hogasen et al. 1995; Hughes et al. 1995]. TGF-β1 has been shown to down-regulate expression of certain adhesion receptors including scavenger receptors and CR3 in macrophages [Bottalico et al. 1991; Hogasen et al. 1995]. We also investigated the effect of mesangial cell-derived conditioned medium on the expression of the β2-integrin LFA-1 (CD11a/CD18), CR3 (CD11b/CD18) and p150/95 (CD11c/CD18) by macrophages. Although our results were inconclusive, together with our present finding that TGF-β1 produced by mesangial cells is a negative regulator for macrophage adhesiveness, inhibited expression of these adhesion receptors by TGF-β1 might explain the suppressive effect of mesangial cell-derived medium on macrophage adhesiveness.

Using the anti-TGF-β1 neutralising antibody, we found that the contribution of TGF-β1 was partial, since an excess of anti-TGF-β1 antibody inhibited only 35% of the activity of mesangial cell-derived medium. Furthermore, total activity of mesangial cell-derived medium was decreased by heat treatment, suggesting the involvement of other thermolabile molecules. IL-10 or TNF-α could be the candidates [van Lenten and Fogelman, 1992; Moore et al. 1993], but expression of these cytokines is not normally detected in SM43 mesangial cells [Kitamura et al. 1996]. Further investigation will be required to elucidate the unidentified components in mesangial cell-derived medium.

In this study, we utilised plastic plates coated with different ECM components which are found in the normal glomerulus. Mesangial cell medium significantly reduced adhesiveness of macrophages seeded on laminin, collagen type IV or basement membrane Matrigel as well as on plain plastic. Interestingly, the effect of mesangial cell-derived medium was most pronounced on macrophages adhered to plastic, rather
than those on ECM-coated plates. This difference may be explained by the enhancement of macrophage adhesiveness by cell-ECM interaction since expression of integrins may be upregulated by TGF-β1 [Wahl et al. 1993].

Adherence is a priming trigger for a wide range of monocyte/macrophage functions. For example, adhesion induces differentiation of monocytes to tissue macrophages, facilitates migration, phagocytosis, respiratory oxidative burst, and expression of certain cytokines and proto-oncogenes [Springer and Anderson, 1986; Haskill et al. 1988; Newman and Tucci, 1990; Shaw et al. 1990; Azavedo and de Souza, 1992; Xing et al. 1992]. Based upon these findings, adhesiveness, possibly, controls function of macrophages within the glomerulus. We, therefore, examined the relationship between macrophage adhesiveness and activity following the exposure to mesangial cell-derived medium. Compared to adherent cells, non-adherent macrophages showed reduced mitogenic activity and blunted expression of cytokines in response to LPS. These findings indicate that mesangial cell-derived factors, including TGF-β1, induce detachment of macrophages followed by their blunted responses to a specific stimulus.

In the acute, reversible model of anti-Thy 1 nephritis in the rat, a transient accumulation of monocytes/macrophages is observed within 24 hours following injection of the anti-Thy 1 antibody [Kawachi et al. 1992a]. The increased number of glomerular macrophages is sustained up to day 7 and declines thereafter. At day 14, the majority of the inflammatory macrophages disappear from the glomerulus [Kawachi et al. 1992a]. The mechanism involved in this process may include trafficking of inflammatory macrophages to draining lymph nodes [Lan et al. 1993b]. In the acute model of anti-Thy 1 glomerulonephritis, upregulation of glomerular TGF-β1 is detected from day 4 and plateaus until at least day 14 [Okuda et al. 1990]. Interestingly, the anti-Thy 1 antibody re-injected at day 14 does not induce macrophage accumulation [Kawachi et al. 1992a]. Upregulation of TGF-β1 is, thus, closely correlated not with accumulation but with reduced macrophage retention in the
glomerulus. The negative effect of mesangial cell-derived factors including TGF-β1 on macrophage adhesiveness may play a role in the clearance of activated macrophages from the nephritic glomeruli and, in part, participate in the recovery of the glomerulus from acute inflammation.

4.2. MESANGIAL CELL-DERIVED TGF-β1 INHIBITS SYNTHESIS OF PROINFLAMMATORY CYTOKINES BY ACTIVATED MACROPHAGES

Activated macrophages are capable of producing a variety of inflammatory cytokines. These cytokines induce glomerular hypercellularity and secretion of pro-inflammatory mediators by glomerular cells, and thereby, contribute to glomerular injury. In this thesis, we demonstrated that mesangial cells secrete a macrophage deactivator which inhibits production of IL-1β, IL-6, and TNF-α by activated macrophages. We identified this inhibitory factor as TGF-β1.

The pathogenic actions of IL-1β, IL-6 and TNF-α in glomerular diseases have been extensively investigated [Holdsworth and Tipping, 1985; Sedor, 1992]. It is now known that IL-1β is released by many cell types including macrophages [Dinarello, 1991]. Locally, IL-1β stimulates cells to undergo profound morphological and functional changes that are involved in glomerular inflammation and injury. IL-1β, produced mainly by macrophages, is a mitogen for mesangial cells [Sedor et al. 1992]. It enhances mesangial production of biologically active molecules including reactive oxygen metabolites and prostaglandin E₂ [Shah, 1995], which may contribute to alterations in the glomerular microcirculation during inflammation. IL-1β stimulates production of fibrinolytic activities in cultured endothelial and mesangial cells [Zoja et
al. 1991], suggesting that IL-1β plays a crucial role in thrombogenesis. IL-1β has been shown to facilitate mesangial production of collagen and GBM-degrading neutral protease which play an important role in glomerular remodelling [Sedor, 1992]. IL-1β induces chemotaxis and activation of macrophages, polymorphonuclear leukocytes and mesangial cells [Sarén et al. 1996; Yokoo and Kitamura, 1996b]. Inhibition of IL-1β action has been shown to attenuate various forms of experimental glomerulonephritidies, suggesting that IL-1β plays an important role in the generation of glomerular disease [Sedor et al. 1992; Sedor, 1992].

IL-6 is a multifunctional cytokine that has been implicated in the pathogenesis of glomerulonephritis [Chen et al. 1995]. IL-6 is produced by a variety of cell types including macrophages [Sedor, 1992]. Production of IL-6 by macrophages is increased by inflammatory stimuli including LPS, IL-1β and TNF-α [Zoja et al. 1991]. Enhanced IL-6 expression by macrophages has been detected in various forms of glomerulonephritis; e.g., lupus nephritis and mesangial proliferative glomerulonephritis [Takemura et al. 1994]. Previous reports have shown that IL-6 has the ability to activate lymphocytes and induce proliferation of mesangial cells [Ruef et al. 1990]. Furthermore, IL-6 has been reported to stimulate mesangial production of proinflammatory mediators; e.g., reactive oxygen intermediates and PAF [Chen et al. 1995]. IL-6 receptor blockade attenuates lupus nephritis [Kiberd, 1993], suggesting that IL-6 has a role in the pathogenesis of certain types of glomerulonephritis.

Interleukins act synergistically with other cytokines, particularly TNF-α [Baud et al. 1992]. TNF-α is produced by various cells including macrophages [Baud et al. 1992]. Glomeruli from rats injected with TNF-α show a transient influx of leukocytes [Tomosugi et al. 1989]. Locally, TNF-α promotes haemodynamic changes and inflammatory responses. It induces production of reactive oxygen species by human mesangial cells [Radeke et al. 1990]. TNF-α stimulates resident cells to expression of
ICAM-1, an adhesion receptor for leukocytes [Brennan et al. 1990]. TNF-α alone or synergistically with IL-1β induces mesangial production of inflammatory mediators including prostaglandin E2 [Baud et al. 1992] and PAF [Sedor, 1992]. Expression of TNF-α is detectable in various forms of glomerulonephritides [Tang et al. 1994]. Blockade of TNF-α action attenuates experimental glomerulonephritis [Sedor, 1992], suggesting that TNF-α contributes to the pathogenesis of glomerular disease.

The findings described above suggest pathological roles for macrophage-derived IL-1β, IL-6 and TNF-α in glomerular injury. Our current data suggest that TGF-β1, produced by mesangial cells, may exert therapeutically relevant effects via suppression of cytokine production by activated macrophages.

4.3. MESANGIAL CELL-DERIVED TGF-β1 INHIBITS EXPRESSION OF GELATINASE B AND iNOS BY ACTIVATED MACROPHAGES

Using cross-feeding and co-culture studies, we elucidated that; i) activated macrophages induce mesangial cell production of inflammatory mediators including gelatinase B and iNOS, and ii) this stimulatory effect is counteracted by activated mesangial cells via TGF-β1.

Gelatinase B is a matrix-degrading proteinase that is synthesised by a wide range of cell types including macrophages [Baricos and Shah, 1991]. In contrast to gelatinase A, expression of gelatinase B is inducible in response to several proinflammatory stimuli including IL-1β and TNF-α [Sarén et al. 1996; Yokoo and Kitamura, 1996b].
Gelatinases may play an important role in the activation of cells through several possible mechanisms; e.g., release of cells from ECM-mediated cell cycle arrest and activation and release of growth factors stored in ECM [Alexander and Werb, 1989; Shapiro, 1998]. Gelatinase B mainly degrades type IV and V collagens that are major constituents of glomerular ECM [Couchman et al. 1994]. Expression of this proteinase is upregulated in certain glomerular diseases [Maynard et al. 1994; Koide et al. 1996; McMillan et al. 1996], and enhanced gelatinase B activity possibly induces degradation of glomerular matrix, i.e., mesangial matrix and basement membrane, and thereby contributes to structural and functional alteration [Baricos and Shah, 1991], leading to proteinuria [Bruijn et al. 1994].

Nitric oxide (NO) has been identified as an important regulator of many vital biological processes, including the control of vascular tone, neurotransmission, ventilation, hormone secretion, platelet aggregation, inflammation, immunity, mitochondrial respiration and RNA synthesis [Kone, 1997]. In the glomerulus, basal production of NO by the constitutively expressed nitric oxide synthase may play a role in the regulation of glomerular haemodynamics [Raij and Baylis, 1995]. Increased NO production by iNOSs may cause tissue damage as a result of the interaction of NO with superoxide anion, leading to formation of injurious metabolites including peroxynitrite and hydroxyl radical [Hogg et al. 1992; Cattell and Cook, 1993]. Enhanced expression of iNOS is observed in various forms of immune-mediated glomerulonephritidies [reviewed in Cattell and Cook, 1993]. Clinical and histopathological data demonstrated that there is a positive correlation between deterioration of glomerular function and enhanced iNOS expression [Kashem et al. 1996]. The major source of iNOS in nephritic glomeruli is macrophages [Furusu et al. 1998]. It has been reported that blockade of iNOS action attenuates autoimmune glomerulonephritis and anti-Thy 1 glomerulonephritis [Weinberg et al. 1994; Narita et al. 1995].
Taken together, these data suggest that in vivo production of gelatinase B and iNOS by activated macrophages might be involved in the pathogenesis of glomerular injury. Our findings suggest that mesangial cell-derived TGF-β may attenuate glomerular injury via suppression of metalloproteinase and iNOS expression in activated macrophages.

The molecular mechanisms involved in the inhibitory action of TGF-β1 on gelatinase B is not well understood. In the human gelatinase B gene, a TGF-β inhibitory element (TIE) is located in its 5'-flanking region [Huhtala et al. 1991]. Although information is limited on the regulatory region of the rat gelatinase B gene, TGF-β1 could inhibit gelatinase B expression via TIE. Another potential mechanism is that TGF-β1 may attenuate gelatinase B expression indirectly via downregulation of IL-1β (or other stimulators including TNF-α) in macrophages [Kitamura et al. 1996; Sütö et al. 1996b]. Our unpublished results showed that NR8383 cells co-cultured with mesangial cells exhibited blunted expression of IL-1β mRNA in response to LPS.

4.4. CROSS-TALK BETWEEN MACROPHAGES AND MESANGIAL CELLS IN THE GLOMERULUS

To investigate further the cross-talk between macrophages and resident glomerular cells, we have developed a novel approach by transfer of genetically-engineered macrophages into the glomerulus [Kitamura and Sütö, 1997b]. Using this method, we elucidated that delivery of activated macrophages induces expression of gelatinase B, iNOS and stromelysin in resident glomerular cells [Sütö et al. 1997; Sütö and Kitamura, 1997; Kitamura, 1998]. This is consistent with the results from in vitro co-
culture and cross-feeding studies using mesangial cells and activated macrophages, as shown in Figures 3.13., 3.14., and 3.18. This result provides the first, direct evidence that activated macrophages have the ability to induce expression of certain genes in the normal glomerulus.

Molecular mechanisms involved in the triggering action of macrophages are not fully elucidated, but LPS-stimulated macrophages abundantly express IL-1β (Figure 3.15A.). Externally added IL-1β induces gelatinase B and stromelysin in both cultured mesangial cells (Figure 3.15.) and normal glomeruli [Kitamura et al. 1995; Kitamura, 1998]. These results suggest that macrophage-derived IL-1β is a paracrine stimulator that induces glomerular expression of gelatinase B and stromelysin [Sütö et al. 1996b; Kitamura, 1998]

Other molecules elaborated by activated macrophages may be involved in the induction of gelatinase B, iNOS and stromelysin. TNF-α might be another candidate since; i) macrophages produce TNF-α in response to LPS [Kitamura et al. 1996] and ii) TNF-α is a general inducer of gelatinase B, iNOS and stromelysin in mammalian cells [Sarén et al. 1996; Galis et al. 1995].

The central result in the present cell transfer study was that, when stimulated macrophages were transferred into nephritic glomeruli expressing TGF-β1, induction of gelatinase B, iNOS and stromelysin was dramatically repressed, when compared to its induction in normal glomeruli. Based on the fact that TGF-β1 is the suppressor of macrophage-mediated induction of gelatinase B, iNOS and stromelysin in cultured mesangial cells (Figures 3.17. and 3.20.), the insensitivity of the nephritic glomeruli to the stimulatory action of macrophages is likely to be due to the local effect of TGF-β1. Indeed, using an in vivo gene transfer approach [Kitamura et al. 1994c], it has been previously demonstrated that introduction of a mutated gene that encodes the active
form of TGF-β1 suppressed glomerular gelatinase B expression in response to exogenous IL-1β [Kitamura et al. 1995]. These results suggest a suppressive effect of endogenous TGF-β1 in the macrophage-mediated glomerular cell activation.

In this study, we postulated the mesangial cell as a source of gelatinase B and stromelysin expression. However, a recent report has suggested that glomerular epithelial cells also produce gelatinase B in a pathologic circumstance [McMillan et al. 1996]. Furthermore, glomerular endothelial and epithelial cells have been shown to express stromelysin in glomerular diseases [Suzuki et al. 1997]. Mesangial cell-derived TGF-β1 may inhibit macrophage-induced expression of gelatinase B and stromelysin in other intrinsic cells.

In the acute model of anti-Thy 1 glomerulonephritis induced by the mAb 1-22-3, accumulation of macrophages is observed within 24 hours. Even 7 days after the induction of the disease, a number of macrophages still remain in the glomerulus [Kawachi et al. 1992a]. However, in the present study, expression of gelatinase B, iNOS and stromelysin was not obviously detected in the nephritic glomeruli (day 7) where active TGF-β1 was abundantly produced. This result implies the possibility that macrophages may be deactivated in the regenerating glomeruli by mesangial cell-derived TGF-β1 [Sütö et al. 1997; Sütö and Kitamura, 1997].

4.5. TGF-β AS A DEFENDING FACTOR AGAINST MACROPHAGE-MEDIATED GLOMERULAR INJURY

The pathologic outcome of glomerular disease is dependent on the balance between offending factors and defending mechanisms. Several studies have focused on
offending factors that are involved in the initiation of glomerular injury [Border and Ruoslahti, 1992; Border and Noble, 1993; Border and Noble, 1994a; Border and Noble, 1994b]. However, little attention has been paid to elucidate defending factors during the course of the disease.

A wide range of mechanisms may be involved in defence machinery. Activated resident cells have the ability to produce extracellular deactivators of leukocytes (TGF-βs, IL-4, IL-6, IL-10, IL-13, prostacyclins, lipoxins, nitric oxide), inhibitors of cytokines and growth factors (IL-1ra, soluble tumour necrosis factor receptor, decorin) and complement regulatory proteins (complement receptor-1, vitronectin, clusterin). Antioxidants (superoxide dismutase, catalase, nitric oxide) and heat shock proteins, which are produced in response to various stress, may also serve as defence machinery. These defending mechanisms are tightly regulated under pathophysiological conditions. These mechanisms may participate in the resolution of diseases.

It is of importance that constitutive expression of TGF-β1 is detected in the normal glomerulus [MacKay et al. 1990; Okuda et al. 1990; Ando et al. 1995] and that physiological levels of TGF-β are essential for protecting various organs from inflammation [Shull et al. 1992; Kulkarni et al. 1993]. TGF-β is upregulated in various forms of glomerulonephritides. Using the in vivo gene transfer technique, Kitamura and coworkers investigated the anti-inflammatory potential of TGF-β in the glomerulus [Kitamura et al. 1995]. They found that mesangial cells, overexpressing active TGF-β1 showed a reduced mitogenic response to FCS and blunted induction of gelatinase B in response to IL-1β in vitro. They further examined IL-1 responses and mitogenesis of normal glomeruli expressing active TGF-β1 in vivo. Isolated, normal glomeruli containing TGF-β1-transfectants exhibited reduced mitogenic activity and repressed gelatinase B expression in response to IL-1β. When TGF-β transfectants were transferred into glomeruli subjected to an in vivo mitogenic stimulus (anti-Thy 1
glomerulonephritis), the mitogenic activity was also significantly attenuated [Kitamura et al. 1995]. These findings, together with our current data, support the anti-inflammatory potential of TGF-β1 in macrophage-mediated glomerular injury.

In this thesis, we provide evidence for the protective role of TGF-β in macrophage-mediated glomerular injury. Our data demonstrate that mesangial cell-derived TGF-β1 impairs adhesiveness of macrophages and confers blunted responses to a specific stimulus. This may be one potential mechanism for macrophage clearance from inflamed glomeruli. During the course of glomerulonephritis, a wide range of proinflammatory mediators are released from infiltrating macrophages, which contribute to glomerular injury. We identified that TGF-β1 is a mesangial cell-derived paracrine factor that inhibits macrophage production of cytokines, metalloproteinase and iNOS. Indeed, using an in vivo macrophage transfer method, we demonstrated that macrophage-triggered glomerular cell activation is repressed in the glomeruli producing TGF-β1.

Taken together, these data suggest a beneficial potential of TGF-β1 in early glomerular inflammation. TGF-β1 may function as a “defender” that protects the glomerulus from macrophage-mediated injury.

4.6. ROLE OF TGF-β IN GLOMERULAR DISEASE: UNANSWERED QUESTIONS

Several studies have been designed to elucidate the role of TGF-β in the pathogenesis of glomerulonephritis by using strategies to block or reduce TGF-β activity in vivo [Border et al. 1990; Border et al. 1992; Akagi et al. 1996; Isaka et al. 1996a; Isaka et al. 1996b]. In the majority of these studies, a rat model of acute mesangial proliferative
glomerulonephritis was utilised. This model, the acute anti-Thy 1 glomerulonephritis, is characterised by mesangial cell proliferation and matrix expansion that reaches maximum level at around 1 - 2 weeks after the initiation of injury. Pathologic changes in the glomeruli then resolve spontaneously [Bagchus et al. 1986; Yamamoto and Wilson, 1987]. Therefore, the acute anti-Thy 1 glomerulonephritis model is considered to be a model of glomerular repair, but not irreversible glomerulosclerosis. It is, therefore, still unclear whether sustained, local expression of TGF-β1 is sufficient for the generation of irreversible glomerulosclerosis. To determine the exact role of TGF-β in progressive glomerulosclerosis, chronic experimental diseases must be tested.

To examine this point, transgenic mice that express TGF-β1 in the liver under the control of an albumin promoter were generated. These mice developed hepatic fibrosis and severe extrahepatic lesions including progressive glomerulosclerosis and chronic renal failure [Sanderson et al. 1995; Kopp et al. 1996]. However, in this case, intense deposition of IgG immunoglobulins was detected in the glomerulus. The glomerulosclerosis observed in this model may be an outcome of immune-complex-mediated glomerular injury.

The pathophysiological role of TGF-β1 has been extensively investigated, but little attention has been paid to other TGF-β isoforms. It is believed that biological activities of the TGF-β isoforms are similar, but differences in potency may exist in particular cell types [Merwin et al. 1991; Pelton et al. 1991]. For example, it has been shown that TGF-β1 and -β2 enhance scarring whereas TGF-β3 reduces scar formation in the skin [Shah et al. 1995]. Using targeted deletions of TGF-β2 and TGF-β3 isoforms in mice, recent reports have provided new insights into the functional differences among TGF-β isoforms [reviewed in Bitzer et al. 1998]. The function of TGF-β2 and -β3 as well as other TGF-β family members, including bone morphogenetic proteins (BMPs) in the glomerulus must be elucidated in the future. Of note, it has been suggested that BMP-
7 plays a pivotal role in the maintenance of glomerular function [Vukicevic et al. 1996].

Although accumulation of macrophages plays a crucial role in the generation of glomerulonephritis, recent studies have identified neutrophils and T cells in smaller numbers in the diseased glomerulus. TGF-β may affect the function of these leukocytes in the acute phase of glomerular inflammation. Indeed, Smith et al. reported that TGF-β inhibits endothelial production of IL-8, a neutrophil chemoattractant [Smith et al. 1996], as well as transmigration of neutrophils into the extravascular space via inhibiting the endothelial expression of E-selectin and/or IL-8 [Couser, 1993; Smith et al. 1996]. Similarly to its effect on macrophages, TGF-β may attenuate certain inflammatory actions of neutrophils via inhibiting their accumulation in the glomerulus.

In summary, the prosclerotic action of TGF-β has been well documented by many investigators. However, this molecule also has anti-inflammatory properties. Further investigation will be required to explore both the prosclerotic and the anti-inflammatory actions of TGF-β, focusing especially on the concentration and the time point at which its anti-inflammatory properties spill over into its prosclerotic actions.
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PUBLICATIONS ARISING FROM THIS THESIS

PEER-REVIEWED PUBLICATIONS


EDITORIALS AND REVIEWS

ABSTRACTS

Kitamura M, Sütő TS. Site-directed targeting of genetically engineered macrophages into the normal rat glomerulus. *Nephrology* 1997; 3(Suppl): S242


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