The normal function of the renal glomerulus depends on the integrity of the glomerular basement membrane (GBM) and of the extracellular matrix (ECM) of the mesangium. These matrices, however, are dynamic structures that are subject to continuous turnover. This turnover is the result of a tightly controlled equilibrium between the synthesis of new matrix constituents and the degradation and removal of old. In renal disease, this balance may be lost with an inappropriate swing toward either matrix degradation or matrix accumulation. In several studies, changes in the amount and composition of matrix have been linked to profound effects on the normal function of cells (1,2). Thus, an understanding of the mechanisms that determine the amount and composition of the glomerular ECM is of paramount importance to understanding normal glomerular function and the consequences of pathologic changes.

The principal enzymes implicated in the remodeling of ECM are the matrix metalloproteinases (MMP), a group of enzymes that, together, are able to degrade all of the constituents of the ECM. Metalloproteinase production by glomerular cells is now well documented (3–5). In particular, human glomerular mesangial cells (HMC) can secrete MMP2 and MMP9, gelatinases that degrade the major constituent of the GBM type IV collagen. These enzymes are zinc-dependent proteinases that are active in the neutral pH of the extracellular environment, and their final activity is carefully controlled at several levels: by the regulation of transcription, the rate of secretion, and the activation of the enzymes. The MMP are secreted as a proenzyme or latent form, and the activation of these enzymes is due to the proteolytic removal of the propeptide exposing the active site (6). The activation of MMP2 seems to be through a different mechanism to that described for the other secreted MMP, and it now seems that the newly described membrane type metalloproteinases, the MT-MMP, are involved in the activation of this enzyme (7). In addition, the active enzymes can be inhibited on a one-to-one basis by their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMP) of which TIMP I and TIMP II are the best described. Thus, the modification of MMP activity at any one of these steps can affect the turnover of the glomerular matrix.

The regulation of the rate of secretion of these enzymes by cytokines has received a great deal of attention (8–10). Less well understood, however, is the effect that matrix itself may have on the degree and kinetics of its own turnover through the control of MMP activity. Changes in the status and composition of the ECM have been shown to affect the function of a variety of different cells and to modulate the synthesis and release of MMP in other systems (11–14). Thus, any change in
the composition of the mesangial matrix or in the degradation of the constituent proteins that cause the release of soluble protein fragments would be expected to have profound effects on the normal activity of these HMC. This study examines the interaction of HMC with matrix proteins and determines the effect of these proteins on the secretion and activation of the MMP.

**Materials and Methods**

Tissue culture media and supplements were obtained from Sera Lab/RBH Biosciences Ltd. (Crawley, UK) or ICN Biomedical (Bas- ingstoke, Hampshire, UK), and Sigma Chemical Company (Poole, Dorset, UK). Tissue culture plastics were purchased from Falcon; Becton Dickixon (Oxford, UK). Type I collagen was prepared from rat tail tendon as described (15) and denatured at 60°C for 30 min to provide gelatin I. Collagen IV, fibronectin, vitronectin, and laminin were of tissue culture grade and were purchased from Sigma. Gelatin IV was prepared from denatured collagen IV as above. The purity of all of the matrix proteins used in this study was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis reagents were purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts, UK). All other chemicals were of the highest quality available.

**Cell Culture and Identification**

Human glomeruli were obtained by the serial sieving of the normal regions of human kidney recovered at nephrectomy. HMC were maintained in RPMI-1640 containing 20% vol/vol fetal calf serum (FCS). Cells in low passage number (between passages 1 and 5) were used in all experiments. Before experimental procedures, HMC were growth-arrested for 48 h by culture in medium containing 0.2% (wt/vol) lactalbumin hydrolysate (Difco Laboratories, Detroit, MI) in the absence of serum (4). The cells were confirmed to be mesangial cells by morphology and the use of immunohistochemistry as described (9). Cells showed positive staining for intracellular myosin fibrils and were negative for factor VIII and cytokeratin. The effect of all subsequent treatments on cell viability was assessed by 3-[4,5-dimethylthiazole-2-yl]-2,4 diphenyltetrazolium bromide assay (10).

**Cell Adhesion to Defined ECM Proteins**

Ninety-six-well plates were precoated with the following matrix proteins overnight at 4°C: collagen IV, gelatin IV, collagen I, gelatin I, fibronectin, vitronectin, and laminin (10 μg/ml in phosphate-buff ered saline [PBS]). Then they were washed with PBS and blocked with PBS/BSA (1 mg/ml) before use. Controls consisted of wells treated with BSA (1 mg/ml) in PBS. HMC were removed from confluent cultures by the use of ethylenediaminetetraacetic acid (EDTA; 2 mM in PBS) alone, washed in RPMI, added to the wells at a concentration of up to 6 × 10^5/ml in RPMI BSA (1 mg/ml), and allowed to adhere for 1 h at 37°C in an atmosphere of 5% CO₂. Nonadhered cells were removed by washing with PBS containing 2 mM MgCl₂. Adhered cells were fixed with paraformaldehyde (3% vol/vol) for 15 min and stained with a solution of crystal violet (0.5% wt/vol) for 3 min. The cells were then washed with PBS, dried, and solubilized with SDS (1 wt/vol), and the absorbance at 540 nm was determined on a Dynatech MR5000 platerreader (Billinghurst, UK).

**Determination of Gelatinolytic Activity**

To determine the effect of matrix proteins on the secretion of gelatinolytic activity by the cells, we growth-arrested confluent wells of mesangial cells by culture for 48 h in serum-free medium supplemented with 0.2% (wt/vol) lactalbumin hydrolysate. Extracellular matrix proteins (10 μg/ml) were added in solution in the above medium, and the incubation was continued for an additional 72 h. The conditioned medium (CM) was then collected and subjected to zymography. Gelatinolytic activity was determined by subjecting a sample of CM to SDS-PAGE using either a 7.5% gel or a 5 to 12% gradient gel, incorporating gelatin (1 mg/ml) under nonreducing conditions, as described previously (4). To determine net gelatinolytic activity (not inhibited by TIMP) in the supernatants, we assayed samples of CM for their potential to degrade ¹⁴C-labeled gelatin as described previously (4,5).

**Purification of Latent MMP2**

Gelatinolytic activity was semipurified by passing the CM containing latent MMP2 down a gelatin agarose affinity column (Sigma), equilibrated with 50 mM Tris (pH 7.4), containing 0.5 M NaCl, 0.05% Brij wt/vol, and 10 mM EDTA, and then washed in the same buffer. Activity was eluted with 50 mM Tris, containing 1 M NaCl 0.05% (vol/vol) Brij, 10 mM EDTA, and 5% dimethyl sulfoxide (vol/vol). The enzyme was then dialysed into 50 mM Tris (pH 7.4), 0.5M NaCl, 0.05% Brij, 5 mM CaCl₂, and stored at −20°C until use.

**Detection of Cell-Associated MT1-MMP Protein**

HMC were lysed in SDS-PAGE sample buffer, and whole-cell lysates were separated on 7.5% SDS-PAGE gels. After electrophoretic transfer to nitrocellulose and blocking in 5% skimmed milk in PBS, 0.5% Tween 20, the blots were incubated with 10 μg/ml anti-MT1-MMP antibody (TCS Biologicals Ltd., Bodolph Claydon, UK) in PBS, 0.5% Tween 20, 1% BSA overnight. Primary antibody binding was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, UK).

**Preparation of Mesangial Cell Membranes**

HMC were scraped off flasks and pelleted by centrifugation in PBS containing a cocktail of proteinase inhibitors (pepsatin A, aprotinin and leupeptin at 10 μg/ml, phenylmethylsulfonyl fluoride at 0.5 μM; Sigma). Cells were resuspended in hypotonic buffer (10 mM Tris [pH 7.2] containing 1 mM MgCl₂ plus proteinase inhibitor cocktail), mixed with 4 volumes of sucrose buffer (0.25 M sucrose dissolved in hypotonic buffer) and homogenized in this buffer using a Dounce pestle.

The homogenate was centrifuged at 500 × g for 5 min to remove debris. A membrane-enriched fragment was prepared by centrifuging the supernatant at 25,000 × g for 30 min at 4°C, and the resulting pellet was resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride. The protein content of the membrane samples was determined by the method of Bradford (16). Membranes were stored at −70°C until use.

To determine the activating properties of the membrane preparations, latent MMP2 was added to membrane fractions (10 μg protein) and the mixture was incubated for 24 h at 37°C. The degree of MMP activation was then determined by gelatin zymography as described.

**Reverse Transcription-PCR**

Confluent wells of mesangial cells were growth-arrested as described and cultured in the presence of fibronectin for an additional 6 h. Total RNA was extracted using RNA Isolator (Genosys Ltd., Cambridge, UK) according to the manufacturer’s instructions. cDNA was prepared by the reverse transcription of 1 μg of RNA using random primers, and the equivalent of 0.05 μg was amplified by PCR.
using the following primers specific for MT1-MMP, MT2-MMP, MT3-MMP, and \( \alpha \) actin (Genosys Ltd., Cambridge, UK):

**MMP2**

\[ 5' \text{TCTTCGCAATCAGGATGY} \ 3' \]
\[ 5' \text{CTGGTGCAAGCTCATATT} \ 3' \]

**MMP9**

\[ 5' \text{AGCATGGAGAACATCTCAG} \ 3' \]
\[ 5' \text{GGACCGATGGTACATTAA} \ 3' \]

**MT1-MMP**

\[ 5' \text{GCCGCCCGATGTGGTGTTC} \ 3' \]
\[ 5' \text{TGCCCCGCGGTCATCATC} \ 3' \]

**MT2-MMP**

\[ 5' \text{GCCCCCACACCGCTCTATTC} \ 3' \]
\[ 5' \text{CCGACGTCCTCCCACCAA} \ 3' \]

**MT3-MMP**

\[ 5' \text{TATTCGCCGTGCCTTTGATGT} \ 3' \]
\[ 5' \text{TGGGGGCACTGTCGGTAGAG} \ 3' \]

\( \alpha \) actin

\[ 5' \text{GGAGCAATGATCTTGATTT} \ 3' \]
\[ 5' \text{CCTTCCTGGGCATGGAGTCCT} \ 3' \]

\( \beta \) actin

\[ 5' \text{CTGGGCCATTCTCCTTAG} \ 3' \]
\[ 5' \text{ATCCCCCAAAGTTCACAA} \ 3' \]

Northern Blot Analysis

Total RNA (up to 10 \( \mu \)g) was run on a denaturing agarose gel and transferred by vacuum blotting onto a Hybond nylon membrane (Amersham). mRNA for MT-MMP was detected by hybridization with a \( ^{32} \)P-labeled probe prepared from PCR products and detected as described previously (9).

Analysis of TIMP I and TIMP II

TIMP I and TIMP II were measured by enzyme-linked immunosorbent assay on suitably diluted samples using reagents generously supplied by Dr. A. J. P. Docherty (Celltech Ltd., Slough, UK) essentially as described previously (10,17). Standards of TIMP I and TIMP II were prepared using purified TIMP kindly supplied by Prof. T. E. Cawston (Department of Medicine [Rheumatology], University of Newcastle, UK) in the range of 0.15 to 150 ng/ml for TIMP 1 and in the range of 0.15 to 300 ng/ml for TIMP II.

Statistical Analyses

Results are expressed as mean ± SEM of \( n \) experiments. All data were analyzed using the Wilcoxon signed rank test.

Results

HMC Binding to Matrix Proteins

After seeding in the wells of 96-well plates, which had been precoated with individual matrix proteins, there was significantly increased HMC adherence to collagens I and IV (\( P < 0.01 \)) and to denatured type IV collagen (gelatin IV) and fibronectin (\( P < 0.05 \); Figure 1). Cells also bound to vitronectin, laminin, and denatured collagen I, but the number bound was not significantly increased above control.

Matrix Protein Stimulation of MMP Release

The addition of soluble matrix proteins to HMC resulted in the selective modulation of MMP release, detected by zymography. This release, however, was not directly associated with the degree of HMC binding that each protein supported. Figure 2 demonstrates that of the matrix proteins added, collagen I and fibronectin stimulated an increase in the amount of latent MMP2 present in the CM, and, in a similar manner to collagen IV, they also induced its conversion to a lower molecular weight band (66 kD) on the zymogram. In addition, fibronectin induced the secretion and activation of MMP9 seen as bands of lysis at 92 and 95 kD. The effect of fibronectin was dose-dependent with maximal activation of MMP2 and induction of MMP9 occurring above 10 \( \mu \)g/ml (Figure 3). The increase in MMP protein levels was paralleled by an increase in the mRNA (as shown by reverse transcription-PCR [RT-PCR]) for MMP2 and the induction of the mRNA for MMP9 (data not shown). This effect was mimicked by incubation with anti-CD49e monoclonal antibody specific for the \( \alpha 5 \) integrin chain (Immunotech, Birmingham, UK; Figure 4), suggesting that the response to fibronectin was mediated through this integrin.

Changes in the amount of MMP detected were not related to changes in proliferation, and there was no difference in cell number in response to co-culture with any of the ECM proteins under the conditions used (data not shown). Furthermore, analysis (Cytosets Antibody Pair kits, Biosource International, Nivelle, Belgium) of the CM from stimulated HMC demonstrated that there were no detectable levels of cytokines previ-
It has been previously described as potential promoters of MMP secretion (interleukin-6 [IL-6] and IL-8 below 15 pg/ml, IL-1β and tumor necrosis factor-α below 10 pg/ml, transforming growth factor-β below 5 pg/ml; data not shown).

MMP Activation by HMC

MMP2 is now believed to be activated in vivo by the action of the membrane bound metalloproteinases, the MT-MMP. To determine whether this might be occurring in this instance, we incubated latent MMP2 with cell membranes isolated from HMC cultured with different concentrations of fibronectin. HMC membranes were shown to activate latent MMP2, and this activity was markedly increased in the membranes of cells that had been incubated in the presence of fibronectin or FCS (Figure 5). This effect was dose-dependent and became apparent at 10 µg/ml fibronectin (Figure 6).

To ascertain whether this result could be due to an increase in the amount of the MT-MMP synthesized by the HMC, we examined the levels of mRNA for MT1-MMP, MT2-MMP, and MT3-MMP relative to actin by RT-PCR (Figure 7) and Northern blotting (Figure 8). The mRNA for all three MT-MMP was detected in HMC. There was, however, no increase in the levels of mRNA. Densitometric analysis of these figures...
confirmed that there was no increase in the amount of mRNA for these MT-MMP relative to actin, confirming that there was no increased expression of these potential MMP2 activators. Analysis of whole-cell lysates by SDS-PAGE and immunoblotting, however, demonstrated an increase in the ratio of the lower molecular weight form of MT1-MMP over the larger form with increasing concentrations of fibronectin (Figure 9).

Effect of Exogenous Fibronectin Added to HMC on TIMP Release

The consequence of the addition of fibronectin to HMC on the secretion of the inhibitors of MMP was examined by enzyme-linked immunosorbent assay. There was no statistically significant effect on the secretion of either TIMP I or TIMP II in response to fibronectin (Figure 10), although there was a trend toward an increase in TIMP II secretion with increasing concentrations of fibronectin in some experiments. Whether this trend reflected a real change in the total gelatinolytic activity released by HMC was investigated in a radiometric assay of net activity in the CM of stimulated cells (4,5). In response to 10 μg/ml fibronectin or 4 μg/ml anti-α5 integrin antibody, however, there was a 1.5-fold increase or a 3.6-fold increase in activity, respectively, over unstimulated cells, suggesting that the observed trends in TIMP II expression were not sufficient to inhibit the increased gelatinolytic activity released from the cells.

Discussion

The present study demonstrates that HMC bound specifically to several of the matrix proteins found in the glomerular ECM and to others not normally present. There was, however, a rank order to the binding, with collagen I, collagen IV, gelatin IV, and fibronectin supporting the strongest binding. These data concur with those of a previous report describing the attachment of rat MC to matrix substrates (18). As a result of incubating HMC with the same proteins, fibronectin, collagen I, and collagen IV were also found to stimulate the specific secretion and activation of MMP2. The activation was seen as an additional band of lysis below the latent enzyme. In addition, fibronectin stimulated the induction and activation of MMP9, suggesting an activating mechanism selectively induced by the interaction with this matrix protein. Matrix components have previously been shown to influence the phenotype of cultured mesangial cells (1,2,19). These alterations included a change in the synthesis of matrix components when the cells were cultured on different types of ECM (20) and

Figure 6. Fibronectin increases the MMP-activating potential of HMC membranes. Purified latent MMP2 was incubated for 24 h with membranes prepared from HMC that had been incubated with increasing concentrations of fibronectin.

Figure 7. The effect of fibronectin on membrane-type (MT)-MMP mRNA levels. Fibronectin (10 μg/ml) was added to growth-arrested HMC and incubated for up to 12 h. The RNA was extracted, and reverse transcription-PCR (RT-PCR) was carried out using primers specific for MT1-MMP (A), MT2-MMP (B), and MT3-MMP (C). Actin was used as the housekeeping gene, and the PCR products were separated on 2% agarose gels.
increased proliferation when the cells were cultured in collagen gel matrices (21). By adding the matrix components under investigation to growth-arrested cells in serum-free conditions, we were able to exclude mesangial cell proliferation from contributing to the altered secretion of the metalloproteinases seen in this study.

Activation of MMP2 occurs through a different mechanism to that for the other MMP. The enzyme is activated in vitro by incubation with mercuric salts such as p-amino phenyl mercuroacetic. It has also been reported that MMP2 may be activated by other proteinases, such as plasmin (22), or by oxygen radicals. Recently, another activation mechanism involving MT-MMP has been described. Five MT-MMP have now been described (23–25), at least two of which (MT1-MMP and MT2-MMP) are capable of activating latent MMP2. MMP2 is secreted by cells in an inactive form and is activated on the cell surface as part of a complex involving the MT-MMP and TIMP II. It is now accepted that the MT1-MMP binds to TIMP II and that this complex acts as a receptor for MMP2 by binding to the carboxyl end of the molecule (7,26,27). MT1-MMP then proteolytically cleaves the propeptide of the MMP2, activating the latent enzyme. However, if TIMP II is present in excess, it will function as a specific inhibitor by binding through its N terminal domain. Therefore the presence of TIMP II in the extracellular milieu can serve either to assist in the activation of the gelatinase or to inhibit the active enzyme in a 1:1 manner. Such a role for this inhibitor would seem to ensure that the MMP2 will be active locally but be inhibited before it can degrade matrix proteins more distal from the cell surface. In vivo, other mechanisms may also be involved in MMP activation. For example, urokinase plasminogen activator induces the activation of MT1-MMP in HMC (28). In addition, the activation of MMP9 that was observed after the addition of fibronectin to HMC may be secondary to the production of activated MMP2 as active MMP2 has been demonstrated to activate the latent MMP9 in vitro (29) and this enzyme is known to have a different activation mechanism to MMP2.

In the present study, membrane-associated activity, selectively induced by fibronectin, was implicated in the activation of the secreted MMP2. There was, however, no increase detected in the level of mRNA coding for MT1-MMP, MT2-MMP, or MT3-MMP either by RT-PCR or by Northern blots. This finding suggests that the increase in activity seen in the membrane preparation may be due to a modification of the MT-MMP rather than to an increase in the amount produced by the cell. This is further supported by the demonstration that the ratio of the smaller molecular weight (active) form to the larger (latent) form increases with increasing concentrations of fibronectin. This is in agreement with the work of Stanton et al. (30). These workers demonstrated an increase in the activity of MT1-MMP that was paralleled by a decrease in its molecular weight in the absence of any increase in the amount of mRNA present in HT1080 fibrosarcoma cells after their culture on...
fibronectin. Only a slight increase in total MT1-MMP protein production was seen, whereas there was a marked increase in the amount of activated MT1-MMP present. Similarly, Preaux et al. (31) recently demonstrated an activation of MMP2 from myofibroblasts cultured on collagen I with no increase in MT1-MMP mRNA. However, Haas et al. (32) demonstrated an increase in the transcription of MT1-MMP by proliferating endothelial cells in a three-dimensional collagen matrix.

There have been other reports of matrix proteins influencing MMP secretion. For example, it has been shown (11,33) that the ligation of the fibronectin receptor induced the gene expression of both collagenase and stromelysin by fibroblasts. This seemed to be mediated through the 120-kD cell binding fragment. In addition, Larjava et al. (34) described how antibody binding to $\beta_1$ integrin in human keratinocytes induced the 92-kD gelatinase (MMP9), although there was no effect of the extracellular proteins themselves—possibly because these workers cultured the cells on these substrates rather than adding them in solution to cells in culture. Brooks et al. (35) demonstrated that an anti-integrin $\alpha_{V}\beta_3$ antibody was able to block human breast cancer growth possibly through an effect on MMP activity. Furthermore, the use of an antibody to this vitronectin receptor or the addition of vitronectin itself resulted in the increased secretion of MMP2 by a melanoma cell line (12). Later work, however, showed that the ligation of integrin $\alpha_5\beta_1$ seemed to be more important than that of $\alpha_{V}\beta_3$ for stimulating MMP2 secretion in a different melanoma cell line (36). The present study demonstrating the induction of MMP by anti-$\alpha_5$ suggests that in HMC, this receptor is responsible for the observed effects of fibronectin, although whether other receptors may also be involved cannot be ruled out.

The variety of responses to matrix binding, mediated by integrins, has been linked to several different signal transduction pathways. For example, binding to $\beta_1$ integrins induces phospholipase activation in rat glomerular epithelial cells (37), which may modulate proliferation. In addition, Malik and Parsons (38) demonstrated the activation of the p70 ribosomal S6 kinase signaling pathway. Fibronectin binds to the $\alpha_5\beta_1$ integrin and, in other systems, induces the activity of the AP1 transcription factor (39,40). It is therefore likely that this is one of the mechanisms operating in the present system because MMP9 possesses an AP1 binding site in its promoter (41).

The work described here demonstrates that the interaction of mesangial cells with specific matrix proteins can modulate the pattern of gelatinolytic activity secreted by the cells. Thus, any change in the composition of the matrix in vivo would be expected to result in a change in the proteolytic activity present in the glomerulus. Such a change in matrix organization is often seen accompanying glomerulosclerosis and interstitial nephritis, where it is directly linked with the progression to end-stage disease. This is seen both in animal models (42) and in human renal diseases in which increased amounts of fibronectin are localized to areas of sclerosis within the mesangium (43,44). Thus, it seems that in certain pathogenic situations in which the normal constituents and quantities of the extracellular matrix have been disturbed, there may be a related change in the MMP profile and/or activation that may result either in a resolution of the abnormality or, conceivably, in a progression to further damage. Because the expression of particular integrins and their capacity for binding individual proteins, the intracellular signals generated, and the responses

Figure 10. Percentage of tissue inhibitors of metalloproteinases (TIMP) release compared with control (100%) from HMC incubated with fibronectin. Fibronectin (5 to 100 $\mu$g/ml) was added to growth-arrested HMC, and the incubation continued for 72 h. TIMP I (A) and TIMP II (B) were then measured in the CM by enzyme-linked immunosorbent assay. The results shown are the mean $\pm$ SEM of four separate experiments.
stimulated in the cells all vary between different cell types, it will be important to investigate the detailed integrin-specific mechanisms involved in the current study. An understanding of whether the same mechanisms apply to other glomerular cells, e.g., epithelial cells, will also allow the more detailed investigation of the role of individual cells in disease progression.

References

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