Characterization of a Glomerular Epithelial Cell Metalloproteinase as Matrix Metalloproteinase-9 with Enhanced Expression in a Model of Membranous Nephropathy

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Abstract

The role of the glomerular visceral epithelial cell in the physiologic turnover and pathologic breakdown of the glomerular extracellular matrix has remained largely unexplored. In this study a 98-kD neutral proteinase secreted by cultured rat visceral glomerular epithelial cells was shown to be a calcium, zinc-dependent enzyme secreted in latent form. In addition, the protein was heavily glycosylated and demonstrated proteolytic activity against Type I gelatin, Type IV collagen gelatin, and fibronectin. The similarity in molecular mass and substrate specificities to the 92-kD human matrix metalloproteinase-9 (MMP-9, or gelatinase B) suggested the identity of this activity, which was confirmed by immunoprecipitation and Northern blot analysis. The differences in molecular mass (98 vs. 92 kD) were not due to species-specific differences in glycosylation patterns, since cultured rat peritoneal macrophages secreted MMP-9 as a 92-kD enzyme. Furthermore, transfection of the human MMP-9 cDNA into rat glomerular epithelial cells yielded the 98-kD product. Using a specific monoclonal anti–MMP-9 antibody and in situ reverse transcription (ISRT) analysis of MMP-9 mRNA, the expression of this enzyme was evaluated in vivo. Normal rat glomeruli expressed little immunohistochemical or ISRT staining for MMP-9, while in rats with passive Heymann nephritis there was a major increase in MMP-9 protein and mRNA staining within the visceral epithelial cells. The temporal patterns of MMP-9 expression correlated with the period of proteinuria associated with this model, suggesting that a causal relationship may exist between GEC MMP-9 expression and changes in glomerular capillary permeability. (*J. Clin. Invest.* 1996. 97:1094–1101.) Key words: gelatinases • kidney glomerulus • epithelium-enzymology • glomerulonephritis-membranous • basement membrane-metabolism

Introduction

The glomerular basement membrane (GBM)[1] serves as a critical ultrafiltration barrier and is comprised of both size and charge-restricting characteristics. Proteinuria, which is the hallmark of most forms of glomerular disease, occurs as a consequence of the loss of normal permselective properties. Although the consequences of this phenomenon are well studied, the actual pathophysiologic mechanisms involved are not fully resolved and may involve several complementary or independent pathways. For example, loss of negative charge in the GBM due to enzymatic cleavage of heparin sulfate proteoglycan or charge neutralization results in rapid development of proteinuria (1, 2). Other studies have pointed to various reactive oxygen species released by inflammatory or intrinsic glomerular cells as direct mediators of the loss of permselectivity (3–5). A further potential mechanism for GBM damage involves the action of proteolytic enzymes (6–10). For example, the direct infusion of neutrophil elastase or cathepsin G resulted in the nearly immediate appearance of massive proteinuria in the absence of obvious structural changes (11).

The role of proteolytic enzymes produced by intrinsic glomerular cells in glomerular inflammatory processes has become the focus of intense investigation, particularly in regard to the mesangial cell (reviewed in references 12 and 13). In contrast, much less has been learned about the spectrum of proteolytic enzymes produced by the visceral epithelial cell, which, by virtue of its location, could be expected to play a major role in the inflammatory processes affecting the GBM. Using cultured rat glomerular visceral epithelial cells, we report that these cells secrete a glycosylated 98-kD neutral metalloproteinase which is the homologue of the human MMP-9 (gelatinase B). Furthermore, induction of visceral glomerular epithelial cell injury in the passive Heymann nephritis model leads to a rapid increase in MMP-9 synthesis in a time period associated with maximal proteinuria, suggesting that a direct link exists between glomerular epithelial cell proteolytic activities and loss of glomerular permselectivity.

Methods

Cell culture. Rat glomerular epithelial cells (GECs) were prepared and extensively characterized, as previously reported (14, 15). Morphologic characterization included a polygonal shape with a cobblestone growth pattern, while by scanning electron microscopy the cells were round to polygonal with large nuclei and numerous short, finger-like projections. Exposure of the cells to puromycin in concentrations ranging from 25 to 100 μg/ml was cytotoxic. Immunofluorescence staining was positive for the Fx1A antigen and podocalyxin, while negative staining was obtained using antibodies to factor VIII

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1. *Abbreviations used in this paper:* APMA, *p*-aminophenyl mercuric acetate; GBM, glomerular basement membrane; GEC, glomerular epithelial cell; hrMMP-9, human recombinant MMP-9 expressed in COS cells; MMP-9, matrix metalloproteinase-9; TIMP, tissue inhibitors of metalloproteinases.

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and Thy 1.1 antigens. Cells were grown on uncoated plastic in defined medium (K1) (16) and passaged by trypsinization. Where indicated, GEC were stimulated with 10^{-7} M phorbol 12-myristate 13-acetate (PMA) during the period of conditioning. Conditioned medium was harvested after 2–3 d of exposure to the cells, clarified by centrifugation, and concentrated with 10 kD cut-off membranes (Amicon). The typical concentration used was 50×.

Rat peritoneal macrophages were harvested from the peritoneal cavities of thioglycollate-injected Sprague-Dawley rats as previously reported in detail (17). After attachment of the cells in RPMI medium containing 5% FBS, the plates were washed three times with warm PBS to remove nonadherent cells, and fresh medium added. After a further wash at 24 h, the medium was replaced with RPMI containing 0.5% FBS and PMA 10^{-7} M. After 20 h the conditioned medium was harvested.

Preparation of GEC microsomes. GECs were washed twice in Ca^{2+}-, Mg^{2+}-free PBS, harvested by scraping and centrifuged for 10 min at 400 *g*. The cellular pellet was resuspended in 20 mM NaB_4O_7, pH 8.0, and disrupted by freeze/thawing. Large cellular debris was removed by centrifugation at 200 *g* for 10 min and the resultant supernatant centrifuged at 15,000 *g* for 20 min. The microsomal pellet was washed twice with 20 mM Hepes, pH 7.4, and stored at −80°C until use.

Expression of MMP-9. Human recombinant MMP-9 (hrMMP-9) was expressed in either COS-7 (American Type Culture Collection, Rockville, MD) or COS-6M cells by transfection of an expression plasmid, pCDM8 (Invitrogen, San Diego, CA), into which the cDNA of the open reading frame of human MMP-9 (gift from Barry L. Marmer, Washington University, St. Louis, MO) had been inserted. Control cDNA for transfection experiments consisted of a sub-pool of non–MMP-9 expressing GEC cDNA cloned into the pCDM8 expression plasmid. Transient transfections were performed using a DEAE dextran method modified from Kriegler (18). COS cells were plated in Dulbecco's modified Eagle's (DME) (containing 10% fetal bovine serum, 0.2 mM glutamine, 10 U/ml penicillin G, and 100 μg/ml streptomycin) onto six-well plates at a density of 1.8×10^5 cells per well. After 24 h, the cells were washed with warm PBS, followed by Tris-buffered saline (pH 7.4). Sterile DNA/DEAE dextran solution (for each well 25 μl of expression plasmid DNA [20 ng/μl in water], 50 μl Tris-buffered saline, and 50 μl of DEAE dextran [1 mg/ml]) was placed on the cells and agitated every 5 min for 1 h at room temperature. The cells were rinsed with Tris-buffered saline twice, after which 10% DMSO in PBS was placed on the cells for 60 s. After a second PBS rinse, complete medium containing 100 μM chloroquine was added and the cells were incubated for 4 h at 37°C. The medium with chloroquine was replaced with DME containing 2.5% fetal bovine serum. After incubation for 18 h, serum-free DME was placed on the cells and the culture continued for 3 d. The medium was harvested, clarified by centrifugation, and stored at −80°C. The same protocol was used for the transient transfection of GEC, with the substitution of K1 medium for DME.

Methods of analysis of enzyme activity. Gelatin zymography was performed on 7.5% polyacrylamide gels containing 2 mg/ml Type I collagen gelatin as reported in detail (19). For inhibition studies, either 10 mM EDTA, 10 mM EGTA, 10 mM 1,10-phenanthroline, 5 mM phenylmethylsulfonyl fluoride or 5 μg/ml recombinant TIMP-1 (gift of G. Murphy, Strangeways Laboratory, UK) were included into the buffers used for detergent exchange and overnight incubation of the gelatin/acrylamide gels.

The time course of *p*-aminophenyl mercuric acetate (APMA) mediated enzymatic activation was performed by incubation of conditioned medium at 37°C with 0.5 mM APMA. The reactions were stopped at various time points by placing the tube containing the mixture at −80°C.

Substrate specificity of GEC 98-kD gelatinase. Composite 7.5% polyacrylamide gels were poured with the left half of the resolving gel containing 2 mg/ml Type I collagen gelatin and the right half containing one of the following substrates in place of gelatin: type IV collagen gelatin (0.4 mg/ml final gel concentration) (Collaborative Research Inc., Waltham, MA), fibronectin (0.6 mg/ml final gel concentration) (Sigma Chemical Co.), or laminin (0.68 mg/ml final gel concentration) (Sigma Chemical Co., St. Louis, MO). To keep the type IV collagen gelatin in solution, urea (3 M final concentration) was included in gels containing this substrate. These substrate gels underwent the same detergent exchange, overnight incubation, fixation and staining steps as for gelatin substrate gels.

Affinity studies. Gelatin, covalently linked to agarose beads, was tumbled end over end for 2 h at 4°C with conditioned medium diluted at least 1 to 10 with a buffer containing 50 mM Tris pH 7.5, 10 mM $CaCl_2$, 600 mM NaCl, and 0.02% NaN_3. The beads were washed 4× with the same buffer without conditioned medium, then eluted with 2× nonreducing SDS-PAGE sample buffer diluted 1:1 with the wash buffer. All samples were concentrated with Centracon-10 units (Amicon, Inc., Beverly, MA) and an equivalent amount from each sample was loaded on the zymogram.

Lectin from *Triticum vulgaris* or *Lens culinaris* (Sigma Chemical Co.), covalently linked to agarose beads, was tumbled end over end for 2 h at 4°C with concentrated conditioned medium diluted at least 1 to 10 with a buffer containing 50 mM Tris pH 7.5, 10 mM $CaCl_2$, and 0.02% NaN_3. The beads were then washed 4× with the same buffer and eluted with 200 mM *N*-acetyl D-glucosamine, for *Triticum vulgaris*, or 200 mM methyl α-D-glucopyranoside, for *Lens culinaris*. Samples were analyzed by zymography as above.

Deglycosylation analysis. Conditioned medium containing recombinant human MMP-9 expressed in COS 6M or GEC was equilibrated by ultrafiltration with 50 mM NaH_2PO_4, pH 7.0, 5 mM 1,10-phenanthrolene. The proteins were denatured by the addition of SDS to a final concentration of 0.1%, after which Triton X-100 was added to a final concentration of 1%. For *O*-deglycosylation, neuraminidase, (Genzyme Corp., Cambridge, MA) 1 U/ml final concentration, was added to the denatured MMP-9 preparations and the mixture incubated for 1 h at 37°C. Thereafter, 5 U/ml final concentration of endo-α-*N*-acetylgalactosaminidase (*O*-glycanase, Genzyme Corp.) was added for an additional incubation at 37°C for 18 h. For *N*-deglycosylation, *N*-glycosidase F (*N*-glycanase; Genzyme Corp.) was added at a final concentration of 10 U/ml, followed by incubation at 37°C for 1 h. For combined *N*- and *O*-deglycosylation, the *N*-deglycosylation step was performed first, followed by the *O*-deglycosylation step as detailed above. Controls for each MMP-9 species and reaction condition were prepared by similar treatment, but without the addition of deglycosylation enzymes. The reactions were ended by the addition of 2× nonreducing SDS-PAGE sample buffer and analyzed by gelatin zymography as detailed above.

Northern blotting. Poly(A)$^+$ RNA from rat macrophages stimulated with 10^{-7} M PMA (5 μg/lane), poly(A)$^+$ RNA from unstimulated GECs (15 μg/lane), and poly(A)$^+$ RNA from GECs stimulated with 10^{-7} M PMA (15 μg/lane) were electrophoresed on a 1.25% denaturing agarose gel and transferred to a nylon membrane. The blot was hybridized with a cDNA probe made from exons 1-4 of human MMP-9 (bases 5–651 of the open reading frame) labeled by incorporating α-^{32}P-dCTP (New England Nuclear, Boston, MA). The blots were washed with 0.2× SSC, 1% SDS at 50°C for 30 min before exposure.

Immunoprecipitation. 10 μg of monoclonal mouse anti–MMP-9 antibody (Oncogene Science, Manhasset, NY) was added to 100 μl PMA-stimulated GEC-conditioned medium or 100 μl unconcentrated medium from COS cells transfected with the MMP-9 expression plasmid. The mixtures were tumbled gently overnight at 4°C. Agarose-coupled Protein A beads (BioRad Laboratories, Richmond, CA) were washed with PBS and a bed volume of ∼ 30 μl was added to each of the above mixtures and tumbled at 4°C for 2 h. The supernatant was removed and beads washed 5× with 50 mM Tris/HCl, pH 8.3, 450 mM NaCl, 0.5% NP-40. To elute the adsorbed antigen/antibody complexes, 30 μl of 2× nonreducing SDS-PAGE sample buffer were pipetted onto the beads and incubated for 1 h at room temperature with frequent agitation. Zymography was performed on starting material and SDS eluted material.

Immunofluorescence staining of cultured GECs. GECs were plated onto etched coverslips, allowed to attach for 2 h at 37°C, then medium was replaced with K1 medium without (control) or with 10^{-7} M PMA. The cells were incubated overnight. Monensin was added to a final concentration of 1 μM for the final 2 h of incubation. The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at 4°C and permeabilized with 0.1% Triton X-100 for 90 s. The cells were blocked with 5% goat serum, then avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA). The cells were then incubated overnight at 4°C with monoclonal mouse anti–human MMP-9 (5 μg/ml), rinsed, and incubated with biotin-F(ab′)2 fragment goat anti–mouse IgG (5 μg/ml) (Zymed Laboratories, S. San Francisco, CA), for 2 h at room temperature. The cells were then incubated with fluorescein-conjugated streptavidin (1:50; Molecular Probes, Eugene, OR) for 30 min at room temperature in the dark. Rinsed coverslips were mounted in glycerol containing *n*-propyl gallate to prevent photobleaching.

Immunostaining of Heymann nephritis tissues. Passive Heymann nephritis was induced in 190–210- gram male Sprague-Dawley rats (Tyler Laboratories, Bellevue, WA) by the intravenous injection of 10 μg of sheep anti-Fx1A IgG as described in detail (20). Kidneys were harvested on days 5 and 15, fixed in formalin, and embedded in paraffin. Sections (5 μm) were hydrated through xylene and graded alcohols to water, and then placed in 0.05% Triton X-100 for 30 min. The slides were blocked with 5% goat serum, avidin, and biotin solutions. Monoclonal mouse anti–MMP-9 antibody and secondary antibody were used as detailed above. The tissues were then post-fixed in Bouin's fixative for 3 min to reduce native renal alkaline phosphatase activity. Streptavidin-alkaline phosphatase reagent (Zymed Laboratories) was added and incubated for 30 min, followed by development with Alkaline Phosphatase Substrate Kit II (Vector Laboratories) and counterstaining with methyl green. Controls included sections stained with biotin-F(ab′)2 fragment goat anti–mouse IgG alone, which demonstrated no cross-reactivity to either sheep or rat IgG.

In situ reverse transcription (ISRT) analysis of MMP-9 mRNA in Heymann nephritis. ISRT was performed as reported in detail (21). Negative controls included performance of ISRT without specific primers or with a sense MMP-9 primer. The antisense primer derived from the murine MMP-9 sequence corresponded to a unique sequence, nucleotides 2072 to 2097 (5′-TCTTCCAGTACCAAGACAAAGCCCTAT-3′). Tissues evaluted included normal controls and kidneys from nephritic animals at 15 days following injection of sheep anti-Fx1A IgG.

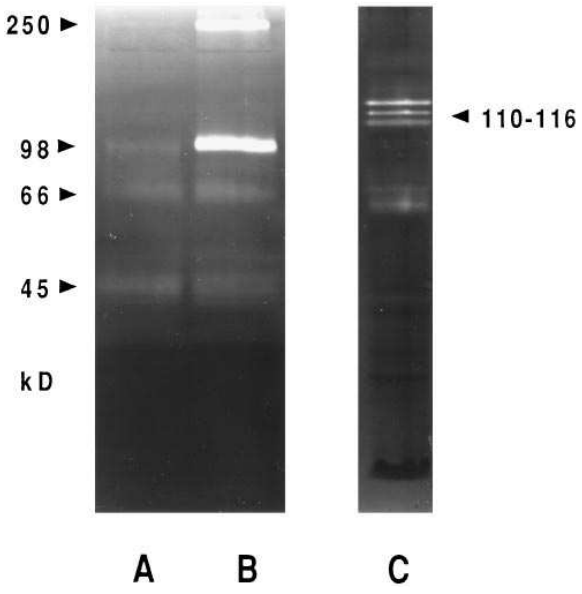

Figure 1. Gelatin zymography (7.5% polyacrylamide) of concentrated GEC-conditioned medium (*A*), concentrated conditioned medium from PMA-stimulated GECs (*B*), and crude microsomal preparation of GEC membranes (*C*). The major gelatin-degrading bands are shown with approximate molecular masses in kD.

Results

Characterization of the GEC gelatinolytic activity. Zymographic analysis of the concentrated supernates of GEC revealed a faint gelatinolytic band with a relative mobility of 98 kD (Fig. 1, lane *A*). This band was significantly enhanced by incorporating 10^{-7} M phorbol ester in the medium of the GECs during the conditioning period (lane *B*). Faint bands were also seen at 250, 66, and 45 kD. Also seen faintly in some preparations was a gelatinolytic band at 150 kD previously described by Johnson et al. (14). A GEC microsomal fraction (lane *C*) also contained gelatinolytic activity, but with a mass of 110–116 kD, which presumably represents the same glomerular membrane-associated enzymatic activity described originally by Le et al. (22). Thus, the 98-kD gelatinolytic activity represents a primary GEC secretory product.

The inhibition profile of the GEC 98-kD gelatinase was identical to that of hrMMP-9 (Fig. 2). Both EDTA and EGTA were nearly completely inhibitory. Likewise, the zinc chelator 1,10-phenanthrolene inhibited both activities (not shown). The inhibitor of serine proteinases, phenylmethylsulfonyl fluoride, did not inhibit either the GEC 98 kD or the hrMMP-9 enzymes, while TIMP-1 was effective in blocking activity of both enzymes. This inhibitor profile identifies the GEC 98-kD gelatinase as a member of the matrix metalloproteinase family.

All members of the matrix metalloproteinase family are secreted in latent, or zymogen form. In each case, activation involves the removal of a portion of the amino terminus with a concomitant loss of ~10 kD in molecular mass. The activation process can be accomplished by incubation of the enzyme with proteolytic enzymes (e.g., trypsin), denaturants (e.g., SDS), or organomercurials (e.g., APMA). We characterized the GEC 98-kD gelatinase with respect to its APMA activation time course and compared it with hrMMP-9 and rat macrophage MMP-9 activation. As shown in Fig. 3, exposure to APMA results in a 10-kD loss in the molecular mass of the 98-kD GEC

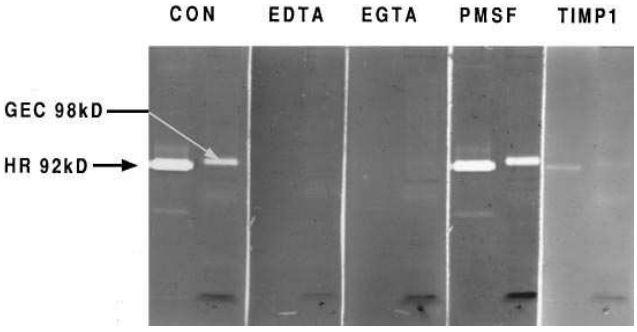

Figure 2. Inhibition studies. After electrophoresis of samples of conditioned medium on 7.5% acrylamide gelatin substrate gels, the gels were cut into equivalent strips, each strip containing hrMMP-9 (HR 92 kD) in the left lane and GEC-conditioned medium (GEC 98 kD) on the right. The strips were processed without (CON) or with the following inhibitors incorporated into incubation buffers: 10 mM EDTA, 10 mM EGTA, 5 mM PMSF, or 5 μg/ml TIMP-1.

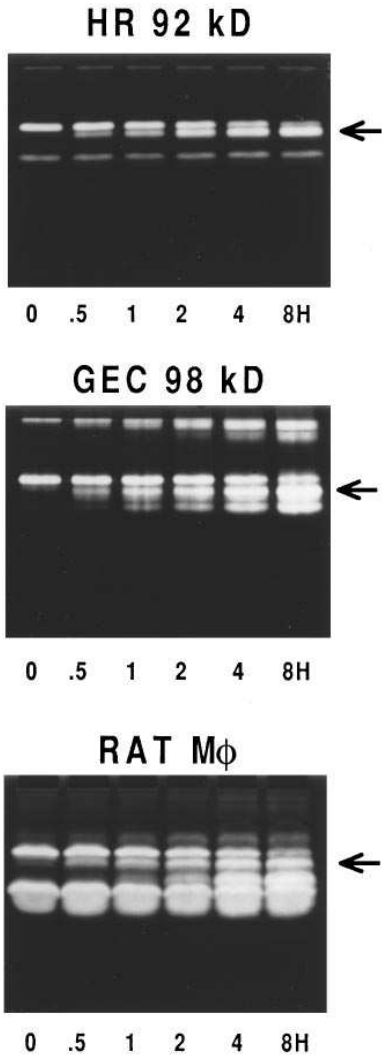

Figure 3. APMA activation kinetics. Aliquots of conditioned medium containing either hrMMP-9 (HR 92 kD) GEC conditioned medium (GEC 98 kD), or rat macrophage conditioned medium (RAT Mϕ) were incubated at 37°C for 0, 0.5, 1, 2, 4, and 8 h with 0.5 mM APMA and the reaction stopped by freezing. Incubation with the organomercurial resulted in an ~ 10-kD reduction in size of all proenzymes with virtually identical kinetic patterns. The major proteolytically active band resulting from activation is shown with an arrow.

gelatinase with a kinetic pattern that is virtually identical to that exhibited by hrMMP-9 and rat macrophage MMP-9.

To further establish the identity of the GEC 98-kD gelatinase, we analyzed by Northern blots the poly(A)$^+$ RNA from PMA-stimulated rat peritoneal macrophages, nonstimulated GEC and from GECs stimulated with PMA (Fig. 4). Two transcript sizes of ~ 2.7 and 2.3 kb were readily detected in the RNA from PMA-stimulated macrophages. MMP-9 transcripts were below detection limits in RNA from nonstimulated GECs; however, exposure of GEC to PMA resulted in the appearance of the same 2.7 and 2.3 transcripts, which are similar in size to the two transcripts at 3.2 and 2.5 kb reported by Masure et al. for mouse MMP-9 (23).

Further confirmation of the identity of the rat GEC 98-kD gelatinase as rat MMP-9 was provided by immunoprecipitation of the enzyme with a monoclonal anti–human MMP-9 antibody. As shown in Fig. 5, the rat GEC enzyme was specifically precipitated by the MMP-9 antibody, as was hrMMP-9.

The extracellular matrix substrate specificities of the GEC 98-kD gelatinase were evaluated by zymographic studies in which either type IV collagen gelatin, fibronectin or laminin were incorporated and compared to identical quantities (in terms of gelatinolytic activity) of hrMMP-9. Both enzymes are capable of degrading type IV collagen gelatin, (Fig. 6), but

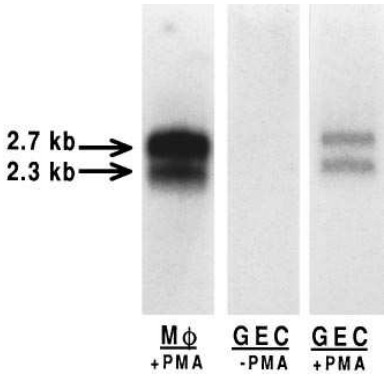

Figure 4. Northern blot analysis. Shown are 5 μg of PMA-stimulated rat macrophage poly(A)$^+$ RNA (Mϕ/+PMA), 15 μg unstimulated GEC poly(A)$^+$ RNA (GEC/−PMA), and 15 μg PMA-stimulated GEC poly(A)$^+$ RNA(GEC/+PMA) electrophoresed on a denaturing agarose gel, transferred to a nylon membrane, probed with human MMP-9 cDNA (bases 5–651 of the open reading frame), and washed at moderate stringency.

only the 98-kD GEC enzyme showed activity against fibronectin. The GEC enzyme also degraded laminin, but the bands were too faint to be photographed, while laminin was not degraded by hrMMP-9 (not shown).

Previous studies with human MMP-9 derived from leukemic cell lines have demonstrated a high affinity for enzyme binding to immobilized gelatin (24, 25). As shown in the upper panel of Fig. 7, the 98-kD GEC gelatinolytic activity is tightly bound to gelatin, in a similar pattern to hrMMP-9. In contrast, the relative affinities of the two enzymes to the lectins *Triticum vulgaris* and *Lens culinaris* differed. While both enzymes bound to each lectin, hrMMP-9 bound more tightly and could not be eluted with the specific glycosides at 200 mM concentration, whereas the GEC 98-kD gelatinase was eluted quite well from both lectins with this concentration of glycoside. The disparity between the lectin affinities of two enzymes is consistent with differences in specific glycosylation patterns.

Although rat macrophage MMP-9 and rat GEC 98-kD gelatinase have the same RNA transcript sizes, MMP-9 from rat macrophages exhibited a relative mobility on gelatin gels of ~ 92 kD (Fig. 8 *A*). We hypothesized that this may be due to differences in post-translational modification (glycosylation) between the two cell types. Thus, we expressed human MMP-9 both in primate COS cells and in rat GECs and compared their relative zymographic mobilities. Human recombinant MMP-9 expressed by COS cells was in the usual position of 92 kD, but

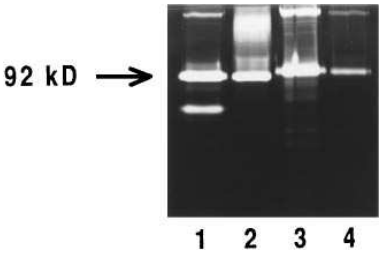

Figure 5. Immunoprecipitation of GEC 98-kD gelatinase by monoclonal mouse anti–human MMP-9. A 7.5% polyacrylamide/gelatin substrate gel was loaded with: conditioned medium from hrMMP-9 expressed in COS cells (lane *1*); and hrMMP-9 immunoprecipitated with mouse monoclonal antibody to human MMP-9 (lane *2*); conditioned medium from PMA-stimulated GEC (lane *3*); and GEC 98-kD gelatinase immunoprecipitated by mouse monoclonal antibody to human MMP-9 (lane *4*).

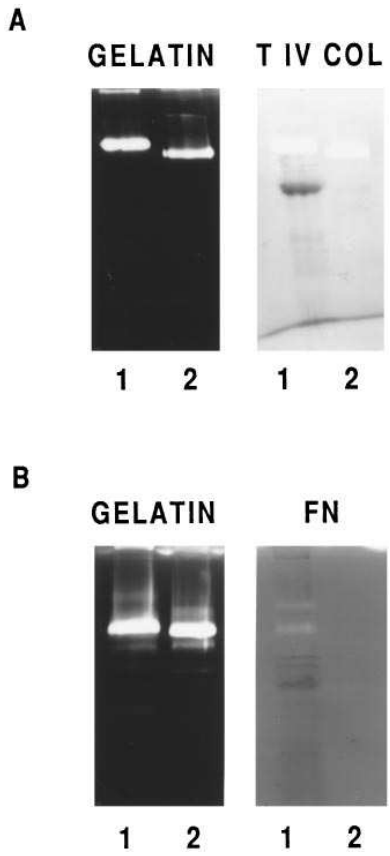

Figure 6. Substrate specificity. (*A*) Type IV collagen. Two halves of a composite substrate gel, the left half containing 2 mg/ml gelatin, and the right half containing 0.4 mg/ml type IV collagen, were loaded with equal gelatinolytic activities of: concentrated GEC-conditioned medium (lane *1*), or hrMMP-9 (lane *2*). (*B*) Fibronectin. Two halves of a composite substrate gel identical to the one shown in *A* except that the substrate in the right half of the gel is 0.6 mg/ml fibronectin.

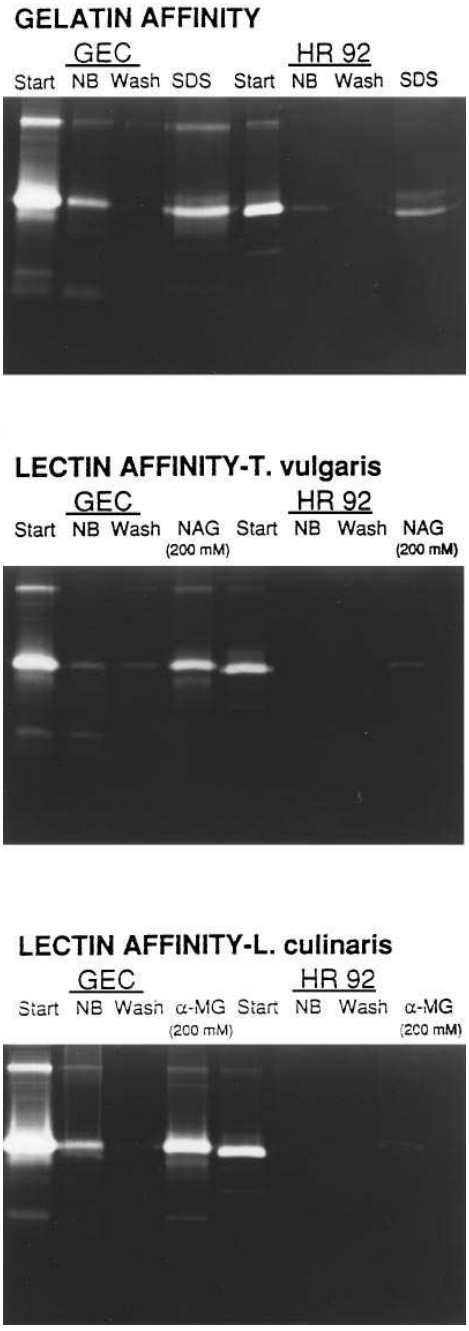

Figure 7. Gelatin and lectin affinity studies. Samples from affinity matrices were loaded on gelatin substrate gels; the first four lanes of each gel were samples from GEC-conditioned medium (*GEC*) and the last four lanes were from hrMMP-9 (*HR-92*). Lanes were loaded with concentrated aliquots of conditioned medium diluted with start buffer (*Start*), nonbound fraction after incubation with the matrix (*NB*), final wash (*wash*), and eluted sample (*SDS*)-for gelatin, 200 mM *N*-acetyl D-glucosamine (*NAG*) for *T. vulgaris*, and 200 mM methyl α-D-glucopyranoside (*α-MG*) for *L. culinaris*.

expression of the human MMP-9 cDNA in rat GECs yielded a 98-kD gelatinolytic product, identical in size to the intrinsic rat GEC 98-kD gelatinase (Fig. 8 *B*). These findings suggest that the differences in relative mobilities between the COS-derived hrMMP-9 and the rat 98-kD GEC gelatinase are not simply due to species differences, but rather represent differences in cell-specific patterns of glycosylation, as rat macrophage MMP-9 migrates with a relative mobility of 92 kD.

To further evaluate these differences, COS-derived hrMMP-9 and rat 98-kD gelatinase were subjected to either *N*- or *O*-deglycosylation analysis as detailed in Materials and Methods. The results of these studies are summarized in Fig. 9. *N*-glycanase treatment of hrMMP-9 results in a change in molecular mass from 92 to 86 kD, a net loss of 6 kD. In contrast, *N*-glycanase treatment of rat GEC gelatinase results in a change from 98 to 96 kD, a net loss of only 2 kD. *O*-glycanase treatment of hrMMP-9 results in a change in molecular mass from 92 to 82 kD, a net loss of 10 kD. The same treatment of GEC gelatinase yields a change in mass from 98 to 78 kD, a net loss of 20 kD. Thus, the GEC gelatinase is more extensively *O*-glycosylated than the hrMMP-9, while hrMMP-9 is more *N*-glycosylated than the GEC gelatinase. Furthermore, the more extensive degree of GEC gelatinase *O*-glycosylation accounts for the increased relative mass of this product.

Immunohistochemical studies. Subconfluent cultures of phorbol ester-stimulated GECs were evaluated by immunofluorescence microscopy for the appearance and distribution of the MMP-9 antigen. The distribution of the MMP-9 antigen in these cultures was heterogeneous and concentrated in smaller cells localized on the periphery of the GEC clusters (Fig. 10), while the larger cells in the interior of these clusters displayed minimal MMP-9 staining.

To extend these studies, immunohistochemical staining for the MMP-9 antigen was performed on paraffin-embedded renal cortical sections taken from normal rats and at days 5 and

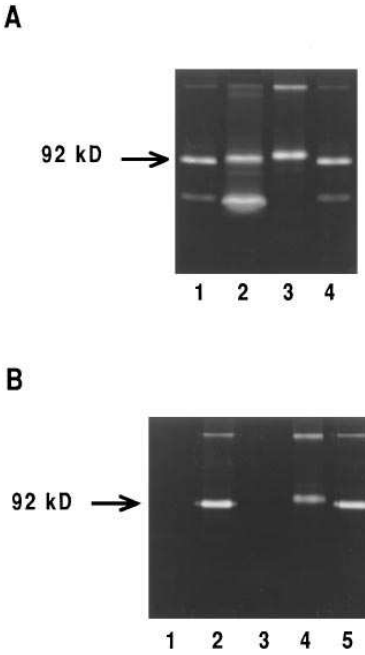

Figure 8. (*A*) Molecular mass comparison of hrMMP-9, rat macrophage MMP-9 and rat GEC MMP-9. A 7.5% acrylamide gelatin substrate gel was loaded with conditioned medium from the following sources: hrMMP-9 expressed in COS cells (lane *1*), PMA-stimulated rat macrophage conditioned medium (lane *2*), PMA-stimulated GEC-conditioned medium (lane *3*), and human recombinant MMP-9 expressed in COS cells (lane *4*, identical to lane *1*). (*B*) Molecular mass comparison of human recombinant MMP-9 expressed in COS cells and GEC. A 7.5% polyacrylamide/gelatin substrate gel was loaded with conditioned medium from the following sources: COS cells transfected with irrelevant control cDNA in pCDM8 (lane *1*), COS cells transfected with human MMP-9 cDNA in pCDM8 (lane *2*), GEC transfected with irrelevant control cDNA in pCDM8 (lane *3*), GEC transfected with human MMP-9 cDNA in pCDM8 (lane *4*). Lane *5* is identical to lane *2* for closer comparison with lane *4*.

15 after induction of visceral epithelial cell injury with injection of anti-Fx1A antibodies (passive Heymann nephritis). Normal renal cortical sections were notable for very low levels of MMP-9 antigen, either within the glomeruli, tubules or interstitium (Fig. 11 *A*). In contrast, the induction of visceral epithelial cell injury was associated with the appearance of dense MMP-9 immunostaining at both days 5 and 15 (Fig. 11, *B* and *C*). The distribution of the MMP-9 antigen is primarily localized to the visceral epithelial cytoplasm, although by day 15 some immunoreactive material was present within capillary lu-

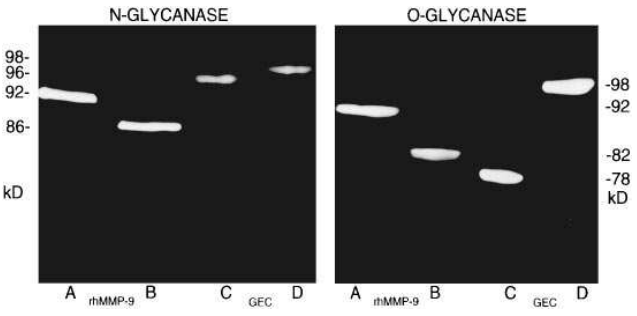

Figure 9. Deglycosylation analysis. In the left panel (*N*-glycanase), the 92-kD hrMMP-9 (lane *A*) was incubated with *N*-glycanase as detailed in Methods, yielding an 86-kD product (lane *B*). *N*-glycanase treatment of the 98-kD rat GEC enzyme (lane *D*), resulted in a 96-kD end product (lane *C*). In the right panel (*O*-glycanase), the 92 kD hrMMP-9 (lane *A*) was incubated with *O*-glycanase, yielding an 82-kD product. Incubation of the 98-kD rat GEC enzyme (lane *D*) with *O*-glycanase results in a 78-kD product (lane *C*).

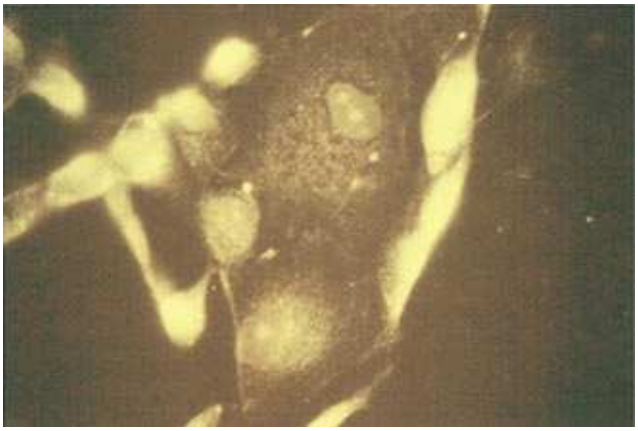

Figure 10. Immunofluorescent staining of rat GEC for MMP-9. Cells were fixed, permeabilized and stained as detailed in Methods. Bright immunofluorescent staining for MMP-9 is seen primarily in the cells on the periphery. Note that the larger, more typically epithelioid cells in the center have minimal staining. Final magnification ×1200.

mina. Controls in which tissues were processed without primary antibody showed no staining in normal rat glomeruli or those exposed to sheep anti-Fx1A antibodies, indicating no cross-reactivity of the secondary antibody to either rat or sheep IgG (not shown).

To confirm the cellular site of MMP-9 synthesis as the visceral epithelial cell in this model, in situ reverse transcription (ISRT) localization of MMP-9 was performed using a technique recently described in detail (21). Control glomeruli demonstrated little, or no histochemical staining for the MMP-9 transcript, while tissues taken from animals at day 5 after induction of Heymann nephritis exhibited dense staining localized to the visceral epithelial cells (Fig. 12). Thus, MMP-9 mRNA transcript and protein are found within the same cellular populations in this model.

Discussion

Prior studies of glomerular epithelial cells have indicated the presence of several, variably characterized neutral metalloproteinases within either culture supernates or whole glomerular extracts. Le et al. (22) described a novel metalloproteinase within the crude membrane extracts of isolated whole glomeruli, which demonstrated a molecular mass of 116–120 kD, and which was not inhibited by TIMP or activated by organomercurial compounds. It is likely that the identical activity is represented in the present studies by the 116–120-kD gelatinolytic activity present in microsomal extracts of cultured GECs. A subsequent study by Johnson et al. (14), used cultured rat GECs maintained without additional cytokine or phorbol ester stimulation, and described a 150-kD neutral metalloproteinase with activity against gelatin and type IV collagen. This activity was not inhibited by TIMP or activated by organomercurial compounds, leading to the suggestion that this particular protease is not a member of the matrix metalloproteinase gene family. Our report characterizes a 98-kD enzyme which is likely the same as an incompletely characterized activity found by Watanabe et al. (26) in the conditioned medium from rat glomeruli and glomerular epithelial cells. Their report indi-

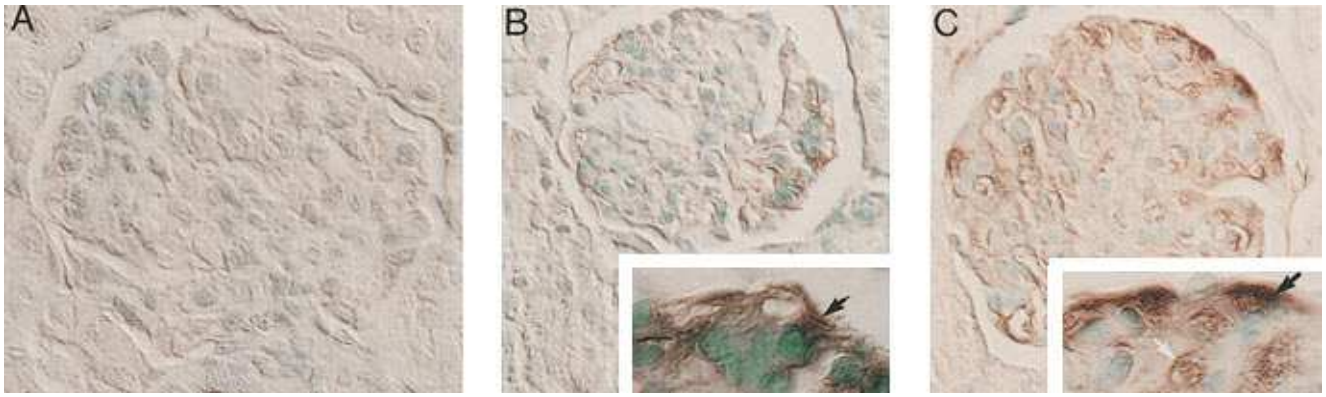

Figure 11. Immunostaining of rat glomeruli using monoclonal mouse anti–human MMP-9. Shown are glomeruli from control rats (*A*); rats with passive Heymann nephritis, day 5 (*B*); and rats with passive Heymann nephritis, day 15 (*C*). Staining for MMP-9 is brown and the blue/green is a counterstain. Dense immunoreaction product is particularly notable in a pattern consistent with MMP-9 expression in visceral GECs (*black arrows*), while at day 15 some endothelial staining is also evident (*white arrow*). Final magnifications: *A* and *C,* ×400; *B* ×300; *insets* ×1400.

cated a ninefold increase in this activity in conditioned medium from glomeruli of rats with Heymann nephritis over medium from normal glomeruli.

The 116–120-kD and 150-kD metalloproteinases differ distinctly in a number of features from the 98-kD activity described in our report. First, significant expression of this activity was not constitutive, but rather was dependent upon stimulation of the cells with phorbol ester. Second, this enzymatic activity was readily inhibited by recombinant TIMP-1 protein. Coupled with the rest of the inhibitor profile and the requirement for organomercurial activation, classification of the 98-kD activity as a matrix metalloproteinase seems evident.

The identity of the 98-kD GEC enzyme as MMP-9 is confirmed by Northern blot analysis of poly(A)$^+$ selected RNA from phorbol-stimulated GECs. The characteristic two transcripts for MMP-9 are seen both in GECs, as well as rat macrophage poly(A)$^+$ RNA. The two mRNA transcript sizes are the result of alternate utilization of two polyadenylation sites and do not affect the open reading frame and the size of the translated protein. Further documentation of the identity of the GEC enzyme is provided by the specific immunoprecipitation of this enzyme by an antibody to MMP-9.

Despite the absence of hybridizing bands in RNA from unstimulated GECs, a small 98-kD gelatinolytic band was seen with zymography of the concentrated supernates from unstimulated GECs. This apparent discrepancy can be explained by the extreme sensitivity of zymography which is capable of detecting picogram quantities of gelatinase (27).

We have shown that the size discrepancy between the 92- and 98-kD forms of this enzyme is due to differences in the post-translational modification of the enzyme secreted by GECs, as compared to COS cells or rat macrophages. The lectin binding and deglycosylation experiments indicate that this mass difference is explained by alternate patterns of glycosylation. The actual functional significance of this difference, particularly in terms of substrate specificities, is currently under investigation.

When cultured, phorbol-simulated GECs were stained for MMP-9, the cells which stained were those located on edges of cell clusters. This result is in direct agreement with the findings of Xie et al. (28), who reported MMP-9 staining exclusively in cells on the periphery of colonies of human epidermoid carcinoma cells. These peripherally located cells were shown to be proliferating, in contrast to more centrally located cells that were not dividing.

The finding that primarily proliferating GECs produce MMP-9 raises the possibility that diseases which damage visceral glomerular epithelial cells leading to cell proliferation and repair might induce production of MMP-9. Passive Heymann nephritis is one glomerular disease involving injury to GECs and is induced by the injection of anti-Fx1A IgG into rats. The disease produces a lesion similar to human membranous nephropathy with proteinuria beginning on day 5 after

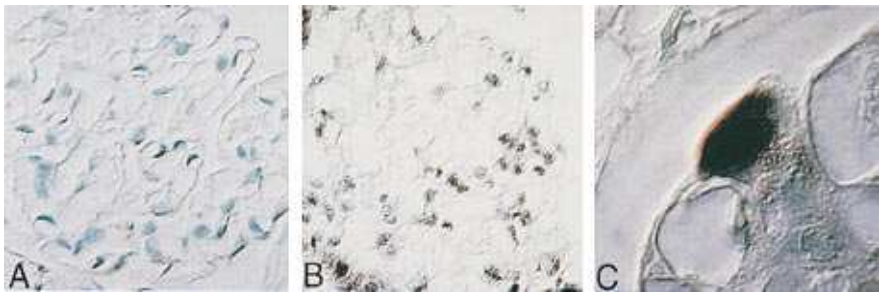

Figure 12. In situ reverse transcription localization of MMP-9 mRNA in glomeruli. Shown are glomeruli from control rats (*A*); and glomeruli from rats with passive Heymann nephritis at day 5 after induction of disease (*B* and *C*). Staining for the MMP-9 mRNA transcript is dark brown. Glomeruli from control animals do not demonstrate any significant MMP-9 transcript expression, while multiple darkly stained cells are seen in the glomerular section from day 5 nephritic animals (*B*). The visceral epithelial cell localization of the MMP-9 transcripts is demonstrated in *C*, which demonstrates intense staining of a large cell located on the external aspects of two discrete glomerular capillary loops. Controls included ISRT performed without a specific primer and ISRT performed with nonhybridizing sense MMP-9 primers; neither demonstrate significant staining (not shown). Final magnifications: *A* and *B* ×250; *C* ×1000.

antibody injection. Floege et al. (15) have shown that visceral glomerular epithelial cells in passive Heymann nephritis have mitotic figures and stain positively for the proliferating cell nuclear antigen, whereas normal visceral GECs do not proliferate. In our studies MMP-9 immunohistochemical staining and ISRT localization of MMP-9 transcripts of renal tissues from rats 5 and 15 d after infusion of anti-Fx1A IgG showed a significant increase in glomerular visceral epithelial cell staining for MMP-9 protein and transcripts, as compared with control glomeruli. This is supportive of the hypothesis that the GEC-secreted proteolytic enzyme might play a role in breakdown of the ultrafiltration barrier as the expression of MMP-9 is temporally correlated with maximal proteinuria.

Other studies have shown metalloproteinases can increase microvascular endothelial permeability. Partridge and co-workers showed that a TNF-α–induced 96-kD gelatinase secreted by bovine pulmonary microvascular endothelial cells (which is presumably MMP-9) increased monolayer permeability to ^{125}I-albumin, an effect which could be inhibited by TIMP or 1,10-phenanthroline. Davies and co-workers (30) used a synthetic metalloproteinase inhibitor (BB-94) to prevent ascites formation in nude mice which had received MMP-9-synthesizing ovarian carcinoma xenografts, an effect which was not seen when the inactive diasterioisomer of the inhibitor was used.

If the GEC MMP-9 is involved in the pathophysiology of membranous nephropathy, pharmacological modification of its activity or secretion could provide a potential therapeutic approach for the treatment of this disease. More work is needed to further define the role of GEC MMP-9 in Heymann nephritis; however, study of the enzyme in this and other models of glomerular epithelial cell injury represents a promising area of research.

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References

1. Rosenzweig, L.J., and Y.S. Kanwar. 1982. Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to 125-I-bovine albumin. *Lab. Invest.* 47:177–184.

2. Kanwar, Y.S., A.L. Linker, and M.G. Farquhar. 1980. Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Biol. Chem.* 86:688–693.

3. Johnson, R.J., W.G. Couser, E.Y. Chi, S. Adler, and S.J. Klebanoff. 1987. New mechanism for glomerular injury: Myeloperoxidase-hydrogen peroxide-halide system. *J. Clin. Invest.* 79:1379–1387.

4. Johnson, R.J., S.J. Klebanoff, R.F. Ochi, S. Adler, P. Baker, L. Sparks, and W.G. Couser. 1987. Participation of the myeloperoxidase-H_2O_2-halide system in immune complex nephritis. *Kidney Int.* 32:342–349.

5. Shah, S.V. 1988. Evidence suggesting a role for hydroxyl radical in passive Heymann nephritis in rats. *Am. J. Physiol.* 254:F337–F344.

6. Johnson, R.J., D.H. Lovett, R.I. Lehrer, W.G. Couser, and S.J. Klebanoff. 1994. Role of oxidants and proteases in glomerular injury. *Kidney Int.* 45: 352–359.

7. Davies, M., A.J. Barrett, J. Travis, E. Sanders, and G.A. Coles. 1978. The degradation of human glomerular basement membrane with purified lysosomal proteinases: evidence for the pathogenic role of the polymorphonuclear leukocyte in glomerulonephritis. *Clin. Sci. Mol. Med.* 54:233–240.

8. Davies, M., G.A. Coles, and K.T. Hughes. 1980. Glomerular basement membrane injury by neutrophil and monocyte neutral proteinases. *Renal Physiol. (Basel)*. 3:106.

9. Vissers, M.C., and C.C. Winterbourn. 1988. Gelatinase contributes to the degradation of glomerular basement membrane collagen by human neutrophils. *Collagen Rel. Res.* 8:113–122.

10. Baricos, W.H., G. Murphy, Y. Zhou, H.H. Nguyen, and S.V. Shah. 1988. Degradation of glomerular basement membrane by purified mammalian metalloproteinases. *Biochem. J.* 254:609–612.

11. Johnson, R.J., W.G. Couser, C.E. Alpers, M. Vissers, M. Schulze, and S.J. Klebanoff. 1988. The human neutrophil serine proteinases, elastase and chathepsin G, can mediate glomerular injury in vivo. *J. Exp. Med.* 168:1169–1174.

12. Baricos, W.H., and S.V. Shah. 1981. Proteolytic enzymes as mediators of glomerular injury. *Kidney Int.* 40:161–173.

13. Davies, M., J. Martin, G.J. Thomas, and D.H. Lovett. 1992. Proteinases and glomerular matrix turnover. *Kidney Int.* 41:671–678.

14. Johnson, R.J., H. Yamabe, Y.P. Chen, C. Campbell, K. Gordon, P. Baker, D. Lovett, and W.G. Couser. 1991. Glomerular epithelial cells secrete a glomerular basement membrane degrading metalloproteinase. *J. Am. Soc. Nephrol.* 2:1388–1397.

15. Floege, J., R.J. Johnson, C.E. Alpers, S. Fatemi-Nainie, C.A. Richardson, K. Gordon, and W.G. Couser. 1993. Visceral glomerular epithelial cells can proliferate in vivo and synthesize platelet-derived growth factor B-chain. *Am. J. Pathol.* 142:637–650.

16. Taub, M., L. Chuman, M.H. Saier, Jr., and G. Sato. 1979. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. *Proc. Natl. Acad. Sci. USA*. 76:3338–3342.

17. Martin, J., D.H. Lovett, D. Gemsa, R.B. Sterzel, and M. Davies. 1986. Enhancement of glomerular mesangial cell neutral proteinase secretion by macrophages: role of Interleukin 1. *J. Immunol.* 137:525–529.

18. Kriegler, M. 1990. DEAE-dextran transfection method. *In* Gene Transfer and Expression: A Laboratory Manual. Stockton Press, New York. 99–100.

19. Marti, H.P., L. Lee, M. Kashgarian, and D.H. Lovett. 1994. Transforming growth factor-β1 stimulates glomerular mesangial cell synthesis of the 72 kD Type IV collagenase. *Am. J. Pathol.* 144:82–94.

20. Salant, D.J., C. Darby, and W.G. Couser. 1980. Experimental membranous glomerulonephritis in rats: quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J. Clin. Invest.* 66:71–81.

21. Lee, L.K., A.S. Pollock, and D.H. Lovett. 1993. The asymmetric origins of the mature glomerular basement membrane. *J. Cell. Physiol.* 157:169–177.

22. Le, Q., S. Shah, H. Nguyen, S. Cortez, and W. Baricos. 1991. A novel metalloproteinase present in freshly isolated rat glomeruli. *Am. J. Physiol.* 260: F555–F561.

23. Masure, S., G. Nys, P. Fiten, J. VanDamme, and G. Oppdenakker. 1993. Mouse gelatinase B: cDNA cloning, regulation of expression and glycosylation in WEHI-3 macrophages and gene organization. *Eur. J. Biochem.* 218:129–141.

24. Okada, Y., Y. Gonoji, K. Naka, K. Tomita, I. Nakanishi, K. Iwata, K. Yamashita, and T. Hayakawa. 1992. Matrix metalloproteinase 9 (92 kD type IV collagenase) from HT 1080 human fibrosarcoma cells. *J. Biol. Chem.* 267: 21712–21719.

25. Morodomi, T., Y. Ogata, Y. Sasaguri, M. Morimatsu, and H. Nagase. 1992. Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukemia and HT 1080 fibrosarcoma cells. *Biochem. J.* 285: 603–611.

26. Watabe, K., S. Kinoshita, and H. Nakagawa. 1990. Gelatinase secretion by glomerular epithelial cells. *Nephron.* 56:405–409.

27. Kleiner, D.E., W.G. Stetler-Stevenson. 1994. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.* 218:325–329.

28. Xie, B., C.D. Bucana, and I.J. Fiedler. 1994. Density-dependent induction of 92 kD type IV collagenase activity in cultures of A431 human epidermoid carcinoma cells. *Am. J. Pathol.* 144:1058–1067.

29. Partridge, C.A., J.J. Jeffrey, and A.B. Malik. 1993. A 96-kD gelatinase induced by TNF-α contributes to increased microvascular endothelial permeability. *Am. J. Physiol.* 265:L438jj–L447.

30. Davies, B., P.D. Brown, N. East, M.J. Crimmin, and F.R. Balkwill. 1993. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. *Cancer Res.* 53:2087–2091.