Glomerular Endothelial Cells Synthesize Collagens but Little Gelatinase A and B

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Abstract. Mesangial sclerosis is a major feature of progressive renal disease. The mesangium contains mesangial cells and is bounded by the peripheral glomerular basement membrane and endothelial cells. Mesangial cells synthesize and degrade extracellular matrix. Whereas both mesangial and endothelial cells synthesize extracellular matrix components, the degradative pathway, well studied in the former, has not been investigated in endothelial cells. This study examines lines of all three glomerular cell types derived from female B6SJLF1/J mice, as well as mRNA levels for collagens α1 (I), α1 (IV), α3 (IV), α5 (IV), and α1 (VI), laminin, tenascin, matrix metalloproteinase-2 (MMP-2), and MMP-9. Type I and IV collagen synthesis was confirmed by enzyme-linked immunosorbent assay. MMP-2 and MMP-9 enzyme activity was measured by zymography. It was found that glomerular endothelial cells are a significant source of collagens, laminin, and tenascin. However, they express only low levels of MMP-2 and no detectable MMP-9. Stimulation with exogenous transforming growth factor-β1 leads to a significant increase in collagen I, tissue inhibitors of metalloproteinase-1, and MMP-9 in conditioned media. These data suggest that glomerular endothelial cells may play an active role in extracellular matrix remodeling in glomerular disease.

The striking feature of progressive renal diseases is mesangial sclerosis, a condition characterized by increased deposition of extracellular matrix due to an imbalance between synthesis and degradation. The mesangial area is bounded by endothelial cells, whose contribution to mesangial sclerosis has yet to be fully elucidated. However, vascular endothelial cells in other vessels are known to play an important role in sclerosing processes, such as atherosclerosis (1,2). Glomerular endothelial cells synthesize several extracellular matrix components (3), and recent evidence suggests that they may play an important role in the pathogenesis of progressive glomerular disease (4). The aim of this study was to examine the contribution of glomerular endothelial cells (EN) to extracellular matrix synthesis and degradation in vitro by comparing them with mesangial cells (MC) and podocytes (EP). We examined components found in normal and disease states: collagens type I and IV, laminin-β1 and tenascin, matrix metalloproteinase-2 (MMP-2) and MMP-9, and transforming growth factor-β1 (TGF-β1) (4–6). We measured mRNA levels for α1 (I), α1 (IV), α3 (IV), and α5 (IV) collagens, laminin-β1, tenascin, MMP-2 and MMP-9, and TGF-β1. In addition, we assessed the synthesis of collagen types I and IV by enzyme-linked immunosorbent assay (ELISA), the activity of the gelatinases by zymography, and the expression of tissue inhibitors of metalloproteinases (TIMP) by reverse zymography. Finally, we examined the effect of exogenous TGF-β1 on the production of collagens type I and IV, gelatinases, and TIMP. This information provided an overall estimate of the ability of glomerular endothelial cells to contribute to extracellular matrix turnover in vitro.

Materials and Methods

Cell Culture
Glomerular endothelial, epithelial, and mesangial cell lines, derived from normal 4- to 6-wk-old female B6SJLF1/J mice (The Jackson Laboratory, Bar Harbor, ME), were used at passages 20 to 25. These cells have been described in great detail in previous studies (3,7,8). The identification criteria for these cells included morphology, immunofluorescence staining for factor VIII-related antigen, cytokeratin, and actin, and evaluation of acetylated LDL uptake, as described previously (9). Basal mesangial cell culture medium (B medium; National Institutes of Health [NIH], Bethesda, MD) was supplemented with fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) to reach a final concentration of 20% in complete medium (3). Cells were grown in fibronectin (200 µg/ml)-coated, 25-cm² cell culture flasks (Nunc, Naperville, IL) and examined by phase-contrast microscopy on a daily basis. All glomerular cells were growing rapidly with doubling times of 17 h (EN), 23 h (MC), and 26 h (EP), respectively.
Stimulation of Glomerular Endothelial Cells with Recombinant Human TGF-β1

For the stimulation with TGF-β1 (Sigma, St. Louis, MO), 100,000 endothelial cells were plated into 6-well dishes. For each group (control, 1 ng/ml, and 10 ng/ml TGF-β1), 3 wells were plated per experiment. After 4 h in medium containing 20% FBS, cells were rinsed three times with B medium containing 0.1% FBS and incubated for 12 h in this medium. Cells were rinsed again three times, and B medium with 0.1% FBS supplemented with no (control), 1 ng/ml, or 10 ng/ml TGF-β1 was added. The medium was collected after 24 h and stored at -80°C until further analysis by ELISA for collagens type I and IV, zymography for MMP-2 and MMP-9, and reverse zymography for TIMP-1 and TIMP-2. At this point, the cells were trypsinized and counted.

RNA Extraction and Reverse Transcription

Total RNA was extracted from subconfluent cell cultures, using the guanidinium thiocyanate-phenol-chloroform method (10). After DNase I (RNase free grade, Life Technologies) treatment, reverse transcription (RT) was performed using 2 μg of total RNA, 5 mM Mg²⁺, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.6 μg of oligo-dT₁₅ primers, 1 mM deoxyribonucleotide mix, 50 U of RNase inhibitor, and 20 U of avian myeloblastosis virus reverse transcriptase in a total volume of 20 μl (Boehringer Mannheim, Indianapolis, IN). Incubation times were 25°C for 10 min, 42°C for 60 min, and 99°C for 5 min. After adjusting the total volume to 200 μl with redistilled water, 2 μl of the cDNA solution was used as PCR template.

PCR

PCR was performed in a total volume of 50 μl of 1.5 mM Mg²⁺, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 μl of cDNA solution, and 1.5 U of Taq polymerase (Boehringer Mannheim). A PCR reaction in the absence of cDNA was run as negative control. To check for genomic DNA contamination, one RNA sample of each set was used for PCR without prior RT. The PCR products were separated on a 4% agarose gel, visualized with ethidium bromide, and quantified by computer-aided densitometry (NIH Image, National Center for Biotechnology Information, NIH, Bethesda, MD). PCR primer pairs and the number of PCR cycles used for each molecule are shown in Table 1. Differences in expression levels between the different cell lines were sufficient to be detected by standard, rather than competitive, PCR under the described conditions. To determine the assay range, we plotted increasing numbers of PCR cycles against the integrated optical density obtained from computer-aided densitometry (data not shown) (11).

A major problem in the quantification of mRNA levels by RT-PCR has been the potential variation in RT efficiency. Both the use of external RNA standards in the RT assay and normalization of data to so-called “housekeeping” genes have been described. In this study, we chose β-actin as an internal standard and “housekeeping” gene. PCR data, normalized to β-actin for control of interassay variance, are

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Primer Sequences</th>
<th>Product Size (bp)</th>
<th>PCR Cycles</th>
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<tr>
<td>β-actin</td>
<td>TCTAGAGGTTAGTGCCTCTGTCAGG</td>
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<td></td>
<td>TCTAGGCAACCAAGGTGTTG</td>
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<td>GTCCAGGCTGCTCAATATAGG</td>
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Table 1. PCR primers*

* PCR primer pairs, expected product sizes, and number of PCR cycles used. For each molecule, the sequence in the first line is the forward primer and the sequence in the second line is the reverse primer. All sequences are shown 5' to 3'. MMP-2, matrix metalloproteinase-2; TGF-β1, transforming growth factor-β1.
shown as percentage of control. Values for endothelial and epithelial cells are normalized to mesangial cells.

Zymography and Reverse Zymography
Cells were grown in 6-well plates (Nunc) for 72 h. The medium was then replaced by 1 ml of B medium containing 0.1% FBS. After 24 h, the supernatant was removed and stored at −80°C before analysis. The cells were trypsinized and counted. Sample volumes were corrected for cell number. Zymography was performed on precast zymogram gels (Novex, San Diego, CA), as described (12). For reverse zymography, a 15% polyacrylamide gel containing 0.5% porcine gelatin and 6 μg of recombinant gelatinase A was used to separate the samples (13).

ELISA
Cells were grown in 6-well plates (Nunc) for 72 h. The medium was then replaced by 1 ml of B medium containing 0.1% FBS, 50 μg/ml ascorbic acid, and 80 μg/ml BAPN. After 24 h the supernatant was removed, and protease inhibitors (2.5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethysulfonyl fluoride, 10 mM N-ethylmaleimide) were added. Samples were stored at −80°C before analysis. ELISA was performed as described (12). The standard range used was 4 to 64 ng for collagen type I, and 2 to 32 ng for collagen type IV, respectively. For the assessment of TIMP-1 protein levels, a commercial ELISA kit was used according to the manufacturer’s suggestions (Predicta, Genzyme Diagnostics, Cambridge, MA). Media samples were used undiluted, and the standard curve was modified to fit the low concentrations.

Statistical Analyses
Results from duplicates of three or four independent experiments are expressed as means ± SD. Statistical analyses were performed using one-way ANOVA with Bartlett’s test for equal variances and Tukey’s multiple comparison test.

Results
Reverse Transcription-PCR
We compared the expression levels of α1 (I), α1 (IV), α3 (IV), α5 (IV), and α1 (VI) collagen chains (Figure 1A), as well as laminin-β1 and tenasin (Figure 1C), of glomerular endothelial cells to those of mesangial cells and podocytes.

Glomerular endothelial cells expressed all molecules investigated at the mRNA level. Endothelial cells expressed more than a twofold higher (219% of mesangial cells) level of α3 (IV) collagen mRNA compared with mesangial cells. However, they expressed lower levels of the other type IV collagen α chains (α1 = 36% and α5 = 41% of MC), as well as lower levels of types I (19% of MC) and VI (21% of MC) collagen. They also expressed lower levels of tenasin (75% of MC) and laminin (50% of MC) mRNA.

Interestingly, glomerular endothelial cells expressed low levels of both MMP-2 (18% of MC) and MMP-9 (5% of MC) (Figure 2A). Mesangial and epithelial cells expressed both molecules. The levels of MMP-9 were higher in epithelial (130% of MC) than in mesangial cells. However, the level of MMP-2 mRNA was less abundant in epithelial cells (33% of MC).

Extracellular matrix synthesis is influenced by a number of growth factors We chose to investigate the expression of TGF-β1 in the three glomerular cell types, since it may play an important role in glomerulosclerosis (3,4,14). Although all three glomerular cell types expressed TGF-β1 mRNA, the highest levels were found in glomerular endothelial cells (1.7-fold higher than in mesangial and epithelial cells) (Figure 3A). A representative PCR for each molecule is shown in Figure 4B.

Zymography and Reverse Zymography
MMP-2 was synthesized by all three glomerular cell types, but the enzyme activity found in the supernatant of glomerular endothelial cells was only 25% of the enzyme activity found in the supernatant of mesangial cells and 50% of the activity found in the supernatant of epithelial cells, respectively (Figure 2B). MMP-9 activity could barely be detected in glomerular endothelial cell supernatants.

Both TIMP-1 and TIMP-2 were strongly expressed by all three glomerular cell types (Figure 2C). The highest level for TIMP-1 was found in mesangial cells. Endothelial cells also expressed high levels of TIMP-1 (72% of MC), whereas in the supernatant of epithelial cells levels were much lower (23% of MC). For TIMP-2, on the other hand, the highest levels were found in endothelial cells (175% of MC). Epithelial cells also synthesized larger quantities (136% of MC) of TIMP-2 than mesangial cells. A faint band for TIMP-3 could be seen in the supernatant of both mesangial and endothelial cells, but it proved too weak for quantification. A representative gel for both zymography and reverse zymography is shown in Figure 4A.

ELISA
As we noted previously, collagen peptide levels followed mRNA levels obtained by PCR (15). To correct for unequal plating efficiency, we express all protein data as ng/ml per 10⁵ cells. Mesangial cells synthesized approximately twice as much collagen type I as did glomerular endothelial or epithelial cells. The total amount of type IV collagen in the supernatant of endothelial, mesangial, and epithelial cells was similar (Figure 1B).

The ELISA for TGF-β1 (Figure 3B) reflected the findings on the mRNA level, with glomerular endothelial cells expressing three times the amount of TGF-β1 when compared with mesangial or epithelial cells.

Stimulation of Glomerular Endothelial Cells with TGF-β1
After stimulation with 1 ng/ml recombinant human TGF-β1, we found a 1.4-fold increase in collagen I, but only a slight increase in collagen IV in conditioned media of glomerular endothelial cells (Figure 5A). A higher dose (10 ng/ml) of TGF-β1 did not cause a stronger effect. TGF-β1 also showed a stimulatory effect on the expression of MMP-9 (fourfold with 1 ng/ml and threefold with 10 ng/ml TGF-β1); however, it had no effect on MMP-2 levels (Figure 5B). TIMP-1 expression was greatly (1.5-fold) enhanced by both 1 ng/ml and 10 ng/ml TGF-β1, whereas there was no change in the expression levels for TIMP-2 (Figure 5C). An increase in TIMP-3 was also observed but was too low to be quantified.
Figure 1. (A) Comparison of collagen mRNA expression in mesangial (MC; □), glomerular endothelial (EN; ■), and glomerular epithelial (EP; ▪) cells, expressed as percentage of control. Shown are mean and SD of four independent experiments carried out in duplicate. *P < 0.05 for EN versus MC or EP versus MC; #P < 0.05 for EN versus EP. (B) Comparison of collagen protein levels measured by enzyme-linked immunosorbent assay (ELISA) in mesangial (□), glomerular endothelial (■), and glomerular epithelial (▪) cells, expressed as ng/ml per 100,000 cells. Shown are mean and SD of three independent experiments carried out in duplicate. **P < 0.01 for EN versus MC or EP versus MC. (C) Comparison of the expression of laminin and tenascin mRNA in mesangial (□), glomerular endothelial (■), and glomerular epithelial (▪) cells, expressed as percentage of control. Shown are mean and SD of four independent experiments carried out in duplicate. *P < 0.05 for EN versus MC or EP versus MC; #P < 0.05 for EN versus EP.

Discussion
Most progressive glomerular diseases are characterized by an accumulation of extracellular matrix components in the mesangium. The mesangium contains a smooth muscle-like population, the mesangial cells, and is bounded by endothelial cells. Although glomerular endothelial cells are able to synthesize basement membrane components, it is not known whether they release enzymes that degrade these proteins. Because vascular endothelial cells in larger vessels have been shown to contribute to sclerotic lesions (1,2), we examined how glomerular endothelial cells affect extracellular matrix turnover in vitro, and we compared them with mesangial cells and podocytes. Extracellular matrix synthesis by glomerular cells in vitro can be altered by characteristics of the culture surface, the amount of growth factors, metabolic conditions, including ambient glucose concentration, and cell density (3,8,16). Therefore, we used identical culture conditions for all three cell lines, even though epithelial cells are traditionally propagated in low
serum medium. Furthermore, it is known that the genetic background can greatly influence extracellular matrix turnover in vivo (17). Therefore, lines of the three glomerular cell types were all obtained from the same strain, i.e., female B6SJLF1/J mice at the age of 4 to 6 wk, which have been characterized (4,5).

The synthesis of extracellular matrix components in the kidney glomerulus has been studied in both whole glomeruli (18,19) and isolated glomerular cells (20). Mesangial cells in vitro synthesize collagens type I through VI (20,21). Type IV and VI collagens have been detected in bovine glomerular endothelial cells, and rat and bovine glomerular epithelial cells were found to secrete collagens type I, IV, V, and VI (20–22). Both laminin and tenascin expression have been observed in whole glomeruli (14) and isolated mesangial (23,24) and epithelial cells (19). The balance between synthesis and degradation of extracellular matrix components is a crucial element of glomerular homeostasis.

We found that mouse glomerular endothelial cells express multiple extracellular matrix molecules, including several type IV collagen α chains, type I and VI collagens, laminin β1, tenascin, and both MMP-2 and MMP-9. By RT-PCR, mesangial cells have shown higher levels of most collagen chains except for collagen α3 (IV), which is most abundantly expressed by endothelial cells. Endothelial cells expressed high levels of tenascin mRNA, and the message for laminin-β1 was approximately 50% of the level found in mesangial cells.

We found that the synthesis of matrix metalloproteinases was dominated by mesangial cells and podocytes. Endothelial cells expressed low levels of MMP-2 mRNA and almost undetectable amounts of MMP-9 mRNA. The RT-PCR data for the two gelatinases were confirmed by zymography, which showed that the enzyme activity reflects mRNA levels in the three glomerular cell types. We could show by reverse zymography that glomerular endothelial cells expressed high levels of the inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2. TIMP-1 levels were highest in mesangial cells, followed by endothelial cells and epithelial cells, whereas TIMP-2 levels were 1.7-fold higher in endothelial cells than in mesangial cells.

Since net extracellular matrix deposition reflects both synthesis and degradation, we measured types I and IV collagen

![Figure 2.](image)

Figure 2. (A) Comparison of the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 mRNA in mesangial (■), glomerular endothelial (■), and glomerular epithelial (■) cells, expressed as percentage of control. Shown are mean and SD of four independent experiments carried out in duplicate. *P < 0.05 for EN versus MC or EP versus MC; **P < 0.05 for EN versus EP; ***P < 0.05 for EN versus EP. (B) Comparison of the enzyme activity for MMP-2 and MMP-9 in the supernatant of mesangial (■), glomerular endothelial (■), and glomerular epithelial (■) cells, expressed as percentage of control. Shown are mean and SD of two independent experiments carried out in triplicate. ****P < 0.001 for EN versus MC or EP versus MC; ***P < 0.001 for EN versus EP; **P < 0.01 for EP versus MC; *P < 0.05 for EN versus EP. (C) Comparison of the expression of tissue inhibitors of metalloproteinase-1 (TIMP-1) and TIMP-2 by reverse zymography in mesangial (■), glomerular endothelial (■), and glomerular epithelial (■) cells, expressed as percentage of control. Shown are mean and SD two of independent experiments carried out in triplicate. *P < 0.05 for EN versus MC; **P < 0.01 for EP versus MC; *P < 0.05 for EN versus EP.
Figure 3. Comparison of the expression of transforming growth factor-β1 (TGF-β1) mRNA (A) and protein levels (B) in mesangial (□), glomerular endothelial (■), and glomerular epithelial (●) cells, expressed as percentage of control for the PCR data and in ng/ml per 100,000 cells for protein levels. Shown are mean and SD of at least two independent experiments carried out in duplicate. *P < 0.05 for EN versus MC; #P < 0.05 for EN versus EP; **P < 0.01 for EN versus MC or EP versus MC; ***P < 0.01 for EN versus EP.

Figure 4. (A) Representative zymography and reverse zymography. Lane 1, marker; lanes 2 through 4, mesangial cells; lanes 5 through 7, endothelial cells; lanes 8 through 10, epithelial cells. (B) Representative PCR for collagens, tenascin, laminin-β1, MMP-2, MMP-9, TGF-β1, and β-actin. Lane 1, molecular weight standard; lanes 2 and 3, mesangial cells; lanes 4 and 5, endothelial cells; lanes 6 and 7, epithelial cells.

by ELISA. Previously, we have shown that the relative amounts of collagen in mesangial cell supernatants correlated with mRNA levels (15). In this study, collagen type I mRNA levels in endothelial and epithelial cells were less than 20% of that in mesangial cells, whereas type I collagen protein levels were approximately 50% of that in mesangial cells. Similarly, the amount of type IV collagen protein was comparable in mesangial and endothelial cells and only slightly lower in podocytes, even though mRNA levels measured by RT-PCR implicated a clear dominance of mesangial cells in the expression of type IV collagen. One possible explanation for the discrepancies between the net production of collagens type I and IV in these three cell types is the differential expression of MMP-2 and MMP-9 at the mRNA and protein level (25). Thus, it might be expected that the collagens would accumulate in the supernatant of endothelial cells, since they produced small
amounts of MMP-2 and MMP-9. In the case of podocytes, we found that they expressed similar mRNA levels for collagens but higher levels for the two collagenases. The net result was that there were slightly lower amounts of collagen type IV in the supernatant as a result of excess degradation compared to synthesis. Mesangial cells, on the other hand, expressed high levels of collagens as well as MMP-2 and MMP-9 (12), resulting in a net synthesis, which was similar to that of endothelial cells. It may be concluded from these data that glomerular endothelial cells make a significant contribution to extracellular matrix synthesis, but play a minor role in its degradation, based on their low levels of matrix metalloproteinases and relatively high levels of TIMP-1 and TIMP-2. If these data apply to endothelial cells in vivo, they could be of importance in the pathogenesis of glomerulosclerosis.

TGF-β1 is expressed in whole glomeruli (14) and in cultured mesangial cells (5,6). It has been hypothesized that elevated secretion by glomerular endothelial cells may lead to progression of glomerular diseases (4). In the current experiments, TGF-β1 mRNA was expressed by all three glomerular cell types, with the highest levels found in glomerular endothelial cells. This is supported by TGF-β1 protein levels measured by ELISA.

To further investigate the role of glomerular endothelial cells in extracellular matrix turnover, we investigated the effect of exogenous TGF-β1 on the synthesis of collagens type I and IV, MMP-2, MMP-9, TIMP-1, and TIMP-2 in these cells. We chose doses of 1 ng/ml (40 pM) and 10 ng/ml (400 pM), which are above the proposed $K_D$ of approximately 10 pM in previous experiments under similar conditions (3). After the washout period of 24 h and extensive rinsing of the cells with medium containing low serum concentrations, the endothelial cells were almost quiescent. For this reason, we did not observe a significant difference in cell number after 24 h between control and TGF-β1-treated cells. A significant increase in collagen type I production could be observed, but the amount of collagen type IV detected in conditioned media showed no significant change. This is in contrast to previous reports concerning the effect of TGF-β1 on mesangial cells, in which an increase in collagen type IV levels could be found (26,27). Our findings may be explained by the higher MMP-2 activity in conditioned media of TGF-β1-treated cells (three- to fourfold), which was only partly offset by the observed increase in TIMP-1 (1.5-fold). If these findings apply to in vivo conditions, our data suggest that in TGF-β1-mediated glomerular injury, endothelial cells may contribute to extracellular matrix turnover.

In summary, our data suggest that glomerular endothelial cells may have a unique role in the production of extracellular matrix in vivo. A relatively low basal production of MMP-2 and MMP-9, paired with the ability to synthesize various collagen chains, suggests that they may contribute to extracellular matrix synthesis but not degradation under normal conditions. In contrast, after stimulation with exogenous TGF-β1, glomerular endothelial cells show a significant increase in the synthesis of collagen I and MMP-9, which may suggest an active role for glomerular endothelial cells in extracellular matrix remodeling in glomerular disease.
References


