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Transforming Growth Factor-β1 Stimulates Glomerular Mesangial Cell Synthesis of the 72-kd Type IV Collagenase

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Transforming growth factor-β1 (TGF-β1) is generally considered to exert positive effects on the accumulation of extracellular matrices. These occur as the net result of enhanced matrix protein synthesis, diminished matrix metalloproteinase (MMP) synthesis, and augmented production of specific inhibitors, including the tissue inhibitor of metalloproteinases (TIMP-1). Given that glomerular TGF-β1 synthesis is induced by inflammation, the effects of this cytokine on synthesis of the 72-kd type IV collagenase and TIMP-1 by cultured human mesangial cells were evaluated. Concentrations of TGF-β1 of 5 ng/ml and above specifically stimulated the synthesis of the 72-kd type IV collagenase. This effect was independent of the stimulatory effect of TGF-β1 on TIMP-1 synthesis, which was maximal in a lower concentration range (0.1 to 1 ng/ml). Most significantly, the net effect at the higher concentrations of TGF-β1 was an excess of enzyme over the TIMP-1 inhibitor. Northern blot analysis of TGF-β1-stimulated human mesangial cells demonstrated a specific increase in the abundance of the 3.1 kb mRNA transcript encoding the 72-kd type IV collagenase, presumably mediated by a direct stimulation of 72-kd type IV collagenase mRNA transcription observed as early as 3 hours after exposure to TGF-β1. These studies were extended to an analysis of the expression of TGF-β1 and 72-kd type IV collagenase mRNAs in normal and nephritic rats. In normal animals, basal TGF-β1 and 72-kd type IV collagenase mRNA expression was observed in a strictly mesangial distribution. After induction of acute immune complex-mediated glomerulonephritis, there was a major increase in TGF-β1 and 72-kd type IV collagenase mRNA expression, which was strictly limited to the expanded, hypercellular mesangial compartment. Enhanced synthesis of the mesangial type IV collagenase in response to TGF-β1 released during glomerular inflammatory processes could have an important role in the extensive glomerular matrix remodeling that accompanies these disorders. (Am J Pathol 1994, 144:82–94)

Many forms of glomerular disease are associated with changes in extracellular matrix protein content or composition. These changes commonly affect either the glomerular basement membrane or the mesangial matrix compartment. The intrinsic glomerular cells are the primary synthetic sources of these extracellular matrix proteins, which may be induced by the action of proinflammatory cytokines released by infiltrating monocytes or by the glomerular cells, per se. Several recent investigations have emphasized the role of the panregulin, transforming growth factor-β1 (TGF-β1) in these events.[1–3] This interest derives from observations in multiple tissues demonstrating a positive effect of TGF-β1 on the accumulation of extracellular matrix proteins.[4–9] For example, Coimbra et al[10] demonstrated the production of TGF-β1 in a rabbit model of antiglomerular basement membrane disease. This model is associated with severe glomerular sclerosis due to a major increment in collagen synthetic rates by the intrinsic cells. The presence of significant amounts of TGF-β1 protein and mRNA in the glomer-

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uli from these animals suggested that this cytokine mediates the pronounced sclerotic response. These findings have been extended by Border et al[1,2] to the Thy 1.1 model of immune complex-mediated glomerulonephritis. This model is characterized by a marked mesangial proliferative response, coupled with mesangial expansion. Glomerular synthesis of several extracellular matrix proteins was enhanced in this model[1,11] and associated with an elevated glomerular expression of both TGF-β1 mRNA and protein. A direct linkage between glomerular TGF-β1 synthesis and mesangial matrix expansion was demonstrated by the use of TGF-β1 antiserum, which inhibited glomerular matrix accumulation *in vivo*.[2]

The net cumulative effects of cytokines such as TGF-β1 on the glomerular extracellular matrix derive from alterations in both matrix protein synthetic and degradative rates. Studies from our laboratories and others have documented the ability of the intrinsic glomerular cells to secrete enzymes capable of degrading the extracellular matrix.[12–18] The enzymes secreted by the intrinsic mesangial cell population have been extensively characterized at both the functional and structural level. The major members of this gene family secreted by cultured human mesangial cells (HMCs) are the 72-kd type IV collagenase, stromelysin-1, and PUMP-1.[12,16–19] In addition, functional, immunological, and structural studies have demonstrated the ability of HMC to secrete the low molecular weight tissue inhibitors of metalloproteinases (TIMP-1, -2).[18,19]

We have recently evaluated the expression of the 72-kd type IV collagenase and the TIMP-1 protein in the Thy 1.1 model of immune complex-mediated nephritis.[16] Expression of the type IV collagenase by the intrinsic mesangial cells was greatly increased in this model during the period of mesangial hyperplasia and extracellular matrix expansion, which follows injection of Thy 1.1 antiserum. Significantly, the glomerular mesangial expression of the TIMP-1 protein was not increased, suggesting that differential tissue regulation of the proteinases and TIMP molecules occurs. Given these observations and the temporal link between glomerular TGF-β1 and 72-kd type IV collagenase expression observed in the Thy 1.1 model of immune complex nephritis, we postulated that TGF-β1 may directly influence mesangial cell type IV collagenase secretion and resultant levels of proteolytic activity. The studies outlined in this report were designed to test this hypothesis and provide direct evidence for the induction of mesangial cell 72-kd type IV collagenase synthesis by TGF-β1 both *in vitro* and in an animal model of immune complex-mediated glomerulonephritis.

Materials and Methods

Induction of Anti-Thy1.1 Nephritis

The preparation of goat anti-Thy 1.1 IgG has been reported.[16] Nephritis was induced by the intravenous injection of 20 mg anti-Thy 1.1 IgG/100 g body weight into Wistar rats (Charles River, Wilmington, MA). At serial time points after induction of nephritis, the animals were killed and the kidneys fixed by perfusion with buffered 4% paraformaldehyde. Cortical sections from controls and the nephritic animals were snap-frozen in liquid nitrogen-cooled isopentane.

Mesangial Cell Cultures

The methods for the establishment, characterization, and maintenance of homogenous cultures of human glomerular mesangial cells have been reported in detail.[18] For preparation of conditioned media cells (6th to 10th passage) were passaged into 9 cm^2 culture dishes and grown to subconfluency. The cultures were washed three times with phosphate-buffered saline (PBS) and placed for 3 days in a rest medium consisting of RPMI 1640 medium supplemented with 1% nonessential amino acids, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.1% fetal bovine serum. Subsequently, the mesangial cells were incubated for varying time periods in fresh rest medium supplemented as indicated with human TGF-β1 (Collaborative Research, Bedford, MA).

After harvesting the conditioned media, the cell layers were washed twice in 4 C PBS and twice in 4 C 5% trichloroacetic acid, followed by cell layer protein determination. Units of enzymatic activity and antigen levels are expressed in terms of 100 μg cell layer protein. The conditioned media were concentrated 10-fold by ultra/diafiltration, and stored in enzyme assay buffer (40 mM Tris/HCl, 1 mM $CaCl_2$, pH 7.8) at −80 C before assay or use in sodium dodecyl sulfate-gelatin substrate gels (see below). ^{3}H-gelatin was used to quantify type IV collagenase activity as reported in detail.[12,18] The assay incubation mixture included 0.7 mM *p*-aminophenylmercuric acetate to permit complete activation of any latent enzyme. Type IV collagenase units of activity are defined as that

amount that degrades 1 μg substrate/hour under the given assay conditions and are expressed in relation to 100 μg cellular protein.

Enzyme-Linked Immunoassays (ELISA)

A "sandwich" ELISA was established for the quantitative determination of the 72-kd type IV collagenase and TIMP-1 proteins present in HMC culture supernates. An affinity-purified rabbit anti-72 kd type IV collagenase IgG was prepared and characterized as reported.[16,18] An affinity-purified rabbit anti-TIMP-1 IgG was prepared as reported[16] using the synthetic peptide CVPPHPQTAFCNSDL, which corresponds to the NH_2-terminal residues 3–17 of processed human TIMP-1. Both antibodies were biotinylated with N-hydroxysuccinimide-biotin (Zymed, South San Francisco) using standard methodology. For the ELISAs, 96-well plates were coated overnight at 4 C with 0.1 μg anti-type IV collagenase or anti-TIMP-1 IgG/100 μl well. Thereafter, the wells were blocked with PBS containing 0.1% bovine serum albumin (BSA) (ELISA grade, Bio-Rad, Richmond, CA). Serial dilutions of supernate samples and purified antigen standards were incubated in the wells for 4 hours at room temperature, followed by three washes with PBS containing 0.05% Tween 20. Biotinylated anti-type IV collagenase and TIMP-1 IgGs (1 μg/well) were added for 1 hour at room temperature, followed by three washes in PBS/Tween. Horseradish peroxidase-coupled streptavidin (Zymed) was diluted 1:1000 in PBS/Tween and incubated in the wells for 1 hour at room temperature. After five washes with PBS/Tween, 100 μl of ABTS solution (25 μl 3% H_2O_2 in 10 ml of 0.2 mM azinodi-3-ethyl-benzthiozolin sulfonate-6 in 100 mM sodium acetate buffer, pH 4.5) was added for 15 minutes. Thereafter, the development reaction was stopped by the addition of 50 μl 1 N H_2SO_4 and the optical densities read at 410 nm. To determine the amounts of intracellular 72-kd type IV collagenase protein, the cell layers were washed three times in cold PBS, followed by lysis in 0.5% Triton X100 containing a battery of protease inhibitors. The cell lysates were clarified by centrifugation at 10,000 *g* for 10 minutes, followed by ELISA assay as given above. Controls included wells incubated without the primary antibody or the biotinylated primary antibodies. In addition, wells incubated with medium or buffer blanks were included in each assay run. The sensitivity and specificity of the assays were not affected by the addition of recombinant TIMP-1 to the 72-kd type IV collagenase assay and vice versa. All samples were measured in quadruplicate for each dilution. The lower limits of sensitivity were 0.5 to 1 ng/ml; the upper limits within a linear range were 40 to 50 ng/ml for each antigen.

Electrophoretic Procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis substrate gels (10% acrylamide) were prepared by including either 1 mg/ml gelatin (from rat tail type I collagen, Sigma, St. Louis) or 1 mg/ml casein (Sigma) in the polymerization mixture. Enzyme samples were solubilized in nonreducing sample buffer, gently heated at 37 C for 5 minutes, and electrophoresed at 7 C using 25 mA/gel. After electrophoresis, the gels were incubated at room temperature in 2.5% Triton X-100, 50 mM Tris/HCl, pH 8.0, for 30 minutes, then overnight at 37 C in 50 mM Tris/HCl, pH 8.0, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. Thereafter, the gels were stained with Coomassie blue and zones of lysis visualized. Standard proteins were used for assignment of molecular weight.

Immunohistochemical Techniques

The individual cellular expression of the 72-kd type IV collagenase and TIMP-1 proteins was determined by histochemical analysis of cultured HMC. Cells were grown to subconfluence on etched glass coverslips then washed three time in warm PBS and placed in rest medium for 48 hours. Thereafter, the cultures were exposed to increasing concentrations of TGF-β1 for 48 hours. During the last 3 hours of this period, 1 μM monensin (Sigma, prepared as a 1 mM stock in ethanol) was added to block export of the antigens from the cells. The coverslips were washed three times in 4 C PBS and fixed for 20 minutes at 4 C in 4% buffered paraformaldehyde. Washed coverslips were permeabilized with 0.1% Triton X-100 for 90 seconds then blocked with 5% goat serum for 30 minutes and incubated overnight at 4 C with 1 μg/ml in 0.1% BSA of anti-72 kd type IV collagenase IgG or anti-TIMP-1 IgG. Washed coverslips were incubated for 4 hours at 4 C with 5 μg/ml in 0.1% BSA of affinity-purified biotinylated goat anti-rabbit IgG (Zymed). After three washes, the coverslips were incubated with a 1:50 dilution (in 0.1% BSA) of streptavidin-rhodamine (Molecular Probes, Eugene, OR) then washed and mounted in glycerol-containing n-propyl gallate to reduce photobleaching.

Northern Blot Analysis

Poly(A)$^+$ RNA (2.5 μg/lane) from quiescent HMC and cultures incubated for varying time periods with increasing concentrations of TGF-β1 was electrophoresed on 1.2% denaturing agarose gels and transferred to nylon membranes. A 900 bp human 72-kd type IV collagenase cDNA insert spanning the activation locus-zinc binding site was labeled by the random hexamer method with α-^{32}P-dCTP (New England Nuclear, Boston). Other probes that were labeled in an identical manner included a full-length human TIMP-1 cDNA, human stromelysin-1, and the full-length cDNA for human PUMP-1.[17] A full-length human β-actin cDNA probe was used as a normalization control. Hybridizations were performed under standard conditions, followed by washing at high stringency in 1% sodium dodecyl sulfate, 0.1× SSC at 65 C for 45 to 60 minutes. Densitometry and normalization of the exposed films were performed as reported.[16]

In Situ *Reverse Transcription*

The expression of the mRNA of the 72-kd type IV collagenase by cultured HMCs was determined using a modification of a recently published method from our laboratory.[20] HMCs were grown on etched coverslips to subconfluency then washed and placed in rest medium for 3 days as detailed above. Subsequently, the cells were incubated for 3 hours in fresh rest medium supplemented with 10 ng/ml TGF-β1. Control, nonstimulated cells were maintained in rest medium alone. A separate group of cultures was preincubated for 20 minutes in rest medium containing 10 μg/ml cycloheximide, followed by addition of 10 ng/ml TGF-β1. This concentration of cycloheximide inhibits mesangial cell total protein synthesis by greater than 95%. Thereafter, the cells were fixed at 4 C for 20 minutes in buffered 4% paraformaldehyde then rinsed and dehydrated in 50 and 75% ethanol and air-dried, followed by rehydration in 100 mM Tris/HCl, pH 7.5. An antisense primer for human 72-kd type IV collagenase (nucleotides 1802–1826, 5′-TCCAGGTTATCAGGGATGGCGTTCC-3′) was dissolved at 100 μg/ml in 25% formamide/6× SSC/1× Denhart's solution with 100 μg of salmon sperm DNA/ml and annealed at 37 C for 18 hours to the fixed cells. Controls included cells incubated without primer and a group incubated with the corresponding sense primer of the type IV collagenase. After annealing, excess primer was removed by three washes with 1× SSC at room temperature and one 5-minute wash at 55 C. Primer extension was conducted at 37 C for 2 hours by adding a solution containing KCl (40 mM), Tris/HCl (10 mM, pH 8), actinomycin D (100 μg/ml), $MgCl_2$ (10 mM), dATP, dGTP, dTTP (100 μM each), ^{35}S-labeled α-dCTP (300 μCi/ml, 1000 Ci/mmol, Amersham, Arlington Heights, IL), DTT (3 mM), and AMV reverse transcriptase (200 U/ml). The coverslips were washed five times with 1× SSC at 22 C and 0.1× SSC at 55 C for 5 minutes. After a brief rinse in water, the coverslips were dehydrated in ethanol then dipped in Kodak NTB-2 emulsion and exposed for 48 hours. After development the coverslips were stained with Mayer's hematoxylin and mounted. Additional controls included cells treated with 100 μg/ml RNAse before primer extension.

For evaluation of glomerular 72-kd type IV collagenase and TGF-β1 mRNA expression *in vivo*, 5-μM cryostat sections from normal and nephritic rats were mounted on glass slides, previously subbed with 2% 3-aminopropyltriethoxysilane in acetone, and fixed in 4% paraformaldehyde for 20 minutes, followed by three PBS washes and a brief water rinse. Graded dehydration/delipidation in ethanol (70, 95, and 100% for 5 minutes each) was followed by rehydration and concluded by permeabilization in 0.1% Triton X-100 for 90 seconds. The slides were washed for 5 minutes in 100 mM Tris/HCl (pH 7.4), followed by blocking of endogenous tissue biotin with avidin-biotin blocking solution (Vector, Burlingame, CA). Thereafter, the slides were rinsed and prehybridized for at least 30 minutes at room temperature (prehybridization solution: 20 mM Tris/HCl, pH 7.4, 2 mM EDTA, 0.04% BSA, 150 mM NaCl, 200 μg/ml yeast tRNA, and 200 μg/ml salmon sperm DNA). Hybridization (using 100 ng/ml of each oligonucleotide primer) was conducted as detailed above. The sections were washed three times in 1× SSC, followed by a 5-minute preincubation in 50 mM Tris/HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$. Primer extension was performed at 37 C for 90 minutes using a solution of 50 mM Tris/HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 7.5 mM DTT, 100 μg/ml actinomycin-D, 0.01% Triton X-100, 120 U/ml placental ribonuclease inhibitor, 200 U/ml AMV reverse transcriptase, 50 μM biotin-7-ATP, and 600 μM each of dCTP, dGTP, and dTTP. After reverse transcription, the sections were washed three times in 1× SSC at room temperature, three times with 0.1× SSC, and twice in PBS. The sections were blocked with 3% normal rabbit serum, followed by overnight incubation at 4 C with monoclonal murine anti-biotin IgG (Dako, 2 μg/ml in 0.1% BSA/PBS). After washing in PBS, the sections were incubated for 4 hours with 5 μg/ml affinity-purified rabbit anti-

mouse IgG (Zymed) in 0.1% BSA/2% rat serum/PBS. After washing the sections were developed with alkaline phosphatase-coupled ABC reagent (Vector). To reduce endogenous renal proximal tubule alkaline phosphatase activity, the sections were incubated with Bouin's fixative for 3 minutes before the ABC reaction. Sections were counterstained with methyl green then dehydrated and mounted. The antisense primer for the rat 72-kd type IV collagenase, the cDNA cloning of which was recently reported from our laboratory,[21] corresponded to nucleotides 1761–1785 (5′-TCCAGGTTATCAGGGATGGCATTCC-3′). The antisense primer for rat TGF-β1 corresponded to nucleotides 1422–1446 (5′-TGGTTGTAGAGGGCAAGGACCTTGC-3′). Control, sense primers for the rat type IV collagenase and TGF-β1 were also prepared from the corresponding sequences. Additional controls included sections treated with RNAse.

Statistical Methods

Statistical differences between various study groups and the matching controls were determined by standard multiple comparison and analysis of variance techniques. Values for $P < 0.05$ were considered significant.

Results

The initial series of experiments were designed to evaluate the potential effects of TGF-β1 on metalloprotease and TIMP-1 synthesis by cultured human glomerular mesangial cells. Cultured mesangial cells constitutively release small amounts of type IV collagenase activity into the supernates (Figure 1). Addition of 10 ng/ml TGF-β1 to cultured mesangial cells induced by 72 hours a significant increase in total (active plus latent) type IV collagenase activity present in the culture supernates. It should be noted that the ^{3}H-gelatin assay quantifies net enzyme activity present in excess of that blocked by inhibitory molecules, such as TIMP, present in these crude supernates. To extend these findings, a quantitative ELISA was performed on the culture supernates for the 72-kd type IV collagenase and the TIMP-1 proteins. Such assays quantify the effects of TGF-β1 on total 72-kd type IV collagenase and TIMP-1 antigen concentrations, as opposed to net proteolytic activity detected in the gelatinolytic assay. The results of this analysis are given in Figure 2. Control cultures of HMC contained approximately 10 ng/100μg cell protein of 72-kd type IV collagenase antigen. Incubation with higher concentrations

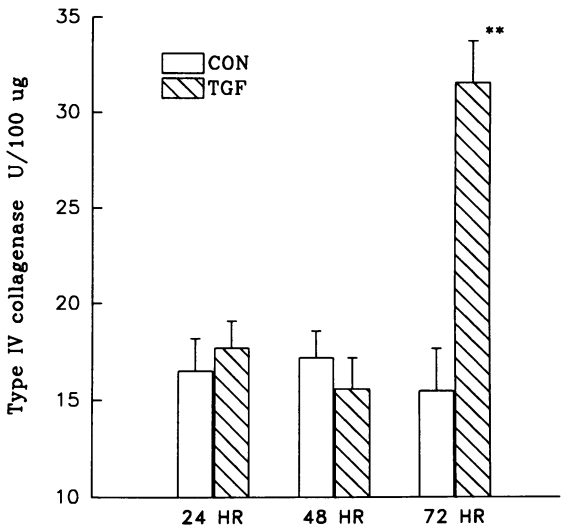

Figure 1. *Stimulation of HMC 72-kd type IV collagenase secretion by TGF-β1: quantitative ^{3}H-gelatinase assay. Subconfluent cultures of HMC were placed in rest medium for 3 days. Cultures were given fresh rest medium supplemented with 10 ng/ml TGF-β1; controls received rest medium alone. Conditioned media were harvested at the times indicated and type IV collagenase enzymatic activity determined. Data given as the means ± 1SD (n = 6). **P < 0.01.*

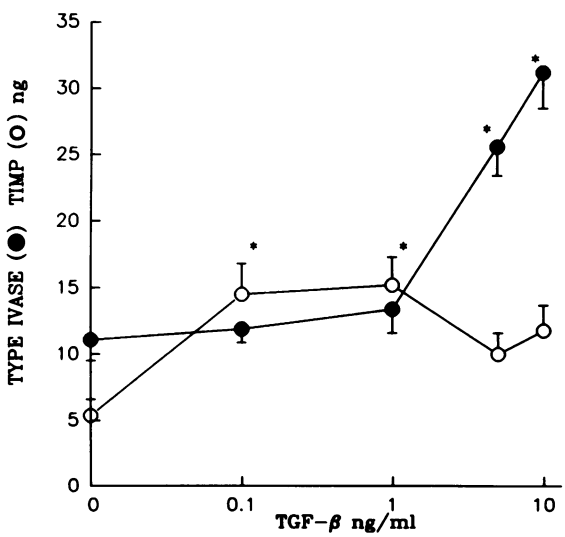

Figure 2. *Quantitation by ELISA of 72-kd type IV collagenase and TIMP-1 antigens in conditioned media of HMC stimulated with TGF-β1. Cultures were prepared as detailed in the legend for Figure 1 and exposed to increasing concentrations of TGF-β1 for 72 hours. Data expressed in terms of ng antigen/100 μg cellular protein and given as the means ± 1SD. *P < 0.05.*

of TGF-β1 (5 ng/ml and greater) for 72 hours led to a dose-dependent, two to threefold increase in the amount of 72 kd enzyme antigen secreted. In contrast, the amount of TIMP-1 antigen secreted by HMC was maximally augmented at a much lower TGF-β1 concentration (0.1 to 1 ng/ml). TIMP-1 secretion was stimulated as soon as 12 hours after exposure to the cytokine (net increase of 5.5 ng/100

μg), whereas 72-kd type IV collagenase antigen levels did not increase significantly until 48 hours of culture (data not shown). At the highest TGF-β1 concentration evaluated, 10 ng/ml, 72-kd type IV collagenase antigen exceeded TIMP-1 antigen levels by nearly threefold. Thus, the quantitative assessment of enzyme and inhibitor antigen levels further document the net positive effect of higher concentrations of TGF-β1 on 72-kd type IV collagenase secretion and ultimate extracellular proteolytic activity.

Gelatin zymograms of TGF-β1-stimulated HMC cultures extended the findings obtained in the ^{3}H-gelatin assay and the specific ELISA (Figure 3). Culture supernates from cells incubated with higher concentrations of TGF-β1 contained significant amounts of the catalytically active 62- and 59-kd fragments of the type IV collagenase, which are formed on removal of the latency-conferring propiece. This indicates that TGF-β1 stimulation of type IV collagenase synthesis is also associated with an increased conversion to the catalytically active form in the culture supernates.

The stimulatory action of TGF-β1 on 72-kd type IV collagenase secretion did not extend to two other members of the matrix metalloproteinase family secreted by HMCs, PUMP-1 (matrilysin), and stromelysin-1. Secretion of stromelysin-1 and PUMP-1, as detected with casein substrate gels (and confirmed by Western blot analysis and ^{3}H-caseinase assay), was readily inhibited by TGF-β1 (Figure 4). Northern blot analysis of TGF-β1-treated cells demonstrated a matching decrease in the steady-state abundance of the mRNAs for PUMP-1 (Figure 5) and stromelysin (not shown), whereas TIMP-1 mRNA levels increased in a dose-dependent manner (Figure 5).

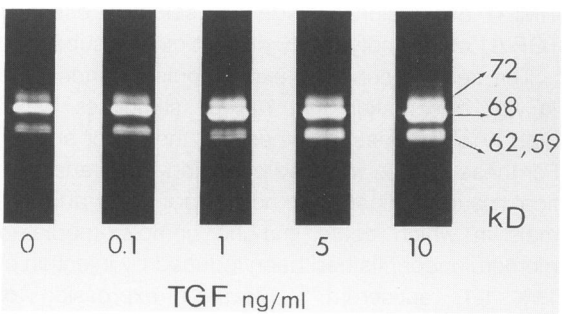

Figure 3. *Gelatin zymogram of conditioned media obtained from control and TGF-β1-stimulated cultures of HMC. Conditioned media were prepared as detailed for Figure 1 and analyzed by electrophoresis. Zones of lysis denote migration of latent and active 72-kd type IV collagenase. With increasing concentrations of TGF-β1 there is an increment in the formation of the catalytically active 62- and 59-kd fragments.*

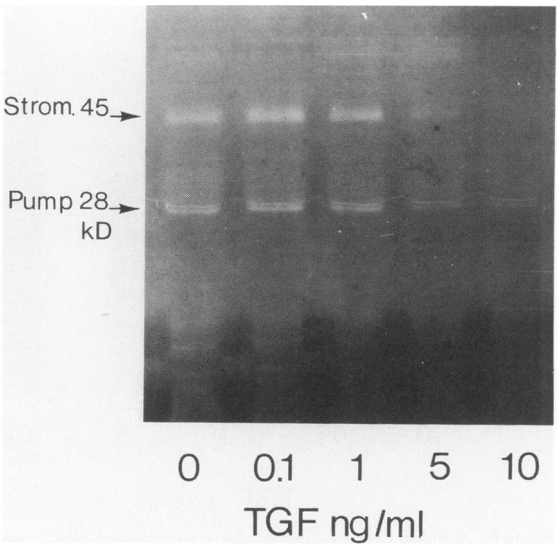

Figure 4. *Casein zymogram of conditioned media obtained from control and TGF-β1-stimulated cultures of HMC. Stromelysin (molecular mass 45 kd) and Pump-1 (molecular mass 28 kd) have a high degree of activity against casein and are detected in the non-stimulated controls. Addition of TGF-β1 inhibits the secretion of both enzymes at concentrations of 5 ng/ml and above.*

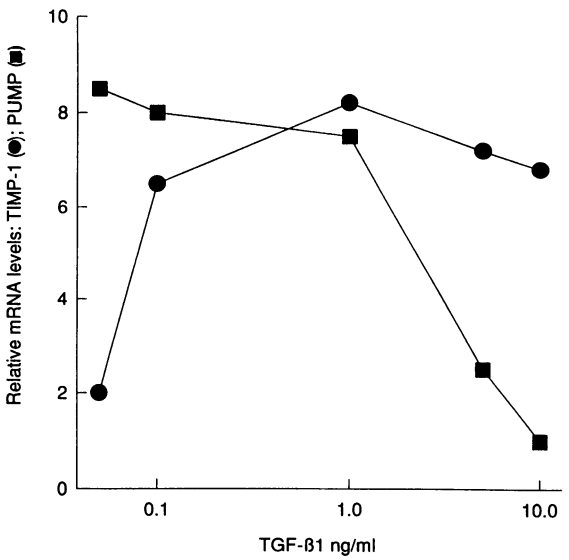

Figure 5. *Effects of TGF-β1 on HMC steady-state mRNA levels of TIMP-1 and PUMP-1. Cells were exposed for 48 hours to increasing concentrations of TGF-β1, followed by RNA extraction and Northern blot analysis. Poly(A)$^+$ RNA (2.5 μg/lane) prepared from control and TGF-β1-stimulated cells was electrophoresed on 1.2% denaturing gels then transferred to nylon membranes, and probed with the labeled cDNAs for TIMP-1, PUMP-1, and β-actin. The results are expressed as the mean (triplicate determinations) relative RNA levels compared with β-actin, which was assigned an arbitrary value of 5.*

Northern blot analyses after 48 hours of TGF-β1 stimulation revealed a progressive increase in the steady-state levels of 72-kd type IV collagenase mRNA at concentrations of 1 ng/ml and greater (Figure 6). Normalization to the abundance of β-actin mRNA revealed stimulation indices of 1.33,

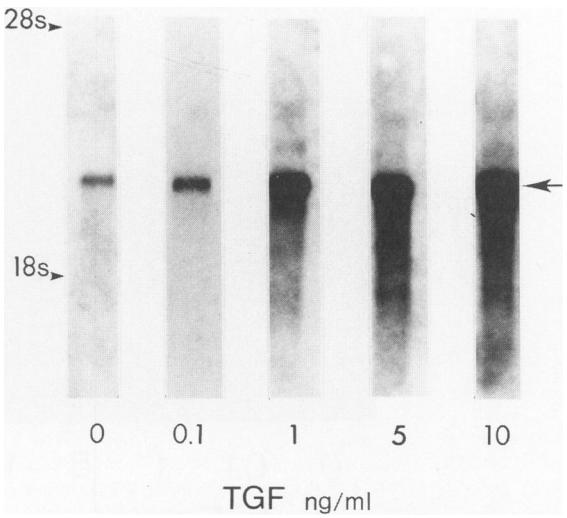

Figure 6. *Northern blot analysis of TGF-β1-stimulated HMC. Poly-(A)$^+$ RNA (2.5 μg/lane) prepared from control and TGF-β1-stimulated cells was electrophoresed on 1.2% denaturing gels then transferred to nylon membranes and probed with the labeled cDNA of the 72-kd type IV collagenase. A single hybridizing band of 3.1 kb was obtained, the abundance of which increased significantly after stimulation for 48 hours with TGF-β1.*

1.93, and 3.14 for concentrations of 1, 5, and 10 ng TGF-β1/ml, respectively. These stimulation indices for mRNA abundance closely match the TGF-β1-mediated increases in total enzymatic activity and antigen concentration detailed above.

To examine whether TGF-β exposure elevates 72-kd type IV collagenase mRNA levels at earlier time points and whether this process affects cells uniformly, *in situ* hybridizations were performed. Cultured HMCs were exposed to 10 ng/ml TGF-β1 for 3 hours and the abundance of the 72-kd type IV collagenase mRNA transcript determined by *in situ* reverse transcription using a method previously reported from our laboratory.[20] The results of these experiments are summarized in Figure 7. No significant signal was observed when a nonhybridizing, sense primer was used (Figure 7A). As shown in Figure 7B, nonstimulated cells contained little detectable 72-kd type IV collagenase mRNA when hybridized with the functional antisense primer. In contrast, there is a major increase in detectable 72-kd type IV collagenase mRNA in mesangial cells after exposure for 3 hours to TGF-β1 (Figure 7C). This TGF-β1-mediated induction of 72-kd type IV collagenase mRNA was not dependent on protein synthesis, because cells preincubated with cycloheximide also demonstrated a significant increase in collagenase mRNA after exposure to TGF-β1 (Figure 7D).

Because of the delay between the TGF-β1-mediated increase in 72-kd type IV collagenase mRNA synthesis (3 hours) and the detection of significant quantities of extracellular enzyme (48 to 72 hours), quantitative ELISA was performed to determine intracellular levels of the collagenase antigen at sequential time points after exposure to 10 ng/ml TGF-β1. These findings are summarized in Figure 8, which indicate that translation of the TGF-β1-induced 72-kd type IV collagenase mRNA is a delayed event, with significant intracellular protein enzyme levels accumulating between 24 and 48 hours after TGF-β1 exposure.

TGF-β1 has been demonstrated to either increase or inhibit mesangial cell proliferation rates, effects that are dependent on plating density or cytokine concentration.[22,23] Although the experiments outlined in this report were performed using subconfluent cultures in a serum-free medium that does not support MC proliferation, additional immunohistochemical studies were performed to directly exclude the potential contribution of TGF-β1-mediated increases in cell number toward the increased release of 72-kd type IV collagenase or TIMP-1. Nonstimulated cells were stained with affinity-purified antibodies against the TIMP-1 and 72-kd type IV collagenase antigens (Figure 9 A and C, respectively), revealing a low level of expression. After stimulation for 48 hours with 1 ng TGF-β1/ml, there was a significant increase in the staining intensity for the TIMP-1 protein and virtually 100% of the cells were positively stained (Figure 9B). In a similar fashion, incubation for 48 hours with 10 ng TGF-β1/ml led to a marked increase in the staining intensity for the 72-kd type IV collagenase, also involving 100% of the cells. These studies indicate that the stimulatory effects of TGF-β1 on TIMP-1 and 72-kd type IV collagenase secretion result from increases in individual cell synthetic rates, as opposed to a simple increase in cell number. The homogeneous patterns of type IV collagenase and TIMP-1 expression exclude any selective effect of TGF-β1 on phenotypically distinct culture subsets.

The second series of experiments extended the *in vitro* observation that TGF-β1 stimulates 72-kd type IV collagenase synthesis to the intact animal. For these studies we used perfusion-fixed renal cortical tissue obtained from normal adult rats and animals in which acute immune complex-mediated glomerulonephritis had been induced by injection of Thy 1.1. antiserum.[16] Elevated expression of TGF-β1 protein in this model has been reported;[2] however, the cellular source(s) have not been defined. To determine the potential linkage between TGF-β1 and 72-kd type IV collagenase expression *in vivo*, *in situ* reverse transcriptional localization of

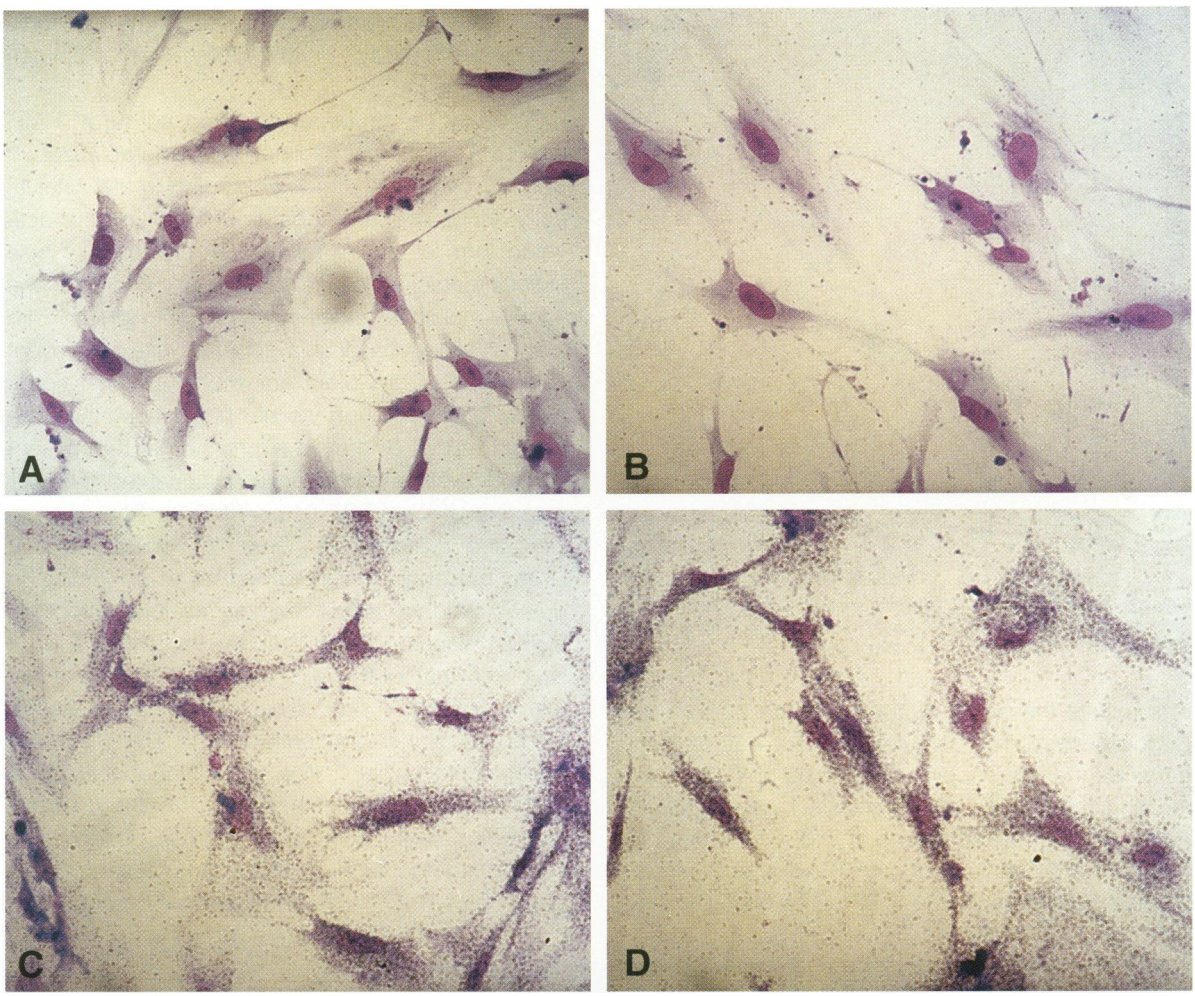

Figure 7. In situ *reverse transcription localization of 72-kd type IV collagenase mRNA in cultured HMC.* A: *Hybridization control using the sense primer for the 72-kd type IV collagenase mRNA. No signal is observable.* B: *Hybridization of nonstimulated HMC with the appropriate antisense primer for the type IV collagenase. Little or no detectable message is present.* C: *Hybridization with the antisense type IV collagenase primer after 3 hours stimulation with 10 ng/ml TGF-β1. A highly significant amount of type IV collagenase mRNA is present in these cells.* D: *Hybridization with the antisense type IV collagenase primer after 3 hours stimulation with TGF-β1 in cultures pretreated with cycloheximide. Protein synthesis inhibition does not block the TGF-β1-mediated expression of 72-kd type IV collagenase mRNA. Final magnification, ×900.*

the corresponding mRNAs was performed. The results of these studies are summarized in Figure 10. Hybridization of perfusion-fixed cortical tissues with a sense primer to the 72-kd type IV collagenase did not yield any detectable signal as expected (Figure 10A). Similarly, a sense primer for rat TGF-β1 also did not yield any detectable signal (not shown). The appropriate hybridizing antisense primer for TGF-β1 localized a significant amount of mRNA strictly within the mesangial regions of normal rat glomeruli (Figure 10B). The distribution of the readily detectable mRNA for the 72-kd type IV collagenase within normal glomeruli was also restricted to the mesangium (Figure 10C). At 5 days after induction of Thy 1.1 nephritis, there is a pronounced mesangial cellular proliferative response and matrix expansion. As shown in Figure 10D, these foci of proliferating mesangial cells express very high levels of TGF-β1 mRNA, a pattern of expression that is identical to the augmented signal for 72-kd type IV collagenase mRNA (Figure 10E and F). As previously demonstrated for the TIMP-1 protein in this model, the TIMP-1 mRNA was not expressed in significant levels at any time point (not shown).

Discussion

The experiments detailed in this report demonstrate the independent regulation of mesangial cell synthesis of TIMP-1 and the 72-kd type IV collagenase. As reported in other tissue culture systems,[4,24] low concentrations of TGF-β1 stimulated the synthesis of the TIMP-1 protein. In contrast, higher concentrations of TGF-β1 (5 ng/ml and above) were required

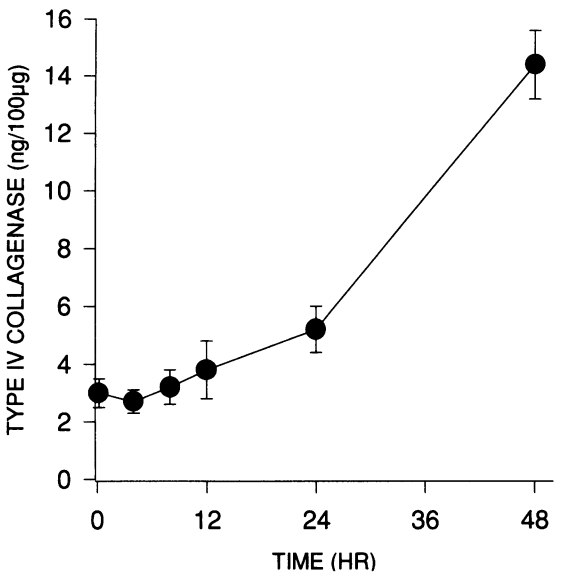

Figure 8. *Quantitation by ELISA of intracellular 72-kd type IV collagenase antigen after stimulation for increasing time periods with 10 ng/ml TGF-β1. Results expressed as the means ± 1SD.*

to stimulate mesangial synthesis of the 72-kd type IV collagenase. This stimulatory event was also independent of the suppressive effects of TGF-β1 on mesangial synthesis of stromelysin-1 and PUMP-1. At the higher concentrations of TGF-β1 the net effect is on the accumulation of 72-kd type IV collagenase in excess of the TIMP-1 inhibitor protein. This effect was demonstrated at both the functional (gelatinase assay, zymography) and antigen (ELISA) levels.

Recently, a structurally related form of TIMP, termed TIMP-2, was isolated and characterized.[25] Both forms of TIMP, through binding to the catalytically active site, are capable of inhibiting the activated form of the 72-kd type IV collagenase in a stoichiometric ratio of 1:1.[25,26] In contrast, the TIMP-2 molecule preferentially binds to latent 72-kd type IV collagenase at a separate site to prevent conversion of the latent enzyme to its catalytically active form. Although we did not directly measure TIMP-2 mRNA or protein expression in the mesangial cell system, others have documented the suppressive effect of TGF-β1 on TIMP-2 mRNA expression and synthesis in human tumor cell lines.[25] Using mesangial cells, Kawanishi et al[19] failed to demonstrate a significant stimulatory effect of TGF-β1 alone on TIMP-2 mRNA expression. Thus, it is unlikely that TIMP-2 expression plays a significant role in the expression of net proteolytic activity induced by higher concentrations of TGF-β1 in our experimental system. The induction of mesangial cell TIMP-1 protein synthesis by TGF-β1 *in vitro* is discordant with our prior *in vivo* observations concerning the lack of significant mesangial expression of TIMP-1 by controls or during acute Thy

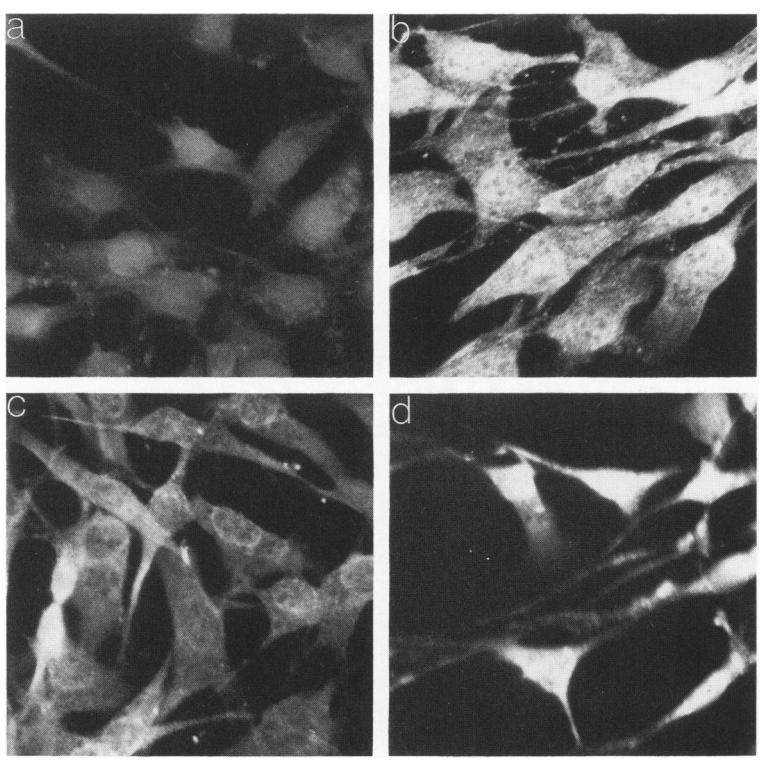

Figure 9. *Immunohistochemical analysis of TIMP-1 and 72-kd type IV collagenase expression by cultured HMC. Cells were prepared and stained as detailed in* Materials and Methods. **A:** *controls stained for TIMP-1;* **B:** *cells incubated with 1 ng/ml TGF-β1 and stained for TIMP-1;* **C:** *controls stained for 72-kd type IV collagenase;* **D:** *cells incubated with 10 ng/ml TGF-β1 and stained for 72-kd type IV collagenase. Final magnification, ×900.*

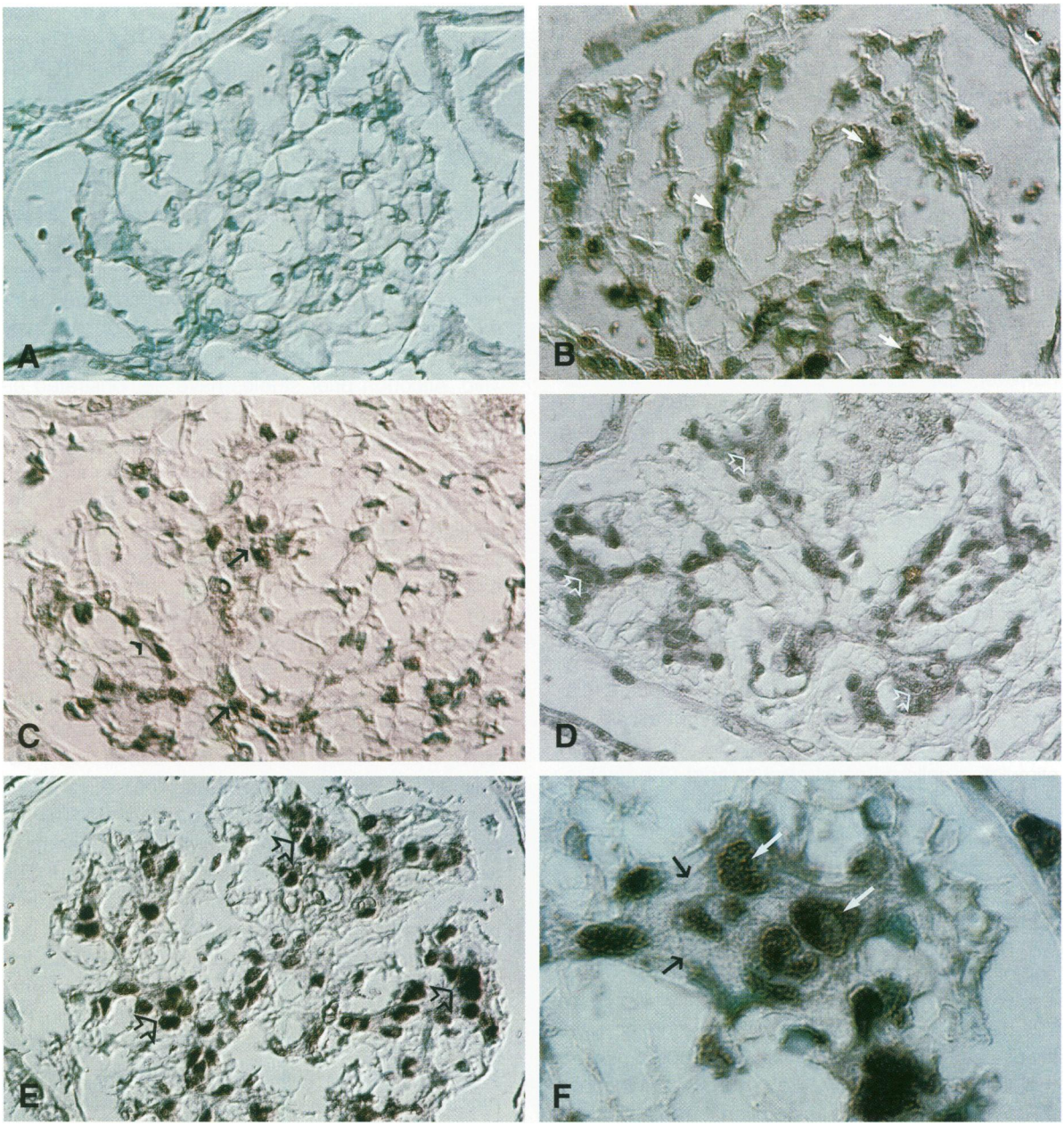

Figure 10. *Localization of glomerular 72-kd type IV collagenase mRNA and TGF-β1 mRNA* in vivo. **A:** *Hybridization of perfusion-fixed glomerulus with a control sense primer for the 72-kd type IV collagenase mRNA.* **B:** *Hybridization of glomeruli from normal rat primed with the antisense oligonucleotide for the type IV collagenase mRNA. Dark brown immunoreactive material localizes the mRNA of the type IV collagenase to the mesangial region of the glomerulus (small white arrows).* **C:** *Localization of TGF-β1 mRNA in the normal glomerulus. Reaction products are confined to the mesangium (dark arrows).* **D:** *Expression of TGF-β1 mRNA 5 days after induction of acute Thy 1.1 nephritis. There is marked proliferation of the intrinsic mesangial cells and these contain abundant TGF-β1 mRNA (open white arrows).* **E:** *Enhanced expression of 72-kd type IV collagenase mRNA at 5 days after induction of Thy 1.1 nephritis. There is intense expression of this mRNA, again strictly localized to the mesangium.* **F:** *Higher power photomicrograph of 72-kd type IV collagenase mRNA expression in the Thy 1.1 nephritic glomerulus. A focus of intensely stained, proliferative mesangial cells (white arrows) is seen embedded within the expanded mesangial matrix (dark arrows). Final magnifications,* **A** to **E** *×500;* **F**, *×1200.*

1.1 nephritis.[16] Firestein et al[27] have noted discordant expression of the stromelysin and TIMP-1 genes by rheumatoid synovial cells *in vivo*, and it may be that a significant element of tissue-specific regulation of TIMP-1 gene expression is lost in the transition to the cultured state.

Variable responses to TGF-β1 exposure, in terms of type IV collagenase synthesis, have been re-

ported in a limited number of cell types. Brown et al[28] did not detect a significant stimulatory action of TGF-β1 on human lung fibroblasts. A slight stimulatory effect was observed by gelatin zymography with one human melanoma cell line, HT-1444. Overall et al[24] also used gelatin zymography and observed a 1.8-fold TGF-β1-mediated increase in 72-kd type IV collagenase secretion by human gingival fibroblasts. In contrast, Salo et al[29] failed to detect a significant induction of 72-kd type IV collagenase secretion by TGF-β1-treated gingival fibroblasts. Exposure of human dermal keratinocytes to TGF-β1 did induce type IV collagenase secretion; however, the primary stimulatory effect was observed with the 92- rather than the 72-kd type IV collagenase. None of these studies used net quantitative assessments of enzymatic activities and antigen levels as were done in this report, nor were immunohistochemical analyses performed to determine whether the type IV collagenase and TIMP-1 proteins were produced homogeneously or by selected cell populations.

The synthesis and release of significant quantities of the 72-kd type IV collagenase protein by mesangial cells after stimulation with TGF-β1 were relatively delayed (48 to 72 hours). In contrast, *in situ* reverse transcriptional analysis of TGF-β1-stimulated cells demonstrated a rapid (3 hours) increase in 72-kd type IV collagenase mRNA abundance, which was not dependent on protein synthesis. It is known from work with other responsive genes that TGF-β1 acts at multiple levels affecting protein synthesis, including stimulation of transcription, increased stability of mRNA, and enhancement of translation.[30–32] Overall et al[33] have demonstrated that TGF-β1 transiently increases 72-kd type IV collagenase gene transcription rates in gingival fibroblasts, which is coupled with a threefold increase in 72-kd type IV collagenase mRNA half-life. The transcriptional regulation induced by TGF-β1 is complex. Although TGF-β1 autoinduction is mediated by the AP-1 complex, transcription of the collagen type I α1 and α2 genes and the plasminogen activator inhibitor gene is mediated via the NF-1 or NF-1-like consensus element.[34–36] We have recently cloned and sequenced 2000 bp of the 5′ untranslated region of the rat 72-kd type IV collagenase gene and identified three NF-1-like consensus elements localized at −1350, −1200, and −800 bp relative to the transcriptional start site (DHL et al, unpublished observations). Experiments using appropriate reporter constructs are currently in progress to determine whether these are the elements that specifically enhance 72-kd type IV collagenase gene transcription by mesangial cells in response to TGF-β1. Given our current observations, it appears that TGF-β1 modulation of mesangial cell synthesis of the 72-kd type IV collagenase is remarkably complex, and acts at both the transcriptional and translational levels. Rapid induction of transcription of a durable mRNA is followed by delayed but persistent translation of the enzyme.

A major increase in 72-kd type IV collagenase mRNA (this report) and protein synthesis by the intrinsic mesangial cells[16] occurs contemporaneously with augmented expression of TGF-β1 mRNA *in vivo*. Although prior studies have demonstrated elevated expression of the TGF-β1 protein in the Thy 1.1 model of nephritis,[1,2] this study defines the synthesizing cell population as mesangial in nature. Elevated synthesis of extracellular matrix proteins, including type IV collagen, fibronectin, and proteoglycans, has been demonstrated in this model during the period of rapid mesangial expansion, and is mediated at least in part by TGF-β1.[3,11] In addition, TGF-β1 mediates a net reduction or inhibition of glomerular plasmin activity during the phase of rapid matrix expansion.[37] Based on this, TGF-β1-mediated expression of the 72-kd type IV collagenase during the same time period would appear at first glance to be paradoxical. A consideration of the normal physiological function of TGF-β1 as a wound healing cytokine may provide the most acceptable resolution of this apparent dilemma. The acute Thy 1.1 model of immune complex nephritis is most notable for the rapid restitution of normal glomerular structure and function, despite rather dramatic early cellular proliferative and matrix expansion responses. Thus, the role of TGF-β1 in this model is consonant with its generalized function as a tissue repair cytokine. Coordinated TGF-β1-mediated stimulation of extracellular matrix synthesis and a critical remodeling enzyme may permit the eventual restitution of normal glomerular basement membrane and mesangial matrix structure. These studies demonstrate the multiple levels of control of extracellular matrix metabolism that exist and may hopefully provide the basis for future insights into the complex nature of glomerular matrix regulation.

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