Regulation of human mesangial cell collagen expression by transforming growth factor-β1

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Poncelet, Anne-Christine, and H. William Schnaper. Regulation of human mesangial cell collagen expression by transforming growth factor-β1. Am. J. Physiol. 275 (Renal Physiol. 44): F458–F466, 1998.—Transforming growth factor (TGF)-β1 has been implicated in glomerular extracellular matrix accumulation. Since the spectrum and mechanism of changes in collagen turnover have not been fully characterized, we evaluated effects of TGF-β1 on collagen expression by human mesangial cells. TGF-β1 induced increased α₁(I), α₂(III), and α₁(IV) collagen mRNA expression. Greater mRNA expression of matrix metalloproteinase (MMP)-2 was compensated by increased tissue inhibitor of metalloproteinases (TIMP)-2 mRNA. There was no change in TIMP-1 or membrane-type MMP mRNA expression, whereas MMP-1 mRNA decreased. Types I and IV collagen protein accumulated in both the cell layer and medium. Changes in collagen mRNA and protein occurred within 4 and 8 h, respectively. MMP-2 and TIMP-1 and -2 activities showed little change. Cycloheximide markedly decreased collagen detection within 4 h and reversed late, but not early, changes in α₁(I) collagen mRNA. In this system, increased synthesis may be more significant than degradation for collagen accumulation, but collagen is short-lived in culture. Diverse TGF-β1 actions on collagen turnover may be either immediate or mediated through synthesis of regulatory molecules.

kidney; extracellular matrix; growth factor; collagen

TGF-β belongs to a family of polypeptides that play an important role in a variety of physiological activities, including growth, differentiation, immunosuppression, proliferation, inflammation tissue remodeling, and wound healing (14). Three TGF-β isoforms (TGF-β1, -β2, and -β3) have been identified in mammalian species. TGF-βs are known to induce matrix accumulation in different cell types by stimulating ECM protein synthesis and in some cases by inhibiting expression of ECM proteases while stimulating synthesis of ECM proteases inhibitors (3). Several in vivo models have been used to investigate the role of TGF-β in kidney diseases. In acute mesangial proliferative glomerulonephritis induced in rats by a single injection of anti-thymocyte serum, increased TGF-β expression is associated with increased plasminogen activator inhibitor (PAI)-1 synthesis, decreased plasminogen activator (PA) activity (36), and elevated matrix deposition (26). ECM accumulation in experimental glomerulonephritis induced by anti-thymocyte serum is suppressed by administration of antibody raised against TGF-β (6), by the natural inhibitor of TGF-β decorin (4), or by TGF-β antisense oligonucleotides (2). In cultured mouse mesangial cells, TGF-β1 stimulates production of type I and type IV collagen and of fibronectin (23). In rat mesangial cells, TGF-β1 was reported to induce increased proteoglycan synthesis without any changes in collagen or fibronectin synthesis (5), whereas another group has demonstrated TGF-β1-induced expression of α₁(I) and α₁(IV) collagen and fibronectin genes (35). TGF-β1 also inhibits PA production while stimulating PAI synthesis by normal rat glomeruli (36, 38). In cultured human mesangial cells, TGF-β1 stimulates type IV collagen and fibronectin mRNA expression (17, 22). These studies suggest that one way TGF-β1 mediates glomerular disease is to alter mesangial matrix turnover. However, the mechanisms of action of TGF-β1 on ECM turnover are not completely defined.

In the present study, we have evaluated the level and timing of TGF-β1-induced changes in human mesangial cell collagen expression of genes involved in collagen turnover, including ECM proteins, relevant ECM proteases, and protease inhibitors. Our results indicate that expression of different proteins and proteases is regulated with distinct kinetics, that a variety of culture conditions modulate TGF-β1 effects, and that TGF-β1 both directly stimulates changes and induces other factors to affect ECM turnover. These results provide a basis for further study of the cellular mechanisms by which TGF-β1 regulates collagen turnover.
MATERIALS AND METHODS

Mesangial cell culture. Human mesangial cells were isolated from glomeruli by differential sieving of minced normal human renal cortex obtained from anonymous surgery or autopsy specimens. Cells were grown in DMEM/F-12 medium supplemented with 20% heat-inactivated fetal bovine serum (HyClone Laboratories), glutamine, penicillin/streptomycin, sodium pyruvate, HEPES buffer, and 8 µg/ml insulin (GIBCO-BRL, Life Technologies) as previously described (30). Cells were confirmed to be mesangial by morphological criteria, by the presence of abundant actin microfilaments, and by absence of staining for cytokeratin and factor VIII-related antigen. They were free of mycoplasma contamination and were used between passages 5 and 8.

Cell treatments. Cells were plated at identical density in 100-mm culture dishes (4–5 × 10⁶ cells/dish). Three to five days later, cells in fresh complete or serum-free medium were treated with various concentrations of active human recombinant TGF-β1 (R & D System; dilutions made from a 4 µg/ml stock solution in 4 mM HCl containing 1 mg/ml BSA) or control vehicle (different time periods leading up to simultaneous harvest. For cycloheximide experiments, cells were washed twice with PBS and pretreated for 30 min with 10 µg/ml cycloheximide (Sigma; 1 mg/ml stock solution in water) in serum-free medium. TGF-β1 (1 ng/ml) was added for different durations before collecting the media and cell lysates.

RNA isolation and Northern blot. Total RNA was isolated by the single-step method of Chomczynski and Sacchi (9). After determination of RNA purity and concentration, 10 µg of total RNA was subjected to denaturing electrophoresis and transferred overnight by capillary action onto nylon membranes. The probes were32P labeled by random priming using the Rediprime kit (Amersham). Hybridization was carried out at 65°C for 4 h in a solution containing 1% SDS, 1 M NaCl, and 10% dextran sulfate. Hybridization was performed overnight at 65°C in the same solution with 104 cpm/ml cDNA probes. The probes were the probes were 32P labeled by random priming using the Rediprime kit (Amersham). After incubation, the blots were washed twice in 2× SSC for 2 min at room temperature, then twice in 2× SSC-1% SDS for 15 min at 60°C, followed by two washes in 0.5× SSC-0.1% SDS for 15 min at room temperature or at 60°C. The washed blots were exposed to X-ray film (Fuji) at −80°C for 1–72 h according to the intensity of the signal. Autoradiograms were scanned with an Arcus II Scanner (Agfa) in transparency mode, and densitometric analysis was performed using the NIH Image 1.61 program for Macintosh. The same blots were successively rehybridized with the other probes after stripping by incubating the membranes three times in 0.01× SSC-0.01% SDS at 100°C for 2 min. Complete stripping was confirmed by exposing the blots to X-ray film.

cDNA probes. cDNAs for human α1(I) (clone Hf677: Ref. 10), α3(I)(II), and α5(Iv) collagen chains were obtained from Dr. Y. Yamada, National Institute of Dental Research, National Institutes of Health (NIH, Bethesda, MD). These cDNAs do not cross-hybridize with other known human collagen chain cDNAs (Y. Yamada, personal communication). cDNA for human matrix metalloproteinase (MMP)-2 (11) was provided by Dr. G. Goldberg, Washington University School of Medicine (St. Louis, MO). The tissue inhibitor of metalloproteinases (TIMP)-1 cDNA (8) was obtained from Dr. D. Carmichael, Synergen (Boulder, CO). cDNAs for human MMP-1 (33) and TIMP-2 (34) were obtained from Dr. W. Stetler-Stevenson, National Cancer Institute, NIH (Bethesda, MD). MT1-MMP cDNA (28) was kindly provided by Drs. Y. Itoh and H. Nagase, University of Kansas Medical Center (Kansas City, KS). Human cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was provided by Dr. L. Bruggeman, Mount Sinai School of Medicine (New York, NY). Bovine cDNA for 28S ribosomal subunit (recognizing human 28S rRNA) was obtained from Dr. H. Sage, University of Washington (Seattle, WA). The signals obtained by hybridization with probes related to ECM turnover were corrected for loading using the signal obtained with the GAPDH or 28S probes. These two control probes yield similar results except in experiments examining the effect of cycloheximide.

Zymogram and reverse zymogram analysis. After treatment, cells were washed twice with PBS and incubated overnight in DMEM/F12 medium. Media were then collected and subjected to electrophoresis under nondenaturing conditions through SDS-polyacrylamide gels (10% acrylamide for zymography and 12% acrylamide for reverse zymography) containing 1 mg/ml gelatin (zymography) or 1 mg/ml gelatin and a source of MMP activity (Reverse Zymography Kit; University Technologies International, University of Calgary, Calgary, Alberta, Canada). The gels were washed in 2.5% Triton X-100, and equilibrated in 10 mM Tris-HCl (pH 8.0) before incubation in 50 mM Tris-HCl (pH 8.0) containing 5 mM CaCl2 and 1 µM ZnCl2 for 16 to 20 h at 37°C. The gels were then stained with Coomassie blue.

Preparation of cell lysates and Western blot analysis. At the end of the treatment, the cells were washed twice with PBS and the medium was replaced with serum-free medium. The media were collected 24 h later, and the cells were washed twice with PBS before being lysed on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 µg/ml aprotinin). In some experiments, cells were treated with TGF-β1 in serum-free medium, and the media and cells lysates were collected directly at the end of the treatment. The cell lysates were centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was collected. The protein content was determined by Bradford protein assay. Fresh or frozen media and cell lysates were analyzed by SDS-PAGE (6% acrylamide gel). All samples were prepared in the Laemmli reducing buffer and boiled for 10 min before loading. Gels were blotted onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in PBS-Tween (0.1%) for 1 h at room temperature followed by incubation with the primary antibody diluted in blocking solution (rabbit anti-human collagen type I and type IV; Biodiesom International; 1:10,000 dilution) for 1 h at room temperature or overnight at 4°C. The blots were washed three times in PBS-Tween for 5 min followed by incubation with the secondary antibody conjugated with horseradish peroxidase (donkey anti-rabbit Ig; Amersham; 1:1,000 dilution) for 1 h at room temperature. After washing, the blots were developed with the enhanced chemiluminescence substrate (ECL) according to manufacturer’s protocol (Amersham). Quantification of the bands on autoradiographs was performed using densitometric analysis.

Statistical analysis. Statistical differences between experimental groups were determined by analysis of variance using InStat 2.03 software program for Macintosh. Values of P < 0.05 were considered significant.

RESULTS

Effects of TGF-β1 on expression of mRNA for ECM components, ECM proteases, and ECM protease inhibitors. To assess the effect of TGF-β1 on ECM turnover by mesangial cells, we first examined how TGF-β1 treat-
ment affects expression of genes for collagens, MMPs, and their inhibitors. Total RNA from human mesangial cells, incubated for 48 h with different concentrations of TGF-β1, was analyzed by Northern blot (Fig. 1). We found a dose-dependent increase in mRNA expression for \( \alpha_1(1) \), \( \alpha_1(III) \), and \( \alpha_1(IV) \) collagen with TGF-β1 treatment. The greatest increase is observed at 5 ng/ml TGF-β1 with 3-, 1.9-, and 2.4-fold stimulation over control for \( \alpha_1(1) \), \( \alpha_1(III) \), and \( \alpha_1(IV) \) collagen, respectively (\( n = 3 \); loading controlled by value of 28S rRNA, similar results were obtained using GAPDH as a control). The larger of the two species of \( \alpha_1(1) \) collagen mRNA is more affected by TGF-β1 treatment than the smaller species. Expression of mRNA for MMP-2 is stimulated 2.7-fold by 5 ng/ml TGF-β1. Messenger RNA levels for the TIMP-2 also are increased in a dose-dependent manner, whereas TIMP-1 mRNA expression is only weakly affected by TGF-β1. MMP-1 mRNA expression decreases with TGF-β1 treatment, with a maximal decrease of 5.5-fold at 1 ng/ml. TGF-β1 has no effect on the membrane-type matrix metalloproteinase MT1-MMP mRNA expression.

We next examined the kinetics of these changes. Cells were treated with 1 ng/ml TGF-β1 for different durations, leading up to simultaneous harvest of total cellular RNA from each of the cultures. This approach was designed to minimize the effect of duration of culture or cell crowding on ECM turnover. By Northern blot analysis, expression of mRNA for \( \alpha_1(1) \) and \( \alpha_1(IV) \) collagen, MMP-2, and TIMP-2 begins to increase by 1 h after adding TGF-β1 to the cultures, with maximal increases at 24 h for \( \alpha_1(1) \) collagen, \( \alpha_1(IV) \) collagen, and TIMP-2. MMP-2 continues to increase up to 48 h (Fig. 2, A and B). The \( \alpha_1(III) \) collagen mRNA expression starts increasing between 4 and 24 h of treatment. MMP-1 mRNA expression starts decreasing significantly between 4 and 24 h of incubation with TGF-β1 (ratio of 24-h treated cells to control cells, 0.60 ± 0.29; \( P < 0.05 \), \( n = 4 \)). A slight decrease in TIMP-1 mRNA levels is observed in cells treated for 48 h (ratio of 48-h treated cells to control cells, 0.77 ± 0.17; \( P < 0.005 \), \( n = 6 \)). These differences in timing and direction of changes suggest that expression of various collagens, MMPs, and TIMPs is not coordinately regulated after TGF-β1 treatment.

Effect of TGF-β1 on collagen production and MMP and TIMP activity. The next experiments were carried out to determine whether the TGF-β1-mediated increases in RNA expression for ECM proteins correlate with protein production. Mesangial cells were treated for 48 h with various concentrations of TGF-β1. After changing to serum-free medium for 24 h, the media were analyzed by Western blot. Staining with antibody to human collagen I revealed two bands of apparent molecular mass of 140 and 190 kDa, corresponding to the sizes of the pro-\( \alpha_1 \) form and \( \beta \) forms of collagen I (Fig. 3A). Longer exposure of the blots shows a 120-kDa band that represents the \( \alpha_1(I) \) form. Developing with anti-collagen IV antibody reveals a doublet band at 200 kDa corresponding to \( \alpha_2(IV) \) and \( \alpha_3(IV) \) collagen, as well as a band over 250 kDa that likely corresponds to aggregates of collagen IV (Fig. 3B). Expression of both collagen types increased significantly with TGF-β1 treatment, with a tendency to maximal accumulation in media at 0.5–1 ng/ml TGF-β1. To determine the duration of exposure to TGF-β1 required to stimulate collagen production, we analyzed the amount of collagen released into media in 24 h after the cells have been treated with 1 ng/ml TGF-β1 for different periods of time, washed, and then cultured for 24 h in serum-free medium (Fig. 4, A and B). Type I and IV collagen accumulation increased after only 1-h exposure to TGF-β1, with peak accumulation after 24- to 48-h exposure. These results are consistent with our Northern blot data and indicate that TGF-β1 induces colla-

"Fig. 1. Effects of transforming growth factor-β1 (TGF-β1) on expression of mRNAs relevant to collagen turnover: dose response. Subconfluent human mesangial cells were treated for 48 h with the indicated doses of TGF-β1. Total cellular RNA was isolated as described in MATERIALS AND METHODS and subjected to denaturing electrophoresis before transfer to a nylon membrane. The same blot was stripped and reprobed for expression of mRNA for the \( \alpha_1 \)-chain of type I, III, and IV collagen, interstitial collagenase (MMP-1), type IV collagenase (MMP-2), membrane-type matrix metalloproteinase (MT-MMP), and tissue inhibitors of metalloproteinase (TIMPs). cDNAs for 28S rRNA and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) were used as a control for loading. Similar results were obtained using 3 different mesangial cell isolates."

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gen production in a dose-dependent and time-dependent manner.

We performed gelatin zymography and reverse zymography on media to determine MMP and TIMP activity. Although in some of our experiments TGF-β1 induced a slight increase in MMP-2 activity, in most cases, MMP-2 activity was not affected by TGF-β1 treatment (Fig. 5A), suggesting that the increase in the steady-state levels of MMP-2 mRNA does not translate into an increase of MMP-2. MMP-1 was not detected in our gelatin zymograms, even after p-aminophenylmercuric acetate activation following the protocol of Daphna-Iken and Morrison (12) (data not shown). TIMP-1 activity is not significantly affected by TGF-β1 treatment, whereas TIMP-2 is hardly detected, and no differences between treated and untreated cells could be discerned (Fig. 5B). Taken together, these results indicate that TGF-β1 induces collagen accumulation by near-confluent human mesangial cells.

Length of time between initial exposure to TGF-β1 and increased production of collagen protein. We next determined how TGF-β1 affects the rate of release of collagen by mesangial cells. Near-confluent mesangial cells were switched to serum-free medium containing

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**Fig. 2.** Time course of TGF-β1 effect on expression of mRNAs relevant to collagen turnover. Mesangial cells were treated with 1 ng/ml TGF-β1 for the time periods indicated, leading up to simultaneous RNA harvest. Northern blot analysis was performed as described in the legend of Fig. 1. Values are expressed as the ratio between treated cells and control cells, after correction from loading using 28S (correction with GAPDH gives similar results). A: α1(I), α1(III), and α1(IV) collagen. B: MMP-1, MMP-2, MT-MMP, TIMP-1, and TIMP-2. Results are given as means ± SE for 3 experiments. *P < 0.05 by Mann-Whitney test.

**Fig. 3.** Western blot for type I and type IV collagen in media of mesangial cell cultures treated with different doses of TGF-β1. Cells were incubated with the indicated dose of TGF-β1 for 48 h before replacing the culture medium with identical amounts of serum-free medium. After 24 h, the medium was harvested and 150 µl/lane was electrophoresed in a 6% acrylamide gel under denaturing conditions and transfer to Immobilon-P membrane. A: blots were developed using anti-human type I collagen. A representative blot is shown at top. Results of densitometric analysis are shown at bottom. Values are expressed as fold increase over control. *P < 0.02 and **P < 0.01 compared with control (n = 5; Mann-Whitney Test). B: Western blot for type IV collagen. A representative blot is shown at top. Results of densitometric analysis are shown at bottom. Values are expressed as fold increase over control. *P < 0.05 compared with control (n = 4; Mann-Whitney test).
1 ng/ml TGF-β1 or control vehicle. Proteins from media and cell layers were harvested at different periods of time and subjected to immunoblot analysis for type I collagen. Collagen in media of control mesangial cells is observed after 16 h, indicating that, in our experiments, 16-h release is necessary for sufficient collagen to be present for detection by immunoblot (Fig. 6A). In contrast, TGF-β1 treatment led to detection of released type I collagen after 8 h. In many cases, a slight decrease of cell-layer type I collagen is observed between 0.5 and 1 h incubation with TGF-β1, perhaps due to release of collagen into the medium (data not shown). The ratio of cell layer collagen I content, comparing TGF-β1 treatment to control, increases significantly beginning after 4 h and continues to a maximum at

![Graph showing the time course for induction of type I and type IV collagen expression in media of mesangial cells treated with TGF-β1.](image)

Fig. 4. Time course for induction of type I and type IV collagen expression in media of mesangial cells treated with TGF-β1. Cells were incubated with 1 ng/ml TGF-β1 for the indicated periods of time leading up to simultaneous replacement of culture medium with serum-free medium. Twenty-four hours later, the media were analyzed by immunoblot as described in Fig. 3. A: type I collagen. B: type IV collagen. Similar results were obtained in 2 additional experiments.

![Diagram showing the effects of TGF-β1 on matrix collagenase and MMP inhibitor activity.](image)

Fig. 5. Effects of TGF-β1 on matrix collagenase and MMP inhibitor activity. Media prepared as described in Fig. 3 were analyzed by gelatin zymogram (A) or reverse zymogram (B). MMP-2 migrates on SDS-PAGE under nonreducing conditions as a 68-kDa species (12). TIMP-1 is detected as a band at 28 kDa, and TIMP-2 is detected as a faint and less distinct band at 21 kDa (19). "Sd" indicates TIMP-1 and TIMP-2 standard. Time course analysis of collagenase and MMP inhibitor activity indicates no difference between TGF-β1-treated and control cells for up to 48 h of treatment (data not shown).

![Graph showing the timing of the release and synthesis of type I collagen by TGF-β1.](image)

Fig. 6. Timing of the release and synthesis of type I collagen by TGF-β1. Near-confluent cells were incubated for the indicated time periods in serum-free medium with 1 ng/ml TGF-β1 or control vehicle. Media and cell lysates were collected as described in MATERIALS AND METHODS. A volume of 150 µl of medium or 50 µg of protein lysate was loaded on a 6% acrylamide gel for immunoblot analysis using anti-type I collagen antibody. A: media. B: cell layers. C: densitometric analysis of cell layer results. Values are expressed as ratio between treated cells and control cells. Kruskal-Wallis nonparametric ANOVA test indicates that the increase ratio of treated to control values over time is very significant (P < 0.005; n = 13; conditions in 5 experiments).
16–24 h (Fig. 6, B and C). These data indicate that TGF-β1 induces increased production and possibly release of collagen beginning between 4 and 8 h. The changes in medium collagen content could therefore reflect both increased synthesis and accelerated release.

To evaluate the relative rates of collagen turnover in human mesangial cells, near-confluent cells were switched to serum-free medium. TGF-β1 or control vehicle was added with or without 10 µg/ml cycloheximide, either immediately (24 h treatment) or 20 h later (4 h treatment), leading up to simultaneous harvest of the cell layers and media. The media and cell layers were analyzed by immunoblotting with anti-collagen I antibody. Representative results are shown in Fig. 7. Consistent with our previous results, treatment with TGF-β1 increases collagen I production and secretion into media (lanes 2 and 6 compared with lanes 1 and 5, respectively). In the presence of cycloheximide, collagen I in media (Fig. 7A) and cell layers (Fig. 7B) is decreased (lanes 3 and 4) compared with control cells (lanes 1 and 2) at 4 h and is undetectable at 24 h (lanes 7 and 8). This result suggests a rapid turnover of collagen I in human mesangial cell culture, regardless of the presence or absence of TGF-β1.

To further examine the timing of collagen I turnover, cells were treated with 1 ng/ml TGF-β1 for 8 h, and cycloheximide was added for different time periods before collecting the cells for analysis by immunoblot. When cycloheximide is added 2 h before harvesting the cell layer (i.e., 6 h after adding TGF-β1), a drastic decrease in cell-associated collagen I is observed (Fig. 8). When cycloheximide is present for longer periods of time (4 h or more), virtually no collagen I is present in the cell layers. These data confirm that collagen I remains in subconfluent cell layers for only short duration.

**Fig. 8. Rapid disappearance of collagen from CHX-treated human mesangial cell layers.** Subconfluent cells were treated with 10 µg/ml CHX or control vehicle, added to different plates at the indicated times prior to simultaneous harvest at the end of a 24 h experiment. Cultures also were incubated with control vehicle or 1 ng/ml TGF-β1 for 8 h prior to harvest. Cell lysates were analyzed by immunoblot with anti-type I collagen antibody.

Requirement for protein synthesis in maintenance of the TGF-β1-induced increase in collagen mRNA expression. To determine the possible role of protein synthesis in TGF-β1-induced collagen gene expression, total RNA was harvested from mesangial cells treated with the same conditions as described for Fig. 7 and was analyzed by Northern blot. At 4 h, the TGF-β1-induced increase in α1(I) collagen mRNA expression was not affected by cycloheximide (data not shown). In contrast, the increase in signal intensity for this mRNA in cells treated with TGF-β1 for 24 h was completely abrogated by cycloheximide (Fig. 9A). As cycloheximide alone does not affect basal levels of α1(I) collagen mRNA, these results suggest that protein synthesis is not required for the initial increase in α1(I) collagen mRNA expression but is necessary for maintenance of that increase. For α2(IV) collagen, 4- or 24 h treatment with cycloheximide alone increased mRNA expression. This induction was further increased when TGF-β1 was added to the cultures (Fig. 9B shows the 24 h data). These results indicate that TGF-β1 induction of α2(IV) collagen mRNA was signaled by proteins already present in the cells and suggest that cycloheximide induces stabilization of mRNA. Cycloheximide did not significantly affect TGF-β1 regulation of TIMP-1 or MMP-2 mRNA expression (data not shown). Taken together, these results suggest that, in human mesangial cells, TGF-β1 induces differential regulation of genes relevant to collagen turnover.

**DISCUSSION**

Glomerulosclerosis is characterized by excessive accumulation of ECM. This process involves deposition of abnormal as well as classic matrix components. In recent years, TGF-β has been identified as an important mediator of the sclerotic process. However, the mechanism(s) of action of TGF-β on ECM turnover is not well understood. Here, we have sought to more precisely characterize the events involved in the effects of TGF-β1 on collagen turnover in human mesangial cells. In near-confluent cells, TGF-β1 induces two- to threefold increases in steady-state levels of mRNA for α1(III), and α1(IV) collagen, with maximal re-
response at 5 ng/ml. Expression of MMP-2 mRNA increases 2.7-fold with 5 ng/ml TGF-β1, whereas MMP-1 mRNA expression decreases by 5.5-fold with a maximal effect at 1 ng/ml. TGF-β1 has only a slight effect on expression of mRNA for the MMP inhibitors TIMP-1 and TIMP-2. These results support the hypothesis that TGF-β1 effects on expression of mRNA relevant to ECM turnover contribute to increased accumulation of collagen. The increase in collagen mRNA expression begins as early as 1 h after TGF-β1 treatment and peaks at 24–48 h. This TGF-β1-mediated induction of collagen mRNA is paralleled by an increase in collagen protein expression, with accumulation in both the medium and cell layer.

TGF-β has been shown to inhibit production of matrix proteases such as tissue-type PA (36) and stromelysin-1 and matrilysin (21) in cultured glomeruli or mesangial cells. Our results indicate that TGF-β1 inhibits expression of mRNA for the interstitial collagenase MMP-1 in cultured human mesangial cells, whereas this factor increases expression of mRNA for another MMP, MMP-2. This stimulatory effect of TGF-β1 on mRNA expression for a MMP is in agreement with results reported by Marti et al. (21). In contrast to the results on Northern blot analysis, zymography indicates that MMP-2, TIMP-1, and TIMP-2 proteins do not significantly change with TGF-β1 treatment. Lovett and colleagues (21) have shown a delayed translation of the TGF-β1-induced MMP-2 mRNA expression, with significant intracellular enzyme levels accumulating between 24 and 48 h after TGF-β1 treatment and detection of significant increased extracellular enzymatic activity after 48 to 72 h. This finding may explain why, in the present set of experiments, increased MMP-2 activity was not detected by zymography despite rapid increases in MMP-2 mRNA expression between 1 and 4 h after TGF-β1 exposure. Similar to the results with MMP-2, neither TIMP-1 nor TIMP-2 activities were changed by TGF-β1 treatment despite changes in TIMP mRNA expression. This finding could be due to a delayed translation as suggested above for MMP-2. Taken together, the data regarding expression of protease and inhibitor mRNA and protein suggest that the ratio of gelatinase to TIMP generally remains the same or decreases during the first 48 h of TGF-β1 treatment. MMP-1 activity was not detected by gelatin zymography. This is not surprising, as interstitial collagenase is considerably less active than gelatinases in gelatin zymograms. Thus we cannot speculate on the effect of MMP-1 activity in our system.

Despite the apparent lack of change in gelatinase activity, collagen protein content of the cultures increases rapidly with TGF-β1 incubation. Treatment of mesangial cells with cycloheximide markedly decreases detectable cell-associated and soluble type I collagen within 4 h, regardless of the presence or absence of TGF-β1, indicating a rapid turnover of collagen in subconfluent human mesangial cell cultures. However, it is possible that, at least for the cell layer collagen content, results are affected by the scorbutic conditions of our assay system. Ascorbate stabilizes collagens through cross-linking (posttranslational modification) but it also increases synthesis of these proteins through stimulation of procollagen gene expression and increased collagen mRNA stability (15, 18, 27). To avoid complicating interpretation of our data, we have chosen not to add ascorbate in our system. Taken together, our data suggest that, at least in subconfluent mesangial cells, the major cause of collagen accumulation is an increased rate of protein synthesis rather than changes in rates of collagen breakdown. However, metabolic labeling data would be necessary to confirm this hypothesis.

By Northern blot, we showed that human mesangial cells express MT-MMP, a newly identified MMP with a potential transmembrane domain (28). Steady-state mRNA levels for MT-MMP were unchanged by TGF-β1 treatment. In rat mesangial cells, MT-MMP mRNA could not be detected by either Northern blot or RT-PCR (37), whereas another study demonstrated the presence of MT-MMP mRNA by RT-PCR (1). This discrepancy might be due to the fact that the cells used...
in the former study were subjected to sequential cloning to isolate a population with high-level, constitutive expression of MMP-2. We did not try to determine whether MT-MMP is present at the protein level in human mesangial cells.

Finally, in the presence of cycloheximide, the TGF-β-1 induction of α1(Ⅰ) collagen mRNA was not affected at 4 h. However, by 24 h, α1(Ⅰ) collagen mRNA expression in the presence of cycloheximide has returned to basal levels, despite the presence of TGF-β-1. Since cycloheximide alone does not affect expression of α1(Ⅰ) collagen mRNA, this response is specific to TGF-β-1 and suggests that some long-term effect(s) of TGF-β-1 on human mesangial cells requires de novo synthesis of proteins such as transcription factors and/or cytokines that might act as autocrine or paracrine mediators (16, 17).

In contrast to the effect on α1(Ⅰ) collagen, cycloheximide alone increases expression of mRNA for α1(ⅠV) collagen and further increases the TGF-β-1-mediated induction of this mRNA at 4 and 24 h. This “superinduction” effect of cycloheximide may result from increased stability of mRNA due to inhibition of synthesis of proteins involved in RNA degradation or relief of transcriptional repression and has been described previously for immediate early genes (24) and other genes such as urokinase receptor (32). In agreement with our results, Hansch et al. (17) showed that short-term treatment with TGF-β-1 in the presence of cycloheximide leads to a superinduction of mRNA for α1(ⅠV) collagen in human mesangial cells. Cycloheximide does not affect TGF-β-1-mediated induction of MMP-2 mRNA, in agreement with Marti et al. (21). These data suggest that the α1(ⅠV) collagen and MMP-2 responses to TGF-β-1 are independent of de novo protein synthesis and may involve altered activity of preexisting transcription factors. The differential regulation and maintenance of increased collagen expression are consistent with the observation that different pathogenetic stimuli, and presumably different mechanisms of mesangial cell activation, are associated with different patterns of collagen protein expression in different glomerular diseases (7, 29).

In summary, we have shown that TGF-β-1 modulates expression of mRNAs relevant to collagen turnover, leading to increased collagen production by human mesangial cells. There is a rapid turnover of type I collagen in this system. The early response triggered by TGF-β-1 is independent of de novo protein synthesis but the sustained increase in mRNA expression for α1(Ⅰ) collagen is mediated by new protein synthesis. In contrast, TGF-β-1-induced expression of α1(ⅠV) collagen and MMP-2 mRNA does not require protein synthesis.

In our experiments, careful consideration was required to account for such issues as cell number, duration of cell culture, and degree of confluence. These conditions could have significant effects on the experimental outcome (39). The rapid rate of collagen turnover in our studies indicates caution in extrapolating from in vitro data to explain in vivo observation. Nonetheless, in vitro studies are essential for understanding the cell biology of glomerulosclerosis. The present data provide a model for further studies of the cellular events involved in TGF-β-1-induced increase collagen production by human mesangial cells.

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