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Mechanical Strain- and High Glucose-Induced Alterations in Mesangial Cell Collagen Metabolism: Role of TGF-β

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Abstract. Cultured mesangial cells (MC) exposed to cyclic mechanical strain or high glucose levels increase their secretion of transforming growth factor-β1 (TGF-β1) and collagen, suggesting possible mechanisms for the development of diabetic renal sclerosis resulting from intraglomerular hypertension and/or hyperglycemia. This study examines whether glucose interacts with mechanical strain to influence collagen metabolism and whether this change is mediated by TGF-β. Accordingly, rat MC were grown on flexible-bottom plates in 8 or 35 mM glucose media, subjected to 2 to 5 d of cyclic stretching, and assayed for TGF-β1 mRNA, TGF-β1 secretion, and the incorporation of ^{14}C-proline into free or protein-associated hydroxyproline to assess the dynamics of collagen metabolism. Stretching or high glucose exposure increased TGF-β1 secretion twofold and TGF-β1 mRNA levels by 30 and 45%, respectively. However, the combination of these stimuli increased secretion greater than fivefold without further elevating mRNA. In 8 mM glucose medium, stretching significantly increased MC collagen synthesis and breakdown, but did not alter accumulation, whereas those stretched in 35 mM glucose markedly increased collagen accumulation. TGF-β neutralization significantly reduced baseline collagen synthesis, breakdown, and accumulation in low glucose, but had no significant effect on the changes induced by stretch. In contrast, the same treatment of MC in high glucose medium greatly reduced stretch-induced synthesis and breakdown of collagen and totally abolished the increase in collagen accumulation. These results indicate that TGF-β plays a positive regulatory role in MC collagen synthesis, breakdown, and accumulation. However, in low glucose there is no stretch-induced collagen accumulation, and the effect of TGF-β is limited to basal collagen turnover. In high glucose media, TGF-β is a critical mediator of stretch-induced collagen synthesis and catabolism, and, most importantly, its net accumulation. These data have important implications for the pathogenesis and treatment of diabetic glomerulosclerosis. (J Am Soc Nephrol 9: 827–836, 1998)

Of the causal factors identified in the progression of diabetic glomerulosclerosis, hyperglycemia and glomerular hypertension predominate (1–3). High concentrations of medium glucose stimulate extracellular matrix (ECM) accumulation by mesangial cells (MC) in culture (4–6). *In situ*, these cells appear largely responsible for mesangial matrix synthesis and therefore are likely contributors to the increased deposition and mesangial expansion that are characteristic of diabetic glomerulosclerosis (7). The pathogenetic mechanisms whereby glomerular hypertension is translated into increased ECM deposition have been only recently identified. We have demonstrated that increased capillary pressure results in glomerular expansion due to the marked compliance of the structures involved (8). The overall glomerular distention causes the outward movement of the paramesangial basement membrane to which MC cytoplasmic projections are firmly attached (9). Thus, the consequence of pressure-induced glomerular distention is MC mechanical strain in the form of stretch. We and others have shown that the application of cyclic mechanical strain to cultured MC enhances the production of matrix molecules, including fibronectin, laminin, and collagen types I and IV (8,10).

Although the mediators of hyperglycemia- and glomerular hypertension-induced ECM formation have not been fully identified, it has been shown that the cytokine transforming growth factor-β (TGF-β) is both produced and bound by MC. One important effect of receptor-mediated binding of active TGF-β1 by cultured MC is the increased synthesis of ECM. Furthermore, high glucose concentrations stimulate MC production of both TGF-β1 and collagen, whereas neutralization of the cytokine reduces glucose-induced secretion of collagen (11).

The pathogenetic role of TGF-β demonstrated *in vitro* also applies *in vivo*, because renal cortical TGF-β mRNA levels are significantly increased after 1 to 3 d of hyperglycemia in the spontaneously diabetic BB rat, nonobese diabetic mouse (12), and streptozotocin-induced diabetic rat (13). In human diabetic nephropathy, a similarly increased glomerular expression of TGF-β has also been reported (14). The treatment of insulin-deficient diabetic mice with an antibody that neutralizes the

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three mammalian isoforms of TGF-β reduces renal overexpression of collagen IV and fibronectin mRNA (15). Similarly, *in vitro* treatment of glomeruli from insulin-deficient diabetic rats with an antibody to TGF-β1 corrects the increased total collagen synthesis (16).

TGF-β may also play a role in the increased ECM production induced in MC by mechanical strain. We have shown that cyclic strain of cultured MC significantly augments the secretion and activation of TGF-β1, a response that is both cytokine- and isoform-specific (17). Although it might be anticipated that such change would dictate altered ECM metabolism, the contribution of TGF-β to stretch-induced ECM production has not been clearly demonstrated. In addition, little is known regarding the action of this cytokine in matrix degradation. We recently reported that cyclic strain stimulates collagen breakdown as well as synthesis (18). The net difference, *i.e.*, accumulation, was influenced by the extracellular concentration of glucose (18). This suggests that net accumulation of ECM may only occur when both hyperglycemia and glomerular hypertension are present. The limited number of studies that have addressed the role of stretch (19), TGF-β (20), or high glucose (21) in MC matrix breakdown have examined changes in the activity of specific metalloproteases or tissue inhibitors of metalloproteases, often yielding conflicting results. This may be due to the complex interplay that occurs among the many proteases and their corresponding inhibitors (7).

In the present study, we investigate the role of TGF-β in stretch- and high glucose-induced MC collagen metabolism by examining separately the influence of these two factors on TGF-β production and by analyzing the effects of TGF-β neutralization on the synthesis, breakdown, and accumulation of collagen. We show that high glucose levels and stretch act in an additive manner to increase TGF-β1 production. In normal glucose, stretch increases collagen synthesis and breakdown independent of TGF-β and without altering collagen accumulation. In high glucose, stretch increases collagen synthesis, breakdown, and net accumulation, mediated by TGF-β.

Materials and Methods

Mesangial Cells

Cells, characterized and described by us previously (22), were obtained from a cloned line derived from outgrowths of Fisher rat glomeruli. These cells express the Thy-1 antigen and demonstrate the same high sensitivity to phorbol-stimulated neutrophil adhesion and lysis, as do early passage MC, indicating continued and like expression of essential cell surface receptors (23).

Antibodies, Standards, and mRNA Probes

Neutralizing antibody to TGF-β1 (chicken antihuman) and recombinant human TGF-β1 were obtained from R&D Systems (Minneapolis, MN) and were used in a biological assay. A monoclonal antibody (1.D11.1) that neutralizes TGF-β1, -2, and -3 was a generous gift of Genzyme Corp. (Cambridge, MA) and was used in the studies of TGF-β blockade on collagen metabolism. A cDNA probe specific for rat TGF-β1 was kindly provided by Drs. Su Wen Qian and Anita Roberts (Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD).

Application of Mechanical Force to Cultured Cells

Mesangial cells were seeded (5×10^4 cells/25-mm diameter well) into six-well plates with flexible elastin-coated bottoms (Flex I plates, Flexercell, McKeesport, PA) and cultured in 1 ml/well growth medium. The medium, RPMI 1640, contained penicillin, streptomycin, 20% Nu-Serum (Collaborative Research, Bedford, MA) and 8 mM glucose. After either a 24- or 48-h incubation, one-half of the plates were subjected to cyclic stretching (experimental group), and the remaining plates were maintained in a static environment under identical conditions (control group). Stretch was mediated by controlled cycles of vacuum applied to the underside of the flexible-bottom culture well, using a computer-assisted system (Flexercell Strain Unit®, Flexercell). This system provides precisely timed, negative pressure cycles of known magnitude, deforming the bottom of the culture well and stretching the cells attached to its surface. In an attempt to mimic conditions of mesangial cell stretch during possible low-frequency oscillations in intraglomerular pressure (24), all experiments were carried out using alternating cycles of 10 s of stretch and 10 s of relaxation (50 mHz). Vacuum intensity was set to provide an average elongation of approximately 8% over the entire culture surface (25). Selected wells received daily additions of 20 μg/ml purified monoclonal TGF-β antibody or control mouse serum over the entire period of study. In experiments in which the effect of high glucose concentration was studied, cells were exposed to 35 mM glucose for a total of 11 to 12 d.

mRNA Analyses

RNA was isolated by guanidinium and phenol extraction (RNA Stat-60, Tel-Test Inc., Friendswood, TX). Samples were denatured in glyoxal/DMSO at 55°C for 1 h, and 20 μg of RNA was used for analysis. Electrophoresis was carried out in 10 mM sodium phosphate/1% agarose gel. Gels were then stained with ethidium bromide and photographed. Gels were blotted onto GeneScreen membranes (Dupont New England Nuclear Research Products, Boston, MA), using standard methods (26). Blots were hybridized and probed for individual mRNA TGF-β isoforms, using the corresponding cDNA that were ^{32}P-labeled by random hexamer priming (Sigma Prime-1 kit, Sigma Chemical, St. Louis, MO). Autoradiograms were quantitatively analyzed by scanning densitometry (Howtek, Scanmaster 3+ Densitometer, Hudson, NH), and image analysis was performed (National Institutes of Health Image, version 1.59, Twilight Clone BBS, Silver Spring, MD). The assayed TGF-β mRNA were normalized to rRNA (18S and 28S) to correct for sample loading differences. This correction yielded values that were similar to those normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

TGF-β Bioassay

The biological activity of TGF-β in the culture medium was assayed 24 h after the last medium change, using a modification of Danielpour's method (17,27). In brief, CCL-64 (mink lung) epithelial cells were seeded into 96-well dishes (1.0×10^4 cells per well) in Dulbecco's modified Eagle's medium containing 0.2% fetal bovine serum. After 1 h, test samples or TGF-β standards, with or without added TGF-β1 antibody, were introduced into cultures. Twenty-two hours later, cells were exposed to 1.0 μCi [^{3}H]thymidine/well in a total of 200 μl of medium for 4 h at 37°C. Cells were then harvested, washed onto filter wells (MultiScreen™ Assay System, Millipore, Bedford, MA), lysed, and fixed with ethanol. The radioactivity contained in the filters was measured in an LS 350 Beckman scintillation counter (Beckman Instruments, Irvine, CA). The TGF-β activity index, expressed as inhibitory units (IU), reflects the activity that is

specifically attributable to TGF-β. This index is calculated from the difference in [^{3}H]thymidine incorporation of a sample assayed with and without antibody divided by radioactivity incorporated in the presence of antibody, then multiplied by 100. In this assay, 1 pg of TGF-β standard was equivalent to 2.4 IU of TGF-β1. Samples were diluted to provide activities in the linear range as determined by dose–response curves with TGF-β standards. The small amount of TGF-β activity detected in the MC growth medium was subtracted from the determined values. Samples were analyzed after acid activation of the latent TGF-β form with 1N HCl, and therefore represent total TGF-β activity (active plus latent).

Collagen Metabolism Studies

Methods described previously (8) were used with minor modifications. The culture medium was changed 24 h before the start of the radiolabeling period to a medium lacking proline (with the exception of that contained in Nu-Serum, which produced a final proline concentration of 40 μM). Radiolabeling was carried out by incubation for 72 h in an identical medium, but containing 0.15 mM β-aminopropionitrile, 210 μM ascorbic acid, and 183 mM [^{14}C]-proline (82.3 mCi/mmol). Previous experiments by us demonstrated that [^{14}C]-proline incorporation into collagen increases linearly over a 72-h period of radiolabeling (8). All tissue culture wells were supplemented every 24 h to provide 140 μM fresh ascorbic acid. At the termination of the radiolabeling period, medium was rapidly aspirated, the plate was placed on ice, and the cell layer was covered with 2 to 6 ml of cold 0.2N perchloric acid. The media and cell layers of six wells were pooled as one sample for analysis.

The total protein contained in the medium samples was precipitated in 75% ethanol at −5°C. After the addition of 89 μCi [^{3}H]-proline as internal standard, medium supernatants were filtered in Centricon™-3 filters (Amicon, Danvers, MA), and the free amino acids were separated by solid-phase extraction, using AG50W-X8 (H^+) columns (Poly-Prep®, Bio-Rad Laboratories, Richmond, CA). Purified amino acids were resuspended in 0.1N HCl, and [^{14}C]-hydroxyproline, total proline, and proline specific activity was determined by reverse-phase HPLC of the amino acid dansylated derivative (8).

Net collagen accumulation in the medium was estimated by two independent methods. The first measurement was obtained according to the ^{14}C incorporation into protein-associated hydroxyproline (8). In this method, the medium protein precipitate, obtained as described above, was hydrolyzed with 6N HCl at 110°C, and amino acids were separated as above by solid-phase extraction after the addition of 3.32 μCi [^{3}H]-hydroxyproline as an internal standard. Amino acids were subsequently analyzed by HPLC for measurement of ^{14}C incorporation into protein-associated hydroxyproline. The second method was based on the amount of total ^{14}C incorporated into collagenase-digestible protein (8). In this method, 1 ml of medium was mixed with a proteinase inhibitor solution (providing per milliliter: 3 μmol phenylmethylsulfonyl fluoride, 0.1 mmol ethylenediaminetetra-acetic acid, 40 μmol *N*-ethylmaleimide). Medium protein was precipitated, and the pellet was washed 5 times with cold 10% trichloroacetic acid. This precipitate was then resuspended in 1N NaOH, and the solution was neutralized with 1N HCl. After adjusting the pH to 7.5, phenylmethylsulfonyl fluoride and *N*-ethylmaleimide were added in the same amounts as before, and $CaCl_2$ was added to provide a final 5 mM solution. For enzymatic digestion, the sample was divided into two equal portions and 140 units/ml collagenase was added to one, whereas the other was used as a control. After incubation for 2 h at 37°C, the undigested protein was removed by precipitation with 10% trichloroacetic acid and 0.5% tannic acid. Finally, the ^{14}C radioactivity in the supernatants and protein precipitate was measured, and the radiolabeled incorporation into collagenase-digestible and collagenase-resistant protein was determined from the difference between the treated and nontreated samples.

Immediately after addition of 0.2N perchloric acid, cell layers were scraped and briefly homogenized in the cold. The precipitates were then lipid-extracted and consecutively subjected to alkaline and acid hydrolysis for extraction of RNA and DNA and the separation of protein (8). The cell layer protein precipitate was hydrolyzed as described above, [^{3}H]-hydroxyproline was added as an internal standard, and amino acids were chromatographically separated for the quantification of [^{14}C] incorporation into hydroxyproline.

Experiments did not include cultures incubated with mannitol as a control for altered medium osmolality because preliminary experiments in which MC were incubated for 5 d in 5 mM glucose or 5 mM glucose plus 15 mM mannitol-containing medium showed no differences in proline incorporation into protein-associated hydroxyproline (glucose: 25.3 ± 4.9, $n = 10$; glucose plus mannitol: 26.1 ± 1.6 nmol/mg DNA/24h, $n = 10$ [mean ± SEM]).

Statistical Analyses

Data are expressed as mean ± SEM. Unless otherwise noted, differences between two groups were evaluated using the unpaired two-tailed *t* test. Multiple-group comparisons were first analyzed by ANOVA. Significant differences among groups were then evaluated by Fisher's protected least significance test.

Results

Effects of Glucose Concentration and Cyclic Stretch on TGF-β1 Production

To examine the effect of glucose concentration, MC that had been continuously grown in 8 mM glucose medium were transferred to 35 mM glucose medium (11 to 12 d total). Although this "low glucose" concentration (8 mM) is higher than the physiologic level (5 mM), it is the minimum concentration required to sustain long-term growth of these cells in culture. To examine the effect of stretch, half of the cultures were subjected to 48 h of cyclic strain. In cells cultured in low glucose medium, stretch induced a greater than twofold increase in the secretion of TGF-β1 (Figure 1). High glucose levels, in the absence of stretch, increased secretion to a similar level. However, mechanical strain of cells exposed to 35 mM glucose elicited a greater than fivefold increase in TGF-β1 activity, compared with nonstretched, static controls cultured in 8 mM glucose. This indicated an additive effect of stretch and high glucose on TGF-β1 secretion. Direct counting of cells at the end of these experiments demonstrated that exposure to high glucose resulted in a significantly lower cell number (8 mM: 7.48 ± 0.63, $n = 6$; 35 mM: 4.93 ± 0.52 × 10^6, $n = 6$, $P < 0.009$). However, stretch did not significantly alter the cell number in either low or high glucose cultures (stretch, 8 mM: 6.47 ± 0.9 × 10^6, $n = 6$; stretch, 35 mM: 4.27 ± 0.33 × 10^6, $n = 6$).

To next determine whether increased secretion was likely due to changes in the level of the specific transcript, MC were cultured as described above, and their RNA was probed for TGF-β1. In cells cultured in low glucose, mechanical strain for 48 h induced a 30% increase in TGF-β1 mRNA (Figure 2).

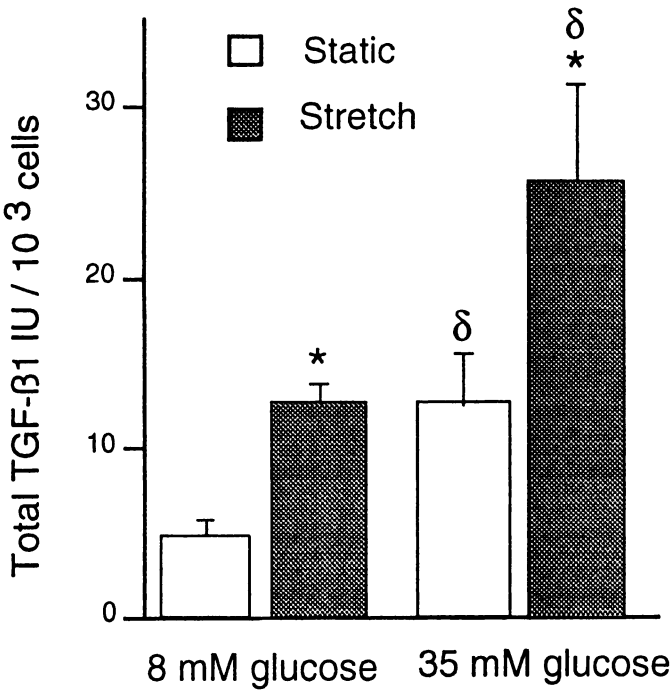

Figure 1. Secretion of transforming growth factor-β (TGF-β) by mesangial cells (MC): effect of stretch and high glucose. Data represent TGF-β1 accumulated during the final 24 h of static or stretch conditions. Significance was determined by two-way ANOVA ($n = 6$ in all groups). Results are mean ± SEM. $^{*}P < 0.005$, effect of stretch; $^{\delta}P < 0.005$, effect of glucose. Glucose and stretch interaction was not significant.

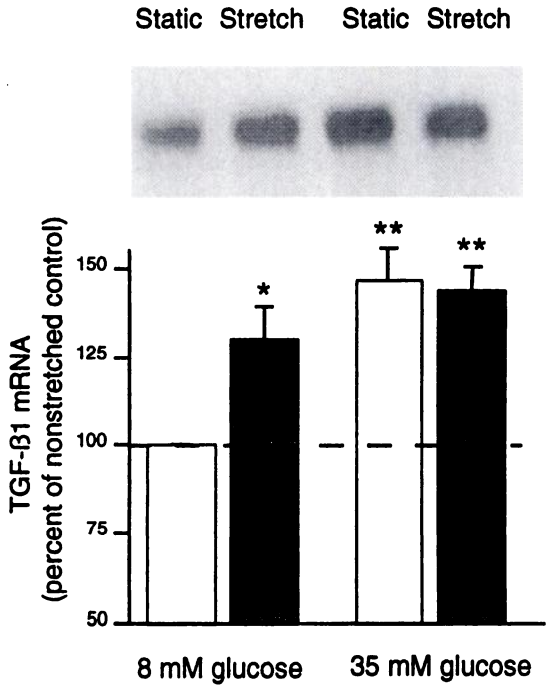

Figure 2. Northern blot analysis of mRNA after exposure to cyclic stretch and/or high glucose medium. (Top Panel) Isolated bands at 2.8 kb, corresponding to TGF-β1. (Bottom Panel) Optical scanning densitometry was used for the quantification of bands from static (open bars) or stretch (shaded bars) cultures. Results shown were normalized to total rRNA and are mean ± SEM from six separate experiments. After results were normalized according to the control value (100%), the data were analyzed using a one-sample *t* test with a hypothesized mean of 100% to compare each of the three test groups with the control. To adjust for multiple comparisons, a Holm's *post hoc* test was then applied. $^{*}P < 0.04$ or $^{**}P < 0.005$ *versus* static, 8 mM control. A paired two-sample *t* test showed no significant differences between the three test groups.

Exposure to high glucose levels increased the transcript level by approximately 45% over that demonstrated in the low glucose controls, a change that was not significantly different from that induced by stretch. Unlike the secretory response, however, mechanical strain in an environment of high glucose failed to increase TGF-β1 mRNA to values greater than those observed in static cultures grown in 35 mM glucose. These findings suggest that the increased secretion of TGF-β1 in response to high glucose concentrations or to mechanical strain alone results from increased transcription or stabilization of the mRNA. However, the further increase in TGF-β1 secretion that occurred when the two stimuli were applied in combination is likely due to regulation at a different level.

Effects of TGF-β Neutralization on Alterations in Collagen Metabolism Induced by Stretch and High Glucose Levels

To determine the role that TGF-β plays in the altered collagen metabolism induced by cyclic stretch, MC were grown in 8 mM glucose medium and were subjected to cyclic strain for 5 d in the presence or absence of a monoclonal antibody that neutralizes TGF-β1, -2, and -3. Fresh antibody was added on each day of stretching, and the medium was replaced with one containing [^{14}C]-proline 72 h before termination of the experiment. Stretching resulted in a small yet significant increase in total medium collagen synthesis (10%), but because collagen breakdown was also increased to a similar degree (18%), no significant change in net accumulation occurred (Figure 3).

In static cultures of cells maintained in 8 mM glucose, blockade of TGF-β activity significantly reduced (27%) baseline collagen synthesis. This treatment also reduced synthesis in the group enhanced by stretch, but the level still remained at values significantly higher than those observed in the antibody-treated static cultures (Figure 3). Stretch-enhanced breakdown of collagen was also diminished by TGF-β neutralization, but again it was not reduced to the level determined in the similarly treated, static cultures. Inhibition of TGF-β activity altered the synthetic/catabolic balance of collagen metabolism, resulting in a substantial decrease in the net amount accumulated (static, 31%; stretched, 21%) (Figure 3). Because the effects of TGF-β neutralization on synthesis and catabolism were approximately of the same magnitude in static and stretched cultures, net collagen accumulation remained similar in both groups. When medium collagen accumulation was determined by a second method based on collagenase sensitivity, the results were similar (static, 105.8 ± 4.0; static + anti-TGF-β, 74.5 ± 2.1, $P <$ 0.001; stretched, 101.4 ± 5.7; stretched + anti-TGF-β, 77.1 ±

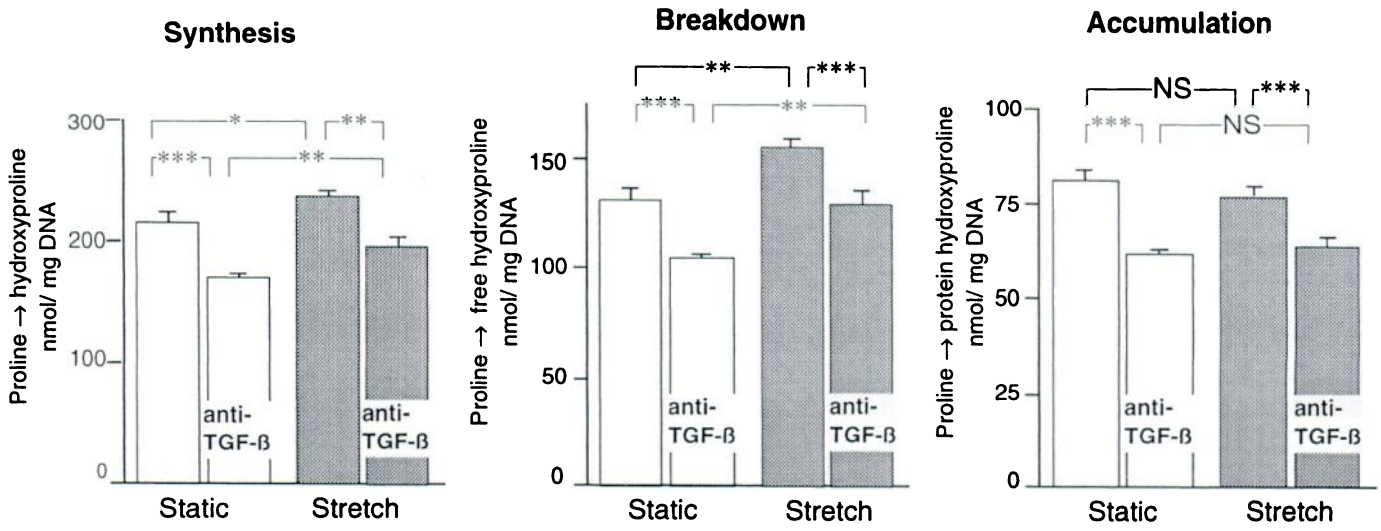

Figure 3. Effects of TGF-β blockade on stretch-altered collagen metabolism. Results are mean ± SEM (n = 6 in all groups). The experiment was repeated with similar results. *$P < 0.02$; **$P < 0.002$; ***$P < 0.0002$.

2.3 nmol proline/mg DNA, $P < 0.001$ [mean ± SEM, n = 6 for all groups]).

In addition to assaying collagen in the medium, we also examined its content within the cell layer. The amount of newly formed collagen associated with the cell layer was only 2 to 3% of the total accumulated (medium plus cell layer). Nevertheless, the effects of stretch and TGF-β neutralization on this collagen fraction mirrored changes in the medium (static, 3.96 ± 0.34; static + anti-TGF-β, 3.18 ± 0.19; stretch, 4.29 ± 0.27; stretch + anti-TGF-β antibody, 3.80 ± 0.36 nmol proline/mg DNA [mean ± SEM]). Differences did not always reach significance in the tissue.

To verify that the above results were not influenced by the incomplete neutralization of TGF-β activity, *i.e.*, insufficient antibody, two tests were carried out. First, we repeated the experiments recorded above using double (40 μg/ml per d), or one-half (10 μg/ml per d) the concentration of neutralizing antibody. Nearly identical results were produced with a two-fold concentration, whereas insignificant differences between treated and nontreated groups were obtained with the lower dose of antibody (data not shown). Second, at the end of experiments using 20 μg/ml per d of antibody, the conditioned medium was tested for biologically active TGF-β1. There was no detectable activity of TGF-β1 in the medium.

Finally, we examined the role of TGF-β in mediating collagen metabolism induced by stretching during exposure to high glucose concentrations. However, the effects of glucose concentration *per se* were first studied in static cultures. MC were continuously grown in 8 mM glucose or switched for the last 11 to 12 d of the experiment to a medium containing 35 mM glucose. As predicted, growth in high glucose medium significantly increased collagen synthesis, breakdown, and accumulation (Figure 4).

Next, we conducted separate experiments in which MC were maintained in 35 mM glucose media only and subjected to cyclic stretch or static conditions in the presence or absence of TGF-β neutralizing antibody. Under these conditions, stretching further stimulated collagen synthesis (31%) and breakdown

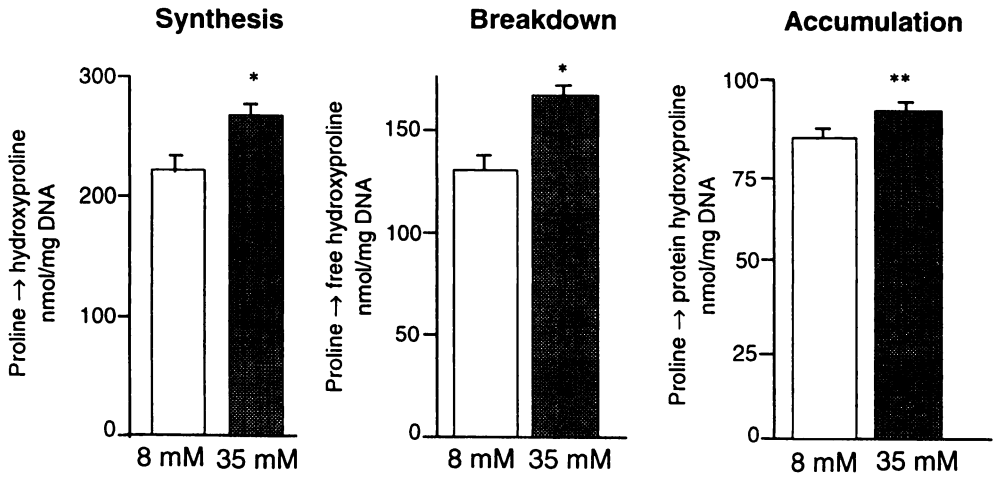

Figure 4. Glucose concentration and collagen metabolism. Results are mean ± SEM (n = 6 in all groups). *$P < 0.001$; **$P = 0.0007$.

(45%) (Figure 5). However, unlike cells grown in 8 mM glucose, the absolute change was greater for synthesis than breakdown, resulting in a marked increase in net collagen accumulation. Also, unlike the results recorded above with 8 mM glucose, TGF-β blockade reduced collagen synthesis to a level not significantly different from that of the antibody-treated, static cultures. In addition, collagen breakdown was also blunted in both stretched and static cultures by TGF-β neutralization (Figure 5). However, in this case, neutralization did not completely abrogate the differences between the groups. The overall result was total elimination of the stretch-induced collagen accumulation.

Quantification of total DNA at the end of the incubation period showed that neither stretch nor TGF-β neutralization altered the DNA content of cultures grown in low or high glucose concentrations (Table 1), suggesting a lack of effect on cell proliferation. However, in all circumstances, exposure to high glucose concentrations significantly reduced DNA (static, 8 mM: 96.3 ± 5.2, $n = 6$; static, 35 mM: 84.0 ± 1.7 μg of DNA, $n = 6$, $P = 0.014$), as observed above in experiments for TGF-β bioactivity in which cell number was determined.

In the experiments presented in Figures 3 and 5, metabolic changes induced by stretch or high glucose concentrations were only a fraction of the basal, unstimulated values. Because of this, evaluation of the role of TGF-β action on the stretch-induced alteration was obscured when results were expressed as a percentage difference between groups. When results were analyzed in terms of absolute changes due to TGF-β, it was evident that in low glucose concentrations the stretch-induced increases in collagen synthesis and breakdown were of equal magnitude (27 *versus* 25 nmol/mg DNA, respectively) and not produced by this cytokine (Figure 6A). TGF-β activity, however, was responsible for a baseline level of collagen turnover and accumulation in both static and stretched cultures. In high glucose concentrations, the majority of the stretch-induced increases in collagen synthesis and accumulation were attributable to enhanced TGF-β activity (Figure 6B). The portion of the increased synthesis and breakdown induced by stretch, but due to factors other than TGF-β, was unchanged by the concentration of glucose.

Discussion

This study demonstrates that mechanical strain and increased glucose concentrations each stimulate TGF-β1 production by MC. However, when mechanical strain occurs in an environment of high glucose concentration, a greater enhancement of TGF-β secretion results, indicating that the magnitude of the stimulation may depend on the coordinate action of several factors. TGF-β secretion was qualitatively, but not quantitatively, related to the level of the corresponding mRNA. In the case of stretch in high glucose, the level of TGF-β transcripts did not rise above that in nonstretched, high glucose control cultures, whereas the amount of TGF-β1 in the medium was substantially increased. This difference suggests that mechanisms other than augmented transcription or decreased degradation of mRNA may be required to produce the additional increment in secreted TGF-β1. In support of this idea, it has been shown in other cell types that increased mRNA levels are not required for increased production of TGF-β (28). Alteration in transcription and/or translation of the TGF-β gene has been shown to be important in modulating the production of this cytokine (29).

The present study confirms our previous observation that cyclic stretching of MC grown under normal glucose concentrations stimulates collagen synthesis, but does not alter its accumulation due to a parallel increase in breakdown (18). Under these conditions, the neutralization of TGF-β in nonstretched cells resulted in significant moderate decreases in collagen synthesis, breakdown, and accumulation. This demonstrates that in the absence of mechanical force, TGF-β plays a positive regulatory role in constitutive collagen turnover. In the presence of cyclic strain, neutralization of TGF-β action

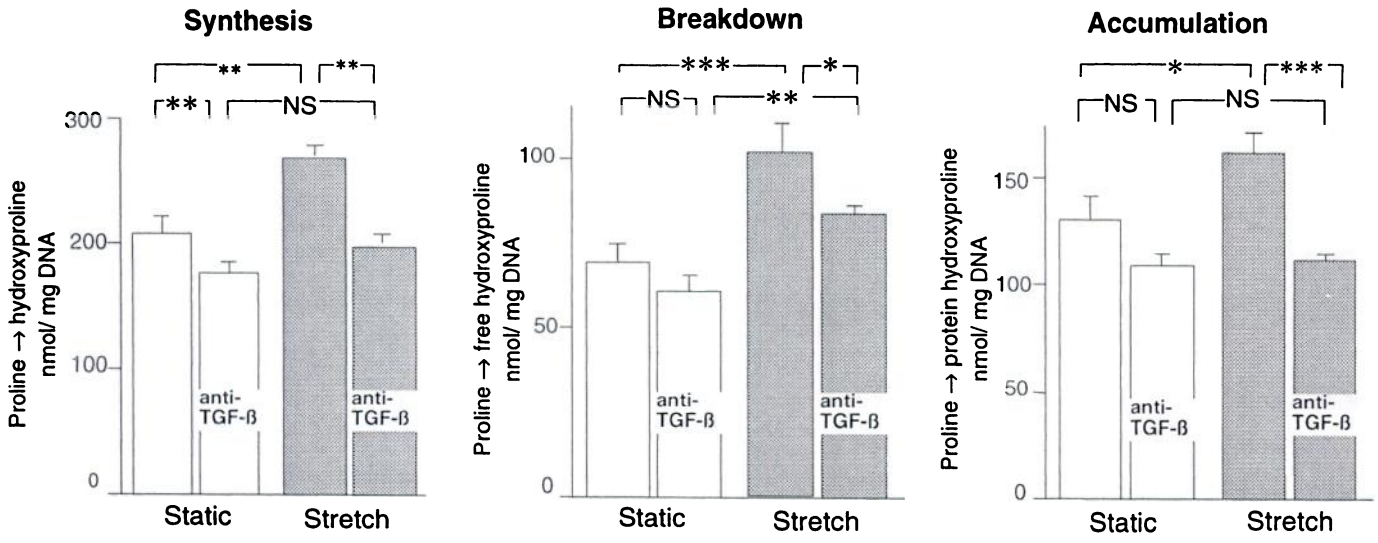

Figure 5. Effects of TGF-β neutralization on stretch-altered collagen metabolism in high concentrations of glucose. Results are mean ± SEM ($n = 6$ in all groups). *$P < 0.02$; **$P < 0.002$; ***$P < 0.0002$.

Table 1. Effects of stretch and transforming growth factor-β (TGF-β) neutralization on DNA content in cultures of mesangial cells[a]

Group	Static	Stretched	Static Anti-TGF-β Antibody	Stretched Anti-TGF-β Antibody
8 mM glucose	100.8 ± 4.0	104.3 ± 1.8	99.3 ± 1.7	96.6 ± 3.5
35 mM glucose	59.9 ± 3.8	55.4 ± 2.9	53.3 ± 2.5	52.2 ± 2.5

[a] Results from two separate experiments carried out at different medium glucose concentrations are presented. Cultures were cyclically stretched and/or treated with neutralizing antibody for 5 d. Fifty thousand cells were plated per culture well. The contents of three wells were pooled per sample. DNA is total in sample at the termination of the experiments, 7 d after plating. Values are mean ± SEM (n = 6 in all groups). Differences between groups within the same experiment were not significant.

also reduced synthesis, catabolism, and accumulation. However, this decrease in collagen metabolic activity was not significantly different from that in static cultures. These data suggest that TGF-β plays little or no role in the stretch-induced alteration of collagen metabolism under normal glucose conditions.

Previous studies have shown that incubation of static MC cultures in high concentrations of glucose increased the synthesis and accumulation of collagen (4–5) through mechanisms other than increased extracellular osmolality (6,30,31). We have also demonstrated this effect of high glucose concentration. In addition, we have observed that the net accumulation of collagen was limited because catabolism was stimulated along with synthesis. When MC were exposed to high glucose, cyclic strain further increased collagen accumulation, the result of a greater stimulation of synthesis than of breakdown. Under these conditions, TGF-β neutralization was highly effective in blocking the accelerated turnover, but its effect on diminishing collagen accumulation was most apparent. Thus, our results demonstrate that enhanced TGF-β activity participates in the metabolic response of MC to stretch only when the prevailing glucose concentrations are elevated. Interestingly, the fraction of the stretch-induced change in metabolism attributable to factors other than TGF-β was not different from that observed in normal glucose. This suggests that multiple pathways, operating in concert, may be important in the translation of a mechanical force into a metabolic event. Because this study did not assay changes in specific collagen types or their proteases and protease inhibitors, the individual components of the metabolic alterations observed remain undetermined. Also undefined was the TGF-β isoform(s) responsible for the observed effect on collagen metabolism.

In apparent contrast to our findings, Hirakata *et al.* (32) reported that in rat MC grown in low (5.55 mM) glucose, the increases in mRNA levels for collagen I, collagen IV, and fibronectin in response to cyclic strain are blocked by anti-TGF-β antibody. Yasuda *et al.* (19), on the other hand, found no significant effect of TGF-β neutralization on the α1 (I) collagen mRNA levels enhanced by stretch. Neither of these two studies examined collagen turnover or the influence of high glucose concentrations on the stretch response. The reason for this ostensible disparity between results of TGF-β neutralization on collagen mRNA levels and collagen metabolic activity may be best explained by a variable relationship between the levels of specific mRNA and the production of the translated protein. This poor correlation is expected to be most evident in the case of proteins, such as collagen, that are subject to extensive posttranslational modification (33).

The relationship between TGF-β concentration in the medium and TGF-β-induced alteration in collagen accumulation may not be a direct one. A specific minimal level of the secreted cytokine may be required for these changes to be effected. More likely, it may require increased TGF-β biological activity through more effective binding. The system we used to apply mechanical force provides a progressive increase in the amplitude of elongation from the center of the circular membrane to the periphery. As a result, cells in the outermost zone receive maximal elongation (19%), whereas those in the innermost zone receive little or no stretch (<5%). Our previous studies on the zonality of cellular changes across the culture well demonstrated that increased deposition of collagen occurred only in zones with significant elongation, but not in areas where cells experienced little or no force (8). Therefore, stimulated collagen deposition is not simply a direct consequence of increased medium TGF-β, which should uniformly affect all cells in the culture well. We have also demonstrated that active TGF-β colocalizes with collagen types I and IV in the areas of maximal strain, further suggesting that TGF-β binding to stimulated cells may be important in the modulation of its actions (17). It has been reported that adrenocorticotropin (34) and angiotensin-converting enzyme inhibition (35) regulate TGF-β receptor expression in adrenocortical cells and LLC-PK_1 porcine kidney cells, respectively, indicating that modulation of this receptor may be a point of control for the response to increased TGF-β.

Other reports have examined the role of TGF-β in MC production of various proteases or tissue inhibitors of metalloproteases. However, our study appears to be the first to investigate the cytokine's role in the actual breakdown of collagen protein. We discovered that although the intensity of the metabolic effect varied with the conditions, TGF-β was invariably a positive regulator of matrix breakdown. The protease(s) responsible for this degradation was not identified. However, the major members of the protease family that are secreted by MC are the 72-kD type IV

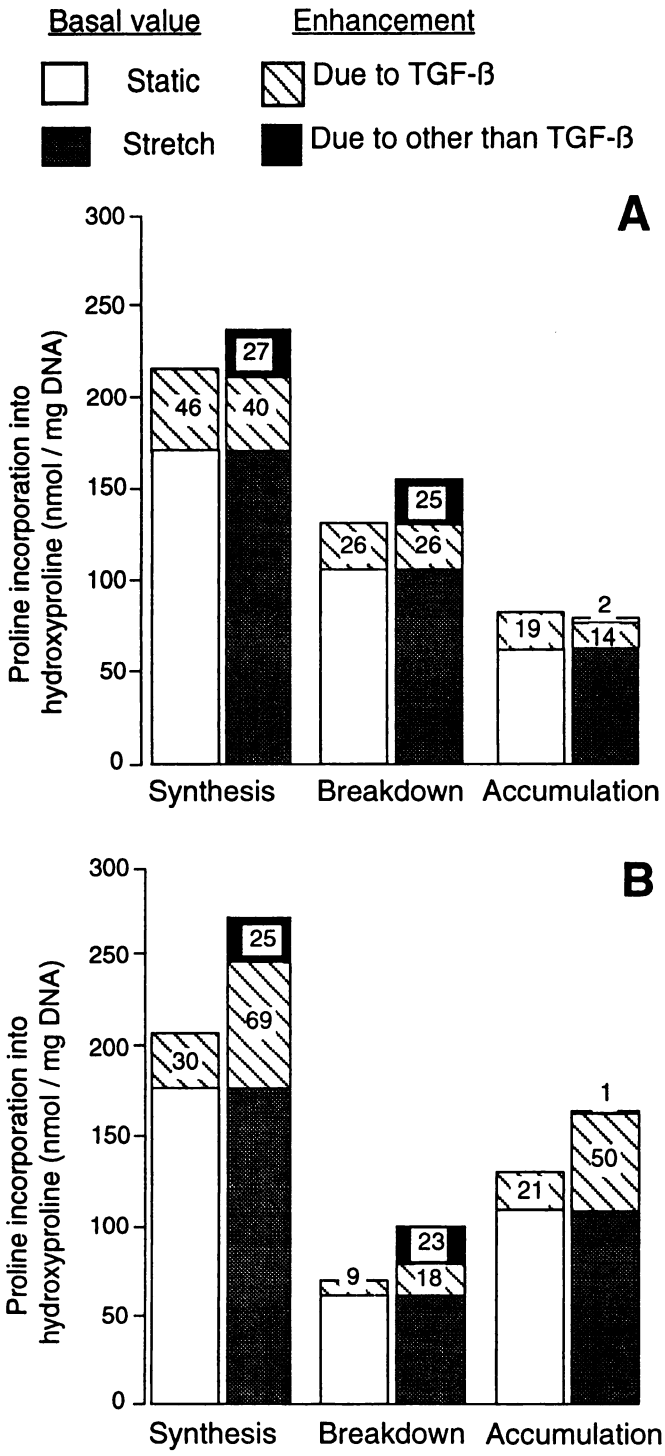

Figure 6. The contribution of TGF-β to stretch-altered collagen synthesis, catabolism, and accumulation under conditions of both high and low glucose concentrations. This figure summarizes the data presented in Figures 3 and 5, and expresses them as the absolute change due to TGF-β. (A) MC grown in 8 mM glucose medium. (B) MC grown in 35 mM glucose medium. Values for total collagen synthesis do not exactly equal the amount catabolized plus accumulated because the small amounts (1 to 2 nmol) of collagen deposited and catabolized in the cell layer were not indicated.

collagenase (MMP-2), stromelysin (MMP-3), and matrilysin (MMP-8) (20). The finding of Marti *et al.* that TGF-β1 stimulates MC synthesis of MMP-2 while decreasing the production of stromelysin and matrilysin (20) suggests that the 72-kD type IV collagenase may be critical in the catabolic activity observed in our studies. In support of this, it has been reported that cyclic strain of cultured MC initially decreases by 6 h, then markedly increases by 24 h the levels of transcript for MMP-2 (19).

In the present study, direct cell counting and DNA determination suggest that the long-term exposure of MC to high glucose concentrations decreases cellular proliferation. This finding is in agreement with the reports of others (36,37). However, the neutralization of TGF-β failed to alter MC number in either high or low glucose. This is in contrast to the finding of Wolf *et al.* (37) that TGF-β neutralization at least partially reversed the high glucose-induced inhibition of MC proliferation. This lack of agreement may be the result of differences in high glucose exposure time (14 d *versus* 3 d). In the present study, cyclic stretching also did not appear to alter cell proliferation. Harris *et al.* (10), using collagen type I-coated plates, observed increased MC proliferation in response to stretch. This inconsistency may not be surprising, because it has been shown that smooth muscle cells seeded on collagen type I-coated plates proliferate in response to stretch, whereas those seeded on elastin-coated plates do not (38).

The observations made in this study using mechanical strain in tissue culture are relevant to pathophysiologic forces that occur *in vivo*. Our studies on the elasticity of isolated perfused glomeruli have shown that the degree of stretching used in the present study may be similar to that which occurs in the glomerular mesangium in experimental diabetes (8,18). Effective autoregulation protects normal glomeruli from exposure to significant variations in systemic BP and the distension it engenders (39,40). However, in experimental diabetes (41) and in remnant glomeruli after extensive renal ablation (42), there is loss of autoregulation at the level of the afferent arteriole, thereby allowing the transmission to the glomerular capillaries of the large (up to 55 mmHg) moment-to-moment variations in systemic BP (24). On the basis of direct measurements of glomerular compliance, we have estimated that these oscillations in intraglomerular pressure may result in repeated distention and contraction of up to 7.3% of mean glomerular volume, compared with 0.4% under normal circumstances (18). This approximates the amplitude (8% average) of stretch used in this study.

Our results support the hypothesis that glomerular hypertension and the wide oscillations in intraglomerular pressure induced by diabetes mellitus may result in repetitive MC mechanical strain and increased collagen turnover. Although this MC strain is associated with increased TGF-β production, TGF-β-mediated collagen deposition becomes significant only in the presence of high glucose concentrations. Therefore, the adverse effects of TGF-β action on the course of diabetic glomerular disease are likely to be best mitigated by the combined correction of altered glomerular hemodynamics and hyperglycemia.

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References

1. Anderson S, Meyer TW, Rennke HG, Brenner BM: Control of glomerular hypertension limits glomerular injury in rats with reduced renal mass. *J Clin Invest* 76: 612–619, 1985
2. Zatz R, Dunn BR, Meyer TW, Anderson S, Rennke HG, Brenner BM: Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 77: 1925–1930, 1986
3. Tuttle KR, Bruton JL, Perusek MC, Lancaster JL, Kopp DT, DeFronzo RA: Effect of strict glycemic control on renal hemodynamic response to amino acids and renal enlargement in insulin-dependent diabetes mellitus. *N Engl J Med* 324: 1626–1632, 1991
4. Ayo SH, Radnik RA, Glass WF II, Garoni J, Rampt ER, Appling DA, Kreisberg JI: Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high glucose medium. *Am J Physiol* 260: F185–F191, 1991
5. Ayo SH, Radnik RA, Garoni JA, Glass WF II, Kreisberg JI: High glucose causes an increase in extracellular matrix proteins in cultured mesangial cells. *Am J Pathol* 136: 1339–1348, 1990
6. Heneda M, Kikakwa R, Horide N, Togawa M, Koya D, Kajiwara N, Ooshima A, Shigeta Y: Glucose enhances type IV collagen production in cultured rat glomerular mesangial cells. *Diabetologia* 34: 198–200, 1991
7. Davies M, Martin J, Tomas GJ, Lovett DH: Proteinases and glomerular matrix turnover. *Kidney Int* 41: 671–678, 1992
8. Riser BL, Cortes P, Zhao X, Bernstein J, Dumler F, Narins RG: Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J Clin Invest* 90: 1932–1943, 1992
9. Kriz W, Elger M, Lemley K, Sakai T: Structure of the glomerular mesangium: A biomechanical interpretation. *Kidney Int* 38[Suppl 30]: S2–S9, 1990
10. Harris R, Haralson M, Badr K: Continuous stretch-relaxation in culture alters rat mesangial cell morphology, growth characteristics, and metabolic activity. *Lab Invest* 66: 548–554, 1992
11. Ziyadeh FN, Sharma K, Ericksen M, Wolf G: Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-β. *J Clin Invest* 93: 536–542, 1994
12. Sharma K, Ziyadeh FN: Renal hypertrophy is associated with upregulation of TGF-β1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol* 267: F1094–F1101, 1994
13. Shankland SJ, Scholey JW: Expression of transforming growth factor-β1 during diabetic renal hypertrophy. *Kidney Int* 46: 430–442, 1994
14. Yamamto T, Nakamura T, Noble N, Ruoslahti E, Border WA: Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci USA* 90: 1814–1818, 1993
15. Sharma K, Jin Y, Guo J, Ziyadeh FN: Neutralization by TGF-β antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45: 522–530, 1996
16. Bollineni JA, Reddi AS: Transforming growth factor-β1 enhances glomerular collagen synthesis in diabetic rats. *Diabetes* 42: 1673–1677, 1993
17. Riser BL, Cortes P, Heilig C, Grondin J, Ladson-Wofford S, Patterson D, Narins RG: Cyclic stretching force selectively upregulates transforming growth factor-β isoforms in cultured rat mesangial cells. *Am J Pathol* 148: 1915–1923, 1996
18. Cortes P, Zhao X, Riser BL, Narins RG: The role of mechanical strain in the pathogenesis of diabetic nephropathy. *Kidney Int* 51: 57–68, 1997
19. Yasuda T, Kondo S, Homma T, Harris RC: Regulation of extracellular matrix by mechanical stress in rat glomerular cells. *J Clin Invest* 98: 1991–2000, 1996
20. Marti HP, Lee L, Kashgarian M, Lovett DH: Transforming growth factor-β1 stimulates glomerular mesangial cell synthesis of the 72-kD type IV collagenase. *Am J Pathol* 144: 82–94, 1994
21. Leehey DJ, Song RH, Alavi N, Singh AK: Decreased degradative enzymes in mesangial cells cultured in high glucose media. *Diabetes* 44: 929–935, 1995
22. Dumler F, Cortes P: Uracil ribonucleotide metabolism in rat and human glomerular epithelial and mesangial cells. *Am J Physiol* 255: C712–C718, 1988
23. Varani J, Taylor CG, Riser BL, Schumaker DK, Yeh K-Y, Dame M, Gibbs DF, Todd RF III, Dumler F, Bromberg J, Killen PD: Mesangial cell killing by leukocytes: Role of leukocyte oxidants and proteolytic enzymes. *Kidney Int* 42: 1169–1177, 1992
24. Bidani AK, Griffin KA, Picken M, Lansky DM: Continuous telemetric blood pressure monitoring and glomerular injury in the rat remnant kidney model. *Am J Physiol* 265: F391–F398, 1993
25. Banes AJ, Link GW, Gilbert JW, Tay RTS, Monbureau O: Culturing cells in a mechanically active environment. *Am Biotechnol Lab* 8: 12–22, 1990
26. Sambrook J, Fritsh E, Maniatis T: Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: *Molecular Cloning Laboratory Manual,* 2nd Ed., Cold Spring Harbor, NY, Cold Spring Harbor Press, 1989, pp 7.37–7.42
27. Danielpour D, Dart LL, Flanders KC, Roberts AB, Sporn MB: Immunodetection and quantitation of two forms of transforming growth factor-beta (TGF-β1 and TGF-β2) secreted by cells in culture. *J Cell Physiol* 138: 79–86, 1989
28. Wakefield L, Kim SJ, Click A, Winokur T, Colletta A, Sporn MB: Regulation of transforming growth factor-β subtypes by members of the steroid hormone superfamily. *J Cell Sci (Suppl)* 13: 139–148, 1990
29. Roberts AB, Kim S, Kondaiah P, Jakowlew SB, Denhez F, Glick AB, Geiser AG, Watanabe S, Noma T, Lechleider R, Sporn MB: Transcriptional control of expression of the TGF-βs. In: *Transforming Growth Factor-βs: Chemistry, Biology, and Therapeutics*, edited by Piez KA, Sporn MB, New York, Annals New York Acadamy of Sciences, 1990, pp 43–50
30. Pugliese G, Pricci F, Locuratolo N, Romano G, Gianni S, Cresci B, Galli G, Rotella CM, DiMario U: Increased activity of the insulin-like growth factor system in mesangial cells cultured in high glucose conditions: Relation to glucose-enhanced extracellular matrix production. *Diabetologica* 39: 775–784, 1996
31. Wakisaka M, Spiro MJ, Spiro RG: Synthesis of type VI collagen by cultured glomerular cells and comparison of its regulation by glucose and other factors with that of type IV collagen. *Diabetes* 43: 95–103, 1994
32. Hirakata M, Kaname S, Chung U, Joki N, Hori Y, Noda M, Takuwa Y, Okazaki T, Fujita T, Katoh T, Kurokawa K: Tyrosine

kinase-dependent expression of TGF-β induced by stretch in mesangial cells. *Kidney Int* 51: 1028–1036, 1997

33. Hostikka SL, Tryggvason K: The complete primary sequence structure of the α2 chain of human type IV collagen and comparison with the α1 (IV) chain. *J Biol Chem* 263: 19488–19493, 1988
34. Cochet C, Feige J-J, Chambaz EM: Bovine adrenocortical cells exhibit high affinity transforming growth factor-β receptors which are regulated by adrenocorticotropin. *J Biol Chem* 263: 5707–5713, 1988
35. Guh J-Y, Yang M-L, Yang Y-L, Chang C-C, Chuang L-Y: Captopril reverses high-glucose-induced growth effects on LLC-PK_1 cells partly by decreasing transforming growth factor-β receptor protein expressions. *J Am Soc Nephrol* 7: 1207–1215, 1996
36. Nahman NS, Leonhart KL, Cosio RG, Hebert CL: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int* 41: 396–402, 1992
37. Wolf G, Sharma K, Chen Y, Ericksen M, Ziyadah FN: High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. *Kidney Int* 42: 647–656, 1992
38. Wilson EK, Sudhir H, Ives E: Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J Clin Invest* 96: 2364–2372, 1995
39. Casellas D, Moore LC: Autoregulation of intravascular pressure in preglomerular juxtamedullary vessels. *Am J Physiol* 264: F315–F324, 1993
40. Brenner BM, Griffin KA, Picken M, Daugharty DM: The dynamics of glomerular ultrafiltration in the rat. *J Clin Invest* 50: 1776–1780, 1971
41. Hayashi K, Epstein M, Loutzenhiser R, Forster H: Impaired myogenic responsiveness of the afferent arteriole in streptozotocin-induced diabetic rats: Role of eicosanoid derangements. *J Am Soc Nephrol* 2: 1578–1586, 1992
42. Pelayo J, Wescott JY: Impaired regulation of glomerular capillary hydrostatic pressure in the rat remnant nephron. *J Clin Invest* 88: 101–105, 1991