Role for Transforming Growth Factor β in Thromboxane-Induced Increases in Mesangial Cell Fibronectin Synthesis

Hilmer Negrete, Rebecca K. Studer, Patricia A. Craven, and Frederick R. DeRubertis

Thromboxane A_2 (TXA_2) has been implicated in the pathogenesis of progressive glomerulosclerosis and stimulates the synthesis of matrix protein by mesangial cells (MCs). This study examined the role of transforming growth factor β (TGF-β) in the mediation of the action of the stable TXA_2/prostaglandin (PG) endoperoxide analog U-46619 to stimulate fibronectin (Fn) synthesis in cultured rat MC. Exogenous TGF-β increased Fn synthesis by MC in a concentration- and time-dependent fashion, as reflected by incorporation of [^{35}S]methionine into immunoprecipitable Fn. Submaximal concentrations of TGF-β (1–2.5 pmol/l) increased Fn synthesis two- to threefold, a response comparable in magnitude to that observed with a maximal stimulatory concentration (1 μmol/l) of U-46619. Anti-TGF-β antibody, but not isotypic IgG, blocked the increases in Fn synthesis induced by both U-46619 and exogenous TGF-β. Endogenous TGF-β bioactivity in MC culture media, assessed by the mink lung epithelial cell system, was significantly increased by 1 μmol/l U-46619 (1.7 ± 0.3 pmol/l) compared with that of control media (0.6 ± 0.1 pmol/l, $P < 0.05$). Total (active plus latent) TGF-β bioactivity, assayed after heat activation of latent TGF-β, was also significantly higher in media of MCs cultured with U-46619 (45 ± 4 pmol/l) compared with control (24 ± 4 pmol/l). Thus, U-46619 increased endogenous TGF-β bioactivity to a level sufficient to account for the enhancement of Fn synthesis observed with U-46619, as reflected by the Fn synthetic response to exogenous TGF-β. This finding, together with the inhibitory effect of anti-TGF-β antibody on Fn synthesis, implicates TGF-β in expression of this action of U-46619. *Diabetes* 44:335–339, 1995

There is now considerable evidence to support an important role for transforming growth factor β (TGF-β) in both the regulation of extracellular matrix accumulation (1–3) and the pathogenesis of glomerulosclerosis (4–8), including that observed in diabetes (9,10). Recently, Isaka et al. (8) used an in vivo renal transfection technique (injection of liposome-bound TGF-β1 cDNA into the rat renal artery), which resulted in overexpression of TGF-β1 in glomeruli and induced marked extracellular matrix accumulation. These and other findings support a direct role for this cytokine in the pathogenesis of glomerulosclerosis. Expression of TGF-β is elevated in human and experimental diabetic nephropathy (9). The mechanisms that lead to increased glomerular TGF-β in diabetes are not known. However, recent studies in cultured rat mesangial cells (MCs) have implicated hyperglycemia per se as one potential signal to enhance TGF-β production by these glomerular cells (11). Cultured MCs exposed to high ambient concentrations of glucose demonstrate increased synthesis of a number of extracellular matrix proteins, including type IV collagen, laminin, and fibronectin (Fn) (12–14). A role for TGF-β in the mediation of the action of glucose on matrix protein synthesis is suggested by *1*) an increase in mRNA levels for TGF-β observed in MCs exposed to high concentrations of glucose (11) and *2*) inhibition by anti-TGF-β antibodies of the effects of glucose to enhance matrix protein synthesis in MC (15,16).

Studies in our own and other laboratories have implicated the vasoconstrictor and platelet aggregator thromboxane A_2 (TXA_2) as a mediator of glomerular injury in experimental diabetes (17–21). Similar to TGF-β, TXA_2 has been linked to the pathogenesis of several forms of human and experimental nephropathy (21). In the streptozotocin (STZ)-induced diabetic rat, the development of albuminuria and mesangial expansion is associated with increased glomerular production and urinary excretion of TXB_2, the stable metabolic product of TXA_2 (17,18). Inhibitors of TXA_2 synthesis attenuate both the development of proteinuria and the glomerular matrix expansion that occur in this diabetic model (17,18). Moreover, similar to the effects of high concentrations of glucose, stable analogs of TXA_2 enhance the synthesis of matrix proteins by cultured MCs (22–24). In this study, we examined the role of TGF-β in the expression of the action of the TXA_2/prostaglandin (PG) endoperoxide analog U-46619 to stimulate the synthesis of matrix protein in MCs.

RESEARCH DESIGN AND METHODS

Cell culture. MC lines were established from glomeruli isolated from the renal cortex of 120- to 160-g female Sprague-Dawley rats using a standard sieving technique, as previously described (25). MCs were maintained in RPMI-1640 medium containing 15% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 10 mmol/l glucose at 37°C in an atmosphere of 5% CO_2 and 95% air. The medium was changed every 2–3 days. MCs were grown to confluence in 12- or 24-well plates, and the serum concentration decreased to 1% before the addition of different agents. Since we have previously found that TXA_2 receptor density in

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Received for publication 25 August 1994 and accepted in revised form 1 December 1994.

FCS, fetal calf serum; Fn, fibronectin; MC, mesangial cell; MEM, minimal essential medium; PG, prostaglandin; PBS, phosphate-buffered saline; STZ, streptozotocin; TGF-β, transforming growth factor β; TCA, trichloroacetic acid; TXA_2, thromboxane A_2.

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cultured MCs is stable up to seven passages (26), studies were conducted in MCs from passages three through seven.

Fn synthesis. Confluent MCs were made quiescent by incubation in RPMI-1640 medium containing 1% FCS. Test agents were then added as described in the figure legends. After incubation for the times noted, MCs were washed with phosphate-buffered saline (PBS). Methionine-cysteine–deficient RPMI-1640 containing 25 μCi/ml [^{35}S]methionine (*trans*-^{35}S label, ICN, Irvine, CA) was added to the wells, and incubation was continued for 4 h at 37°C. [^{35}S]methionine-labeled Fn was extracted from the cells plus matrix, immunoprecipitated with rabbit anti-Fn antibodies (Collaborative Research, Bedford, MA), and assayed as previously described (13,25). Immunoprecipitated Fn is expressed as dpm/10^3 cells or μg protein. Protein was determined by a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO).

Bioassay for TGF-β. Inhibition of [^{3}H]thymidine incorporation into trichloroacetic acid (TCA)-precipitable DNA of mink lung epithelial cells (CCL, American Type Culture Collection, Rockville, MD) was used as a bioassay for TGF-β. This assay system has been described in detail (27,28) and previously used by our laboratory to determine TGF-β bioactivity in MC culture media (29). CCL cells were maintained in minimal essential medium (MEM) (Sigma) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37°C in 5% CO_2 and 95% air and passaged at a density of 2×10^5 cells/25 cm^2 T-flask at 3- to 4-day intervals. For bioassay, cells were seeded at 5×10^6 cells in 48-well plates. After 6–8 h, cells were washed once with PBS, and the medium was changed to MEM containing 0.5% FCS. After an additional incubation of 16 h, medium obtained from MCs grown with or without U-46619 or other test agents was added, and cells were incubated for another 24 h at 37°C. Cells were pulsed with [^{3}H]thymidine, 0.2 μCi/ml, for the final 3 h of the incubation. The wells were then washed with PBS, followed by two additional washings with 6% TCA and one with 100% ethanol. Cells were extracted with 5% sodium dodecyl sulfate and transferred to scintillation vials for determination of [^{3}H]thymidine incorporation into TCA-precipitable material. Conditioned medium was obtained from MCs grown in 12-well plates for 24 h in the presence of 1 μmol/l U-46619 or its ethanol vehicle. The medium was centrifuged to remove cell debris, and 1 mg/ml BSA and 1 μg/ml aprotinin, leupeptin, and pepstatin A were added. Active and total (active plus latent) TGF-β were assayed. Latent TGF-β was activated by heating medium to 80°C for 15 min. TGF-β activities were determined by comparison with a standard curve generated in response to 0.1–10 pmol/l exogenous human TGF-β1. Under these conditions, the percentage of inhibition of [^{3}H]thymidine incorporation ranged from 93% (0.1 pmol/l) to 3% (10 pmol/l), with a K_d of 0.7 pmol/l TGF-β. Media samples were serially diluted to fall within the range of the standard curve. Specificity of the inhibition of [^{3}H]thymidine incorporation mediated by media from MC cultures was confirmed by the complete prevention of this inhibition by rabbit anti-TGF-β antibody but not by normal rabbit IgG. RPMI-1640 media containing BSA, aprotinin, leupeptin, pepstatin A, and U-46619 that had not been exposed to MCs had no effect on [^{3}H]thymidine incorporation by the CCL-64 cells compared with those grown in MEM alone.

Statistical analysis. Experiments were performed at least three times. In a given culture, each incubation condition was represented by either duplicate or triplicate wells. Replicates of the same incubation condition from a single experiment (culture) were averaged and entered as one value for purposes of statistical analysis. Data are presented as means ± SE. Analysis of variance was used for multiple comparisons, and statistical significance between mean values was determined by Student's *t* test.

Materials. Human platelet TGF-β1, rabbit panspecific TGF-β neutralizing antibody, and isotypic rabbit IgG were obtained from R & D Systems (Minneapolis, MN). Other chemicals were obtained from sources noted above or previously reported (24–26).

RESULTS

As shown in Fig. 1, exposure of MCs for 24 h to TGF-β1 (0.5–100 pmol/l) stimulated Fn synthesis in a dose-dependent fashion. The lowest concentration of exogenous TGF-β to increase Fn synthesis significantly over control values was 1.0 pmol/l (control, 14 ± 1 dpm/10^3 cells; 1 pmol/l TGF-β, 28 ± 4 dpm/10^3 cells; $n = 3$, $P < 0.05$). Maximal increases in Fn synthesis over control were observed with 50 pmol/l TGF-β. Addition of 100 pmol/l (Fig. 1) or 500 or 1,000 pmol/l (not shown) did not result in a greater response than that observed with 50 pmol/l. Increases in Fn synthesis in response to TGF-β were evident whether data were expressed on the basis of MC number or culture protein content (Fig. 1). As also shown in Fig. 1, anti-TGF-β antibody (30 μg/ml) abolished the increases in Fn synthesis induced by 10 or 50 pmol/l exogenous TGF-β. Anti-TGF-β antibody did not alter basal Fn synthesis, and isotypic rabbit IgG had no effect on either basal Fn synthesis or the response to exogenous TGF-β (not shown). TGF-β1-induced changes in Fn synthesis as a function of time are shown in Fig. 2. A twofold stimulation of Fn synthesis over control was detectable after 4 h of exposure of MCs to TGF-β1. After 24 or 48 h exposure of MCs to TGF-β, Fn synthesis was three- to fivefold higher than control.

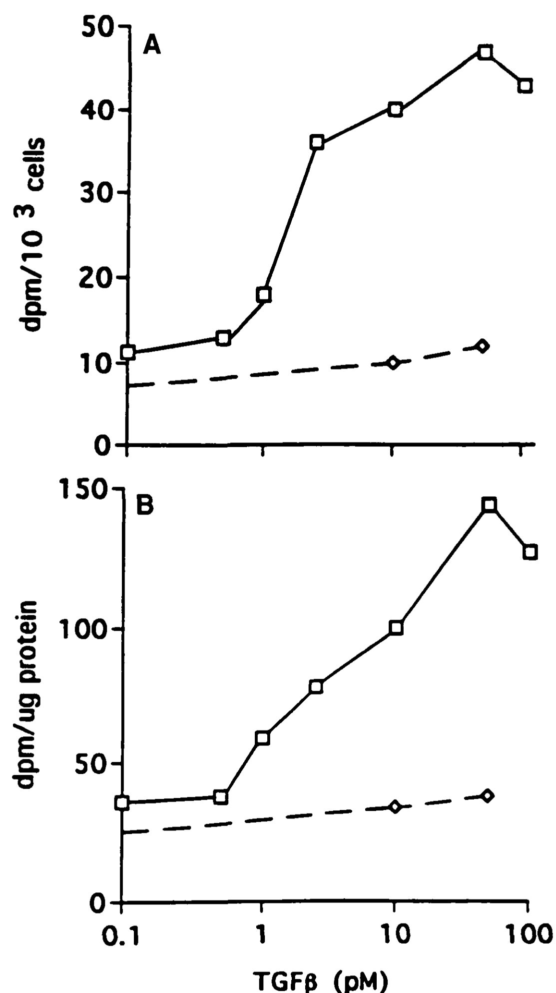

FIG. 1. Concentration response of TGF-β on Fn synthesis in cultured MCs. MCs were grown to confluence and growth-arrested with 1% FCS. After 24 h of quiescence, human TGF-β1 (0.5–100 pmol/l) was added. Fn synthesis was determined on cells plus matrix after 24 h of exposure to TGF-β1. Stimulation of Fn was comparable whether expressed as a function of cell number (*A*) or total protein content (*B*). The results of a representative experiment repeated two times are shown. □, TGF-β1 alone; ◇, TGF-β1 + anti-TGF-β antibody (30 μg/ml).

Fig. 3 shows the effects of TGF-β antibody on U-46619–induced Fn synthesis by MCs. As previously reported (24), U-46619 significantly stimulated Fn synthesis in cultured MCs. The concentration of U-46619 used in these studies (1 μmol/l) gave maximal increases in Fn synthesis after a 24-h culture of MCs (24). Prior addition of anti-TGF-β antibody abolished this U-46619 response. The effect of the anti-TGF-β antibody was specific, since isotypic rabbit IgG failed to

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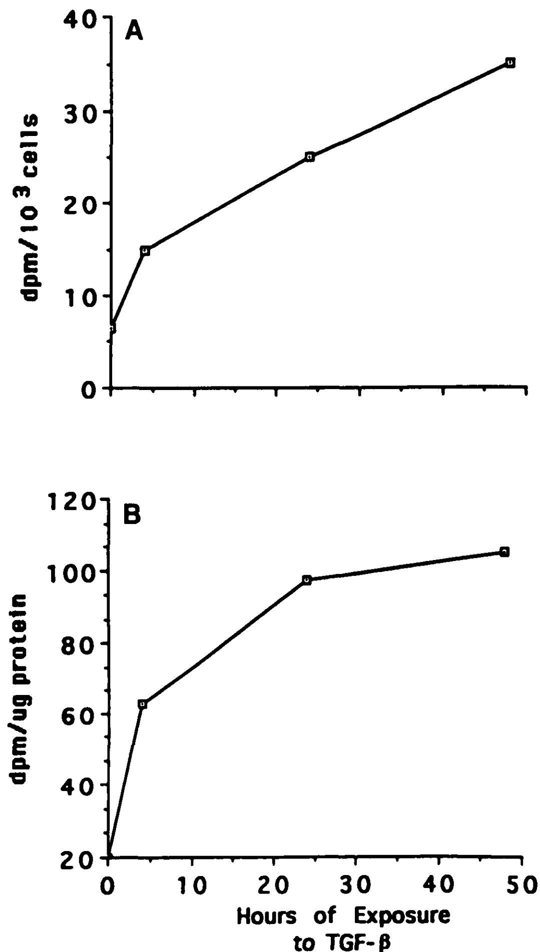

FIG. 2. Time course of effects of TGF-β on Fn synthesis in cultured MCs. MCs were growth-arrested and then cultured for a total of 48 h in media containing 1% FCS. TGF-β (100 pmol/l) was added to some cultures at times 0, 24, and 44 h, and the experiment was terminated at 48 hrs. Fn synthesis is expressed as a function of cell number (*A*) or total protein content (*B*). The results of a representative experiment repeated two times are shown.

inhibit the increased Fn synthesis induced by U-46619 (Fig. 3). Anti-TGF-β antibody and control IgG failed to alter significantly basal Fn synthesis in the absence of U-46619.

To assess whether U-46619 increased active TGF-β, latent

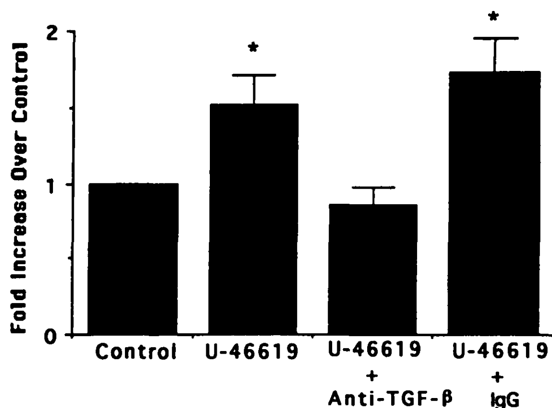

FIG. 3. Effect of TGF-β antibody on Fn synthesis induced by U-46619 in MCs. MCs were treated initially as described in the legend to Fig. 1 and then incubated for 24 h with 1 μmol/l U-46619, with or without 30 μg/ml neutralizing anti-TGF-β antibody or 30 μg/ml isotypic rabbit IgG. Values are expressed as the ratio of the incorporation of [^{35}S]methionine into Fn in cells exposed to test agents over that of control cells. Values shown are means ± SE from five experiments. *$P < 0.05$ vs. control.

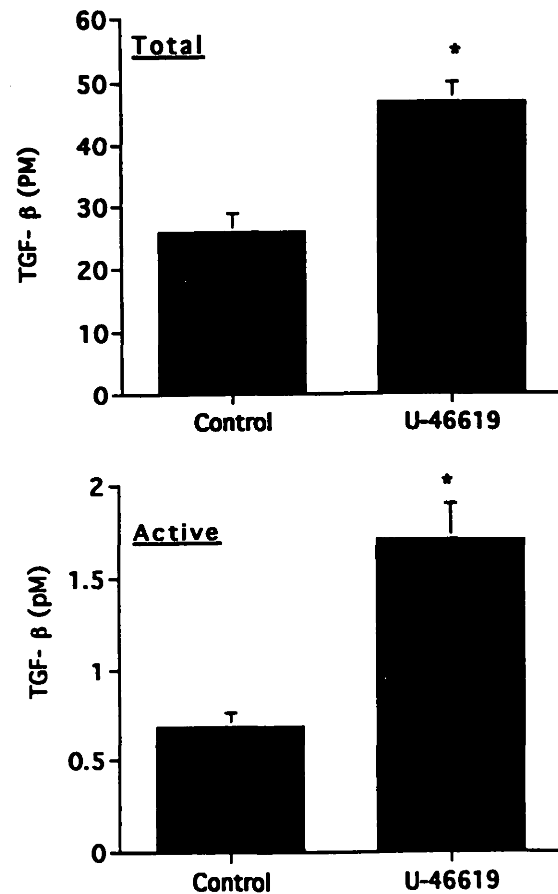

FIG. 4. Effects of U-46619 on TGF-β bioactivity in MC culture media. MCs were initially treated as described in the legend to Fig. 1 and then incubated for an additional 24 h in the presence or absence of 1 μmol/l U-46619. TGF-β bioactivity of media from these cultures was then assayed with the mink lung epithelial cell system as described in the text. Values represent means ± SE from five experiments; *$P < 0.05$ vs. control.

TGF-β, or both, culture medium from MCs incubated with or without U-46619 was tested for TGF-β bioactivities with the mink lung epithelial cell assay before and after heat activation of the latent form. The concentrations of active and total TGF-β bioactivities in media samples from control and U-46619–stimulated MCs are shown in Fig. 4. U-46619 significantly increased the media concentrations of both active and total TGF-β when compared with corresponding control values. U-46619 induced a somewhat greater increase in active TGF-β (approximately threefold over control), compared with the total TGF-β (approximately twofold). Approximately 3% of TGF-β in the control MC media and 5% of TGF-β in the media of MC exposed to U-46619 was in the active form. Both the control and U-46619–responsive TGF-β bioactivities shown in Fig. 4 were abolished by 30 μg/ml anti-TGF-β antibody but not by control IgG, supporting the specificity of the assay system for TGF-β. The TXA_2/PG endoperoxide receptor antagonist SQ-29548 (50 μmol/l) did not alter basal levels of active or latent TGF-β bioactivity in culture media from control MC cultures. However, SQ-29548 completely blocked the increases in these TGF-β bioactivities induced by 1 μmol/l U-46619 (data not shown).

DISCUSSION

Previous studies have implicated TXA_2 in the pathogenesis of glomerulosclerosis in experimental (17–21) and human ne-

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phropathies (21), including those associated with diabetes (17–21). Analogs of TXA_2/PG endoperoxide have also been shown to stimulate matrix protein synthesis in cultured MCs (22–24). The mechanism by which this occurs is uncertain. However, recent studies with angiotensin II (30) and high ambient concentrations of glucose (15) have strongly implicated TGF-β in the mediation of the actions by which each of these two agents stimulates matrix protein synthesis in MCs. The present findings similarly support a role for TGF-β in the mediation of the action of TX to stimulate Fn synthesis in MCs. In agreement with previous reports, both U-46619 (22–24) and TGF-β (31,32) stimulate Fn synthesis in cultured rat MCs. While not examined in the present study, the stimulatory action of both these agents may have occurred at least in part at the transcriptional level, as reflected by increased mRNA levels for Fn and other matrix proteins (22,33). The time course of TGF-β action on Fn synthesis observed in the present study in rat MCs is analogous to that previously observed by this laboratory with U-46619 (24). Moreover, anti-TGF-β antibody, but not control IgG, blocked stimulation of Fn synthesis induced by both exogenous TGF-β and U-46619, an observation which strongly supports a role for TGF-β in the expression of TXA_2 effects on Fn synthesis. Under control culture conditions in the absence of U-46619, addition of anti-TGF-β antibody did not detectably alter basal Fn synthesis. The explanation for this apparent lack of TGF-β neutralization is not known, but two considerations may be relevant. First, while substantial concentrations of latent TGF-β were present in MC culture media under control conditions, concentrations of the active fraction of TGF-β in control media were quite low. The latter were routinely below the lowest concentration of exogenous TGF-β (1 pmol/l) that significantly stimulated Fn synthesis. Second, control MC cultures were exposed to anti-TGF-β antibody for only 24 h. This may have been an insufficient exposure to detect potentially small effects of antibody on Fn synthesis under culture conditions in which the basal rates of MC Fn synthesis were already downregulated by a prior reduction in media serum concentration.

Consistent with the action of anti-TGF-β antibody to block U-46619 stimulation of Fn synthesis, U-46619 increased TGF-β bioactivity in culture media of MCs. This MC response to U-46619 was blocked by SQ-29548, a finding indicative of a receptor-mediated action. SQ-29548 was previously shown to block the Fn synthetic responses to U-46619 (24). With the assay system used, basal levels of both active and total (heat-activated) TGF-β bioactivities observed in the media of rat MCs after 24 h of culture in the present study were lower than corresponding basal media values reported earlier from two other laboratories after 24 or 48 h of rat MC culture (27,30). The proportion of TGF-β in the active form in media from 24-h control MC cultures in the present study (~3%) was comparable to the value reported (4%) after 24 h of rat MC culture by Kaname et al. (27) but lower than that observed by Kagami et al. (30) after 48 h. The reasons for these differences are not clear. Nevertheless, increases in both active and total TGF-β bioactivities were evident in media from MCs exposed to U-46619. These responses are qualitatively similar to but again differ quantitatively from the effects of angiotensin II in cultured rat MCs (30). Thus, Kagami et al. reported 12-fold increases over control of active and 3-fold increases over corresponding control of total TGF-β in MC cultures exposed to angiotensin II for 48 h. There was a resultant increase in the percentage of active TGF-β from 11% in control to 45% in MC cultures exposed to angiotensin II. Based on the disproportionate increase in active compared with total TGF-β in response to angiotensin II, Kagami et al. (30) suggested independent actions of this agent on both TGF-β synthesis and its activation. In response to 1 μmol/l U-46619, active TGF-β increased approximately threefold in 24 h, while total TGF-β increased approximately twofold. Thus, the percentage of total TGF-β in the active form did not differ greatly in the presence and absence of U-46619 (3–5%). The magnitude of the changes in active and total TGF-β in response to U-46619 leaves open the issue of whether this agent has a single or multiple effects on TGF-β metabolism. This will require additional study. However, the absolute concentration of active TGF-β observed in the culture media of MCs exposed to 1 μmol/l U-46619 for 24 h (1.7 ± 0.3 pmol/l) was clearly increased over control (0.6 ± 0.1 pmol/l). Moreover, the range of concentrations of endogenous TGF-β bioactivity observed in MC culture media in response to 1 μmol/l U-46619 (1.3–2.9 pmol/l) were adequate to account for the two- to threefold increase in Fn synthesis induced by this concentration of U-46619, as reflected by the concentration-response relationship of Fn synthesis to exogenous TGF-β (Fig. 1). This consideration, together with the inhibitory effect of anti-TGF-β antibody on U-46619–induced increases in Fn synthesis, indicates that an enhancement of TGF-β bioactivity is an essential step in the expression of this U-46619 action. The present studies do not identify the TGF-β isoform(s) increased when MCs are exposed to U-46619. In cultured murine MCs, mRNA levels for the β1, β2, and β3 isoforms have been detected (34). TGF-β1 is most highly expressed (35), and increases in mRNA levels for this isoform have been reported in rat MCs exposed to high concentrations of glucose (15). By contrast, in MCs exposed to glycosylated proteins, disproportionate increases in mRNA levels for TGF-β2 have been described (34). The anti-TGF-β antibodies used in the present study neutralize both TGF-β1 and -β2, and the bioassay used does not distinguish these isoforms. Thus, additional studies will be required to identify the specific TGF-β isoform(s) that increase in response to TX.

Of note is that expression of TGF-β is enhanced in glomeruli from human diabetic patients and from rats in which diabetes was induced with STZ (9). Glomeruli from STZ-induced diabetic rats produce increased quantities of TXA_2 (17,18). Inhibitors of TXA_2 synthesis attenuate the development of glomerulosclerosis and albuminuria in this (17,18) and other diabetic models (19). It is therefore possible that TX participates in the pathogenesis of glomerulosclerosis in diabetes, at least in part by an effect to enhance autocrine TGF-β in glomeruli, which in turn stimulates MC matrix protein synthesis. High concentrations of glucose may also stimulate MC matrix protein production by a TGF-β–dependent process in diabetes, since studies in MCs have implicated TGF-β in the expression of the action of glucose on matrix protein production (15).

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