1-1-1999

TGF-β receptor expression and binding in rat mesangial cells: Modulation by glucose and cyclic mechanical strain

Bruce L. Riser
Henry Ford Health

Stephanie Ladson-Wofford
Henry Ford Health

Abdulkarim Sharba
Henry Ford Health

Pedro Cortes
Henry Ford Health

Katie Drake
Henry Ford Health

See next page for additional authors

Recommended Citation

Riser BL, Ladson-Wofford S, Sharba A, Cortes P, Drake K, Guerin CJ, Yee J, Choi ME, Segarini PR, Narins RG. TGF-β receptor expression and binding in rat mesangial cells: Modulation by glucose and cyclic mechanical strain. Kidney International 1999; 56(2):428-439.

Authors

Bruce L. Riser, Stephanie Ladson-Wofford, Abdulkarim Sharba, Pedro Cortes, Katie Drake, Christopher J. Guerin, Jerry Yee, Mary E. Choi, Patricia R. Segarini, and Robert G. Narins

Kidney International, Vol. 56 (1999), pp. 428–439

TGF-β receptor expression and binding in rat mesangial cells: Modulation by glucose and cyclic mechanical strain

Bruce L. Riser, Stephanie Ladson-Wofford, Abdulkarim Sharba, Pedro Cortes, Katie Drake, Christopher J. Guerin, Jerry Yee, Mary E. Choi, Patricia R. Segarini, and Robert G. Narins

Department of Internal Medicine, Division of Nephrology and Department of Eye Care Services, Henry Ford Hospital, Detroit, Michigan; Section of Nephrology, Yale University, New Haven, Connecticut; and Celtrix Pharmaceuticals, Santa Clara, California, USA

TGF-β receptor expression and binding in rat mesangial cells: Modulation by glucose and cyclic mechanical strain.

Background. Transforming growth factor-β (TGF-β) is a causal factor in experimental glomerulosclerosis, and it mediates the increased extracellular matrix (ECM) accumulation that occurs in cultured mesangial cells (MCs) exposed to high glucose concentrations and cyclic mechanical strain. This change is associated with increased levels of TGF-β, but may also involve alterations in receptor expression and binding.

Methods. Rat MCs cultured in media containing either 8 or 35 mM glucose were seeded into culture plates with elastin-coated flexible bottoms. Thereafter, they were subjected to cyclic stretch or static conditions and then examined for ^{125}I-TGF-β1 binding and expression of TGF-β receptors at the gene and protein levels.

Results. Kinetic studies showed that MCs bound TGF-β1 in a time- and concentration-dependent manner, expressing 6800 high-affinity receptors per cell, with an apparent dissociation constant (K_d) of 15.4 pM, while cross-linking analysis identified three TGF-β receptors (βR) corresponding to βRI, βRII, and βRIII of 54, 73, and 200 kDa, respectively. Immunocytochemical studies of βRI and βRII protein revealed MC expression in a homogeneous, punctate distribution, whereas Northern analysis demonstrated the presence of the corresponding mRNAs. Exposure to cyclic stretching significantly increased (10%) the overall number of TGF-β receptors, whereas ligands associated with βRs I, II, and III also increased (25 to 50%). The finding of increased (30 to 40%) βRI and βRII transcript levels and immunoreactive protein (163 and 59%, respectively) in the absence of significant changes in the apparent K_d indicated that stretch-induced binding was the result of increased receptor synthesis and expression and not due to a change in binding affinity. In a similar, but more dramatic fashion, exposure to high glucose also elevated (50%) the receptor number, as well as the amount of ligands associated with βRs I, II, and III (100 to 250%). This same treatment also increased the levels of βRI and βRII mRNA (30 to 40%) and the immunoreactive protein (82 and 82%, respectively), without significantly altering the binding affinity of the receptor. A concerted or synergistic effect of both stimuli was not evidenced.

Conclusion. These results suggest that the modulation of TGF-β receptors may be an additional control point in mediating the glucose- and mechanical force-induced increase in ECM deposition by MCs.

Key words: cell elasticity, transforming growth factor-β, extracellular matrix, fibrosis, scarring, tissue repair, glomerulosclerosis, diabetic kidney disease.

Received for publication July 31, 1998
and in revised form January 22, 1999
Accepted for publication March 3, 1999

Transforming growth factor-β (TGF-β) is a family of cytokines that, in mammals, includes TGF-βs 1, 2, and 3. They possess autocrine and paracrine multifunctional activities, one of which is the stimulation of extracellular matrix (ECM) synthesis. This function, although essential for wound healing, is also responsible for mediating fibrosis and scarring when not down-regulated at the termination of normal tissue repair [1]. Such a pathogenetic role of TGF-β has been convincingly demonstrated in a number of experimental models of renal glomerulosclerosis [2–4] and is likely causal in certain forms of this disorder in humans, including that associated with diabetes [5, 6].

The factors responsible for both inducing and sustaining the increased TGF-β activity in diabetic glomerulosclerosis are now beginning to emerge. For example, the incubation of cultured mesangial cells (MCs) in media containing increased glucose levels, simulating the hyperglycemic environment, induces TGF-β secretion and ECM accumulation [7–10]. In addition, inhibition of this TGF-β activity significantly reduces the high-glucose–mediated induction of matrix synthesis [10]. The effect of TGF-β on MC formation of ECM is of significance because these cells are largely responsible for the *in situ* mesangial matrix synthesis and increased deposition observed in diabetic glomerulosclerosis [11, 12].

Another factor that may be important in stimulating and maintaining high TGF-β levels is the mechanical strain resulting from altered glomerular hemodynamics.

Simulating in tissue culture the effects of increased glomerular distention, we have shown that cyclic cell stretching of MCs induces both increased production and activity of TGF-β1 [13], as well as synthesis and accumulation of mesangial matrix components [14]. This stimulation of TGF-β1 activity is manifested as an increase in mRNA levels, secretion of protein, and the activation of the latent molecule. Furthermore, the response is specific for this cytokine because the activities of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and even TGF-β2 remain unchanged [13]. We have also shown that the induction of TGF-β1 activity and collagen accumulation by stretch is further enhanced in the presence of increased glucose concentrations [15]. Finally, the marked increase in collagen accumulation that occurs under these conditions is reversed by the action of a TGF-β–neutralizing antibody [15].

An important factor in the cascade of events leading to augmented ECM production may also be changes in TGF-β receptors. TGF-β1 and TGF-β3 mediate their activity by binding to the TGF-β type II receptor (βRII), a constitutively active transmembrane serine/threonine kinase [16]. This receptor recruits and then phosphorylates another transmembrane kinase, the TGF-β receptor βRI [17], resulting in ternary complex formation and signaling. The TGF-β type III receptor (βRIII) betaglycan is a membrane protein that increases the binding of TGF-βs 1 and 3 to the signaling receptors and may be necessary for equivalent TGF-β2 binding [18]. Changes in expression and/or binding of one or more of these receptors could then conceivably alter the biological response, irrespective of changes in the quantity of available active TGF-β.

Although little is currently known about the regulation of TGF-β binding, it has been demonstrated that cell density [19], as well as exposure to adrenocorticotropic hormone [20], interferon-γ [21], prostaglandin E2 [22], or exogenous TGF-β [23]. can result in altered TGF-β binding in certain cell types. In the rodent kidney, there is an up-regulation in mRNA levels of βRII following the induction of diabetes with streptozotocin [24] and of βRII and βRIII message levels in adriamycin-induced nephrosclerosis [25]. However, the regulation of TGF-β binding in cultured MCs has not been examined. We have previously reported that in cultures of MCs exposed to a gradient of stretching amplitude, only those cells subjected to significant mechanical strain demonstrate intense immunostaining for the active form of TGF-β, as opposed to those in the same culture experiencing little or no strain [13]. This differential localization of active TGF-β occurs even though the conditioned media bathing all cells contains greatly increased amounts of this factor, as compared with unstretched control cultures. This suggests the possibility that TGF-β binding is increased in response to cyclic strain. This study was therefore carried out to determine the effects of exposure to high-glucose medium and/or mechanical strain on TGF-β receptor expression and binding.

METHODS

Mesangial cells

Cells, which we previously characterized and described [26], were obtained from a cloned line derived from outgrowths of Fischer rat glomeruli. These cells continue, with serial passage, to express markers of MCs, including characteristic morphology, ability to grow in media containing D-valine, and growth inhibition in the presence of heparin or mitomycin. They additionally express the Thy-1 antigen and demonstrate the same high sensitivity to phorbol-stimulated neutrophil adhesion and lysis as do early passage MCs, indicating continued expression of essential cell surface receptors [27]. Stock cultures were propagated in the growth medium RPMI 1640, containing antibiotics, 20% Nu-Serum (Collaborative Research, Bedford, MA, USA), and 8 mM glucose.

Tissue culture

Mesangial cells were seeded (5×10^4 cells/25 mm diameter well) into six-well plates with flexible elastin-coated bottoms (Flex I plates; Flexcell International, McKeesport, PA, USA) and cultured in 1 ml/well of growth medium. After either 24 or 48 hours of incubation, experimental cultures were subjected to cyclic stretching, and the control cultures were maintained in a static environment under identical conditions. During this final period, cells were maintained in medium containing 1% fetal calf serum (FCS). 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®; Flexcell International). 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 MC stretch during possible low-frequency oscillations in intraglomerular pressure [28], all experiments were carried out using alternating cycles of 10-second stretch and 10-second relaxation (50 mHz). Vacuum intensity was set to provide an average elongation of approximately 8% over the entire culture surface [29]. Under these mechanical conditions, cellular proliferation was unaffected.

In experiments in which the effect of high glucose concentration was studied, cells that had been maintained in medium containing 8 mM glucose were dispersed and then cultured for seven to eight days in the presence of either 8 or 35 mM glucose. Cells were subsequently dispersed and reseeded in equal densities on Flex I plates and cultures continued in the same 8 or 35 mM glucose media for an additional two to four days. Although 8 mM glucose is higher than "normal physiological" levels

(5 mM), it is substantially lower than those found in usual culture media (11 mM) and is the minimal concentration required for long-term growth of these cells.

Analysis of mRNA

RNA was isolated by a modified guanidinium method in RNA Stat-60 reagent (Tel-Test Inc., Friendswood, TX, USA). Samples were denatured in glyoxal/dimethylsulfoxide (DMSO) at 55°C for 60 minutes and 20 μg of total RNA electrophoresed on 10 mM sodium phosphate/1% agarose gels. Gels were then stained with ethidium bromide (5 μl/ml) and photographed, and the separated RNAs were transferred to Genescreen membranes (NEN Research, DuPont Co., Boston, MA, USA) by capillary action using standard methods [30]. The blots were hybridized and probed for individual TGF-β receptor mRNAs using the corresponding cDNAs that were ^{32}P-labeled by random hexamer priming (Sigma Prime-1 kit; Sigma Chemical Co., St. Louis, MO, USA). Autoradiograms were digitized by scanning densitometry (Scanmaster 3+ Densitometer; Howtek, Hudson, NH, USA) and quantitated with image analysis (vs. 1.59, Twilight Clone BBS; NIH Image, Silver Springs, MD). The assayed TGF-β receptor mRNAs were normalized to rRNA (18S and 28S) to adjust for differences in loading. The probe for βRI was an Xba I fragment of the rat activin receptor-like kinase 5 (ALK5) and hybridized to a 6.0 kb transcript. The βRII probe was a 2.8 kb rat full-length βRII cDNA that hybridizes with a 5.5 kb mRNA. We produced both probes, which have both been previously characterized [31].

Transforming growth factor-β binding assays

Transforming growth factor-β1 was iodinated using the modified chloramine T procedure of Frolik et al [32]. This method generated iodinated TGF-β1 with high specific activity (100 to 150 μCi/μg) that retained its ability to interact with the cell. All assays were performed within two weeks of iodination. Binding assays were performed when cells were 90 to 95% confluent, approximately 4 days after seeding. The medium was aspirated, and cultures were incubated for two minutes at 4°C in a 20 mM glycine buffer (pH 3) containing 135 mM NaCl to remove endogenous TGF-β. After two rinses with binding buffer [Dulbecco's modified Eagle's medium (DMEM), 25 mM HEPES, 1% bovine serum albumin (BSA)], ^{125}I-labeled TGF-β1 was added to the buffer to achieve final concentrations ranging from 2 to 200 pM. Nonspecific binding was determined in wells containing 100-fold or greater excess of unlabeled TGF-β1. Following incubation at 22°C with gentle rotation, the medium was removed for determination of free radioactivity. The cells were then quickly washed twice with binding buffer and with 1% Triton X-100, containing 10% glycerol, 20 mM HEPES, and 0.01% BSA. Triplicate samples were solubilized, and the suspension was counted. Specific TGF-β binding was determined from bound versus free radioactivity after subtracting the amount of nonspecifically bound ligand. Specific binding accounted for 60 to 70% of the total bound radioactivity.

Cross-linking was used to determine specific binding to TGF-β receptor isoforms. In this case, assays were performed as described earlier in this article, except that wells were incubated with 100 pM radiolabeled or radiolabeled plus unlabeled TGF-β1 (10 mM). Following a two-hour incubation at 4°C, the medium was removed, and the bound ligand was cross-linked to receptors by adding cold binding buffer containing 30 mM disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL, USA) freshly dissolved in DMSO. The samples were then incubated at 4°C for 15 minutes with gentle rocking, and the reaction was terminated by three washes with a solution of 250 mM sucrose, 10 mM Tris, and 1 mM ethylenediaminetetraacetic acid (EDTA) to quench the remaining cross-linker. Cells and matrix were then solubilized by the addition of lysis buffer (as described), containing protease inhibitors phenylmethylsulfonyl fluoride (PMSF) 1 mM, Nahrungs Einheit Milch (NEM; milk nutritional unit) 40 mM, and EDTA 1 mM. Samples were centrifuged at 13,000 × *g* for two minutes, and supernatants were collected and boiled for two minutes in 1 × Laemmli buffer [33]. Lysate volumes were adjusted for protein content and then electrophoresed on 4 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and autoradiographed. Band intensities were determined by scanning densitometry, as described earlier here.

Immunocytochemistry for transforming growth factor-β receptors

Mesangial cells cultured on elastin-coated bottoms Flex I plates were removed from 37°C and washed using an infinite dilution protocol. In brief, approximately one half of the culture media were removed from each well, and an equivalent amount of phosphate-buffered saline (PBS) at 37°C was added. This procedure was repeated several times until all traces of media were removed. In the same manner, half of the PBS was removed and replaced with 37°C, 4% (wt/vol) paraformaldehyde in 0.1 M Na Cacodylate buffer, pH 7.4 (both Sigma), again using multiple changes, after which cells were fixed for 30 minutes at room temperature. Samples were then washed with PBT (PBS plus 0.1% Triton X-100) to promote internalization of antibodies. Cells were blocked with diluted goat serum (1:200) and incubated with primary antibodies diluted 1:100 in PBT with 0.5% BSA overnight at 4°C with gentle rocking. Cells were again washed with PBT and incubated overnight at 4°C in goat ant-rabbit IgG conjugated to Cy3 (Amersham Life Sciences, Arlington Heights, IL, USA) diluted 1:200 in

PBS containing 0.5% BSA. After washing, MCs were covered with 5% N-propyl gallate in glycerol, and cover slips were applied. Cells were viewed with a BioRad 1024 (Bio-Rad, Hercules, CA, USA) laser scanning confocal microscope. Digitized 8-bit images from representative areas of the culture membrane were obtained under identical conditions of laser intensity and gain and were a composite of the fluorescent signal from 0.5 μm serial sections. The images from each culture were divided into quadrants and separately analyzed under identical density calibration, and the quantity of fluorescence was determined using image analysis (NIH Image), as we have previously described [13]. Negative controls consisted of replacement of the primary antibody with nonimmune rabbit IgG and preincubation of the antibodies with a 10 M excess of peptide antigen. All controls were without detectable immunostaining. Antibodies to βRI were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The βRI antibody (cat. no. SC 399) was raised against a peptide corresponding to amino acids 482 to 501 mapping at the carboxy terminus of the βRI (also designated ALK-5) of human origin and which is homologous to the corresponding rat primary sequence. Antibody to βRII (cat. no. SC 220; Santa Cruz Biotechnology) was raised against a peptide corresponding to amino acids 550 to 565 mapping within the carboxy terminus of the precursor form of human βRII that differs from the corresponding rat sequence by a single residue. These antibodies show no cross-reactivity on Western blots.

Statistical analysis

Data were expressed as means ± SEM. Unless otherwise noted, differences between two groups were evaluated using paired Student's *t*-test. In the case in which results in these groups 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 the test group with the control. A paired two-sample *t*-test was used to examine differences between the three test groups. In both cases, a Holm's test was then applied *post hoc* to adjust for multiple comparisons [34].

RESULTS

TGF-β1 binding kinetics and the effect of glucose concentration or cyclic strain

We first examined the effect of time on ^{125}I-TGF-β1 binding in control, 8 mM glucose-incubated MCs. Specific binding was time dependent, reaching a maximum after 120 minutes at 22°C (Fig. 1). Therefore, subsequent studies were carried out using a 120-minute binding period. Next, MCs cultured in 8 or 35 mM glucose-containing media were examined for binding characteristics with increasing concentrations of ^{125}I-TGF-β1. Binding occurred in a concentration-dependent manner over the range of

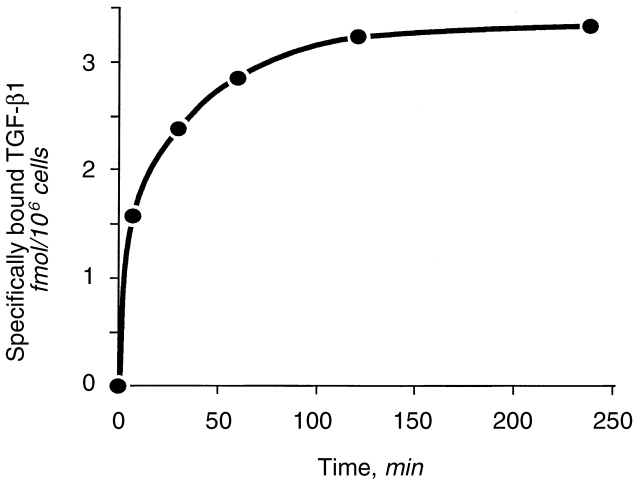

Fig. 1. Time course of ^{125}I-TGF-β1 binding to mesangial cells (MCs). The values represent the average of specifically bound transforming growth factor-β1 (TGF-β1) at each time point, based on triplicate wells in a single experiment. All values were within 10% of the average values.

1 to 35 pM (free molecule) and was saturable (Fig. 2). High-glucose treatment increased the amount of TGF-β1 that was specifically bound. A Scatchard analysis of data (Fig. 2, insert) indicated that a single class of high-affinity binding sites was responsible for the binding and that high-glucose exposure increased the amount of TGF-β1 bound at saturation, but did not alter the slope of the binding curve [35]. Analysis of data from six separate experiments yielded 6755 ± 1465 high-affinity receptors per cell, with an apparent dissociation constant (K_d) of 15.4 ± 1.74 pM under normal glucose conditions. The number of receptors per cell increased 50% to 10,178 ± 2418 ($N = 6$, $P = 0.03$) following exposure to high glucose. There was, however, no significant change in the binding affinity (8 mM, 15.4 ± 1.74; 35 mM, 25.1 ± 5.1, $N = 6$, $P = 0.06$). The observed change was not due to an osmolar effect. In separate experiments performed under identical conditions, the receptor number and apparent K_d of MCs incubated in medium containing 5 mM glucose plus 30 mM mannitol were not different from those incubated in 5 mM glucose alone (93.1 ± 8.6% and 96.4 ± 11.5%, respectively, of control, $N = 4$, $P > 0.5$).

In addition, the increase in receptor number was not the result of a high-glucose–induced change in culture density, as the final cell number in the two groups was not different (8 mM, 2.21 × 10^5/culture; 35 mM, 2.24 × 10^5/culture, $P = 0.84$) under the culture conditions selected. In preliminary experiments, it was shown that the long-term culture of MCs in 30 mM glucose resulted in a reduction in cell replication and changes in receptor expression. MCs seeded at increasing densities and studied after four days demonstrated a significant ($R = 0.729$, $P = 0.0009$, $N = 17$), inverse relationship between the cell number (3500 to 21,000 receptors per cell) and number of

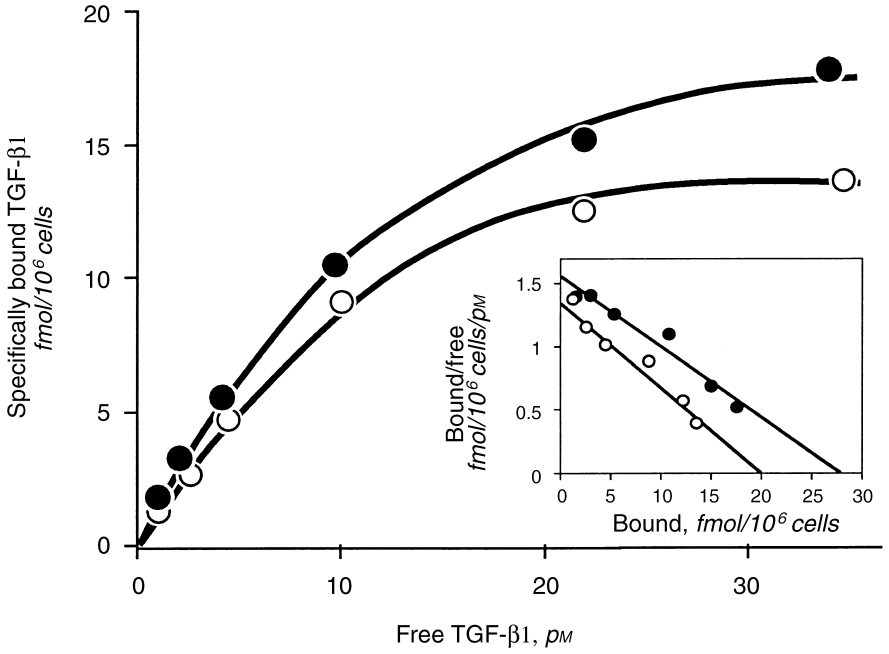

Fig. 2. Concentration-dependent ^{125}I-TGF-β1 binding and the effect of high glucose treatment. Values shown represent specific binding, that is, the difference between total binding of ^{125}I-TGF-β1 and binding in the presence of 100-fold excess unlabeled transforming growth factor-β (TGF-β) and are from a representative experiment. (Inset) Scatchard plot of data. Treatments were either 8 mm (○) or 35 mM glucose (●).

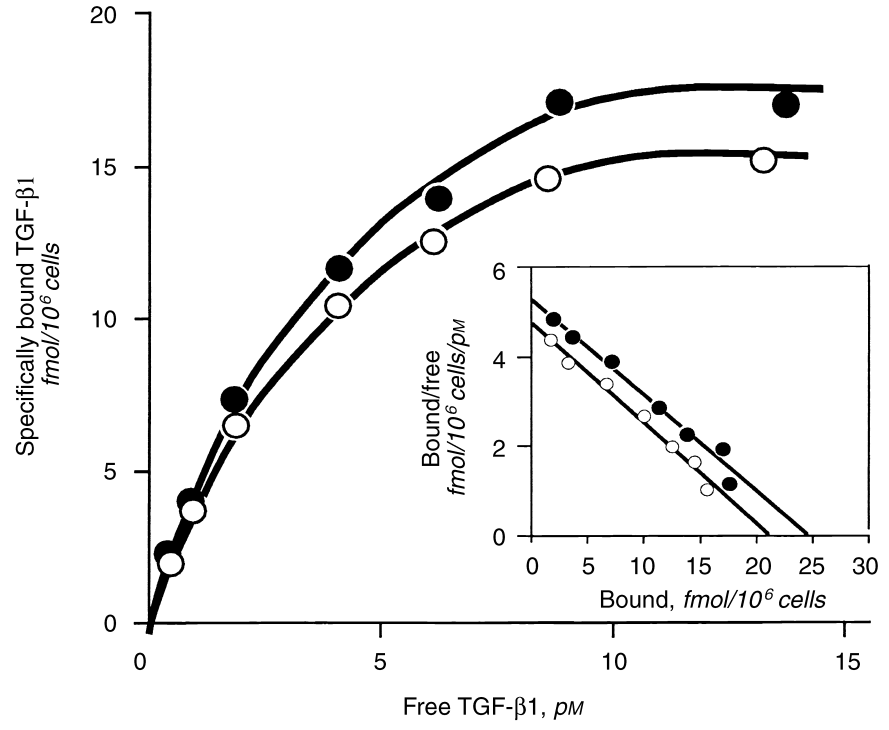

Fig. 3. Effects of cell stretching on concentration-dependent ^{125}I-TGF-β1 binding. The assay was carried out as described in Figure 2, except mesangial cells (MCs) grown in 8 mM glucose were subjected to 48 hours of cyclic stretch or control, static conditions. Results are from a representative experiment. (Inset) Scatchard plot of data. Treatments were static (○) and stretch conditions (●).

calculated TGF-β receptors over a range of 0.5 to 3.5 cells/well.

To next determine if mechanical strain could alter TGF-β1 binding, MCs grown in 8 mM glucose medium were subjected to 24 hours of cyclic stretching. This treatment increased ^{125}I-TGF-β1–specific binding (Fig. 3). The Scatchard analysis of the data (Fig. 3, inset) indicated that this change was due to an increase in the number of receptors and was not attributable to altered binding affinity. Analysis of data from four separate experiments demonstrated a modest, but significant, 10% increase (static, 11,007 ± 1302; stretch, 12,091 ± 1473, $P = 0.039$)

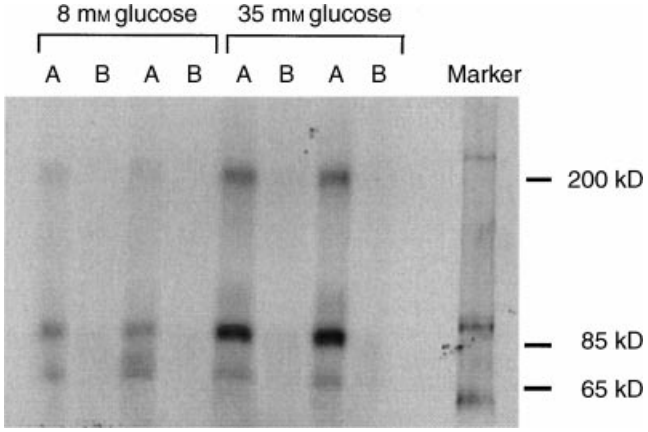

Fig. 4. Electrophoretic protein gel analysis of ^{125}I-TGF-β1 cross-linked to mesangial cell receptors. Binding assays on mesangial cells (MCs) grown in 8 or 35 mM glucose were performed in duplicate, and the bound ^{125}I-TGF-β1 was cross-linked to receptors. Cultures were incubated with radiolabeled transforming growth factor-β1 (TGF-β1; A), or radiolabeled plus 100-fold excess unlabeled TGF-β1 (B). A representative autoradiogram shows three specifically labeled bands observed at 65, 85, and 200 kDa, corresponding to the TGF-β types I, II, and III receptors complexes, respectively.

in the number of receptors. This occurred in the absence of changes in the apparent K_d (static, 5.29 ± 1.9; stretch, 6.08 ± 2.8, $P = 0.43$) or cell number (static, 1.29×10^5/culture ± 0.245; stretch, 1.24 ± 0.219, $P = 0.35$).

Modulation of TGF-β receptor types by high glucose and stretch, determined by cross-linking

The kinetic studies indicated that increased binding resulted from an up-regulation in receptor number. This could involve one or more of the TGF-β receptor types. To identify those receptors responsible, MCs grown in 8 or 35 mM glucose-containing media were incubated with 100 pM ^{125}I-TGF-β1, both with and without 100-fold excess of unlabeled TGF-β1, followed by cross-linking of the ligand to the binding molecule(s). Three specifically labeled bands were observed at 66, 85, and 200 kDa corresponding to the TGF-β type I, II, and III receptor complexes, respectively (Fig. 4). Each complex represents the receptor with an attached monomer of TGF-β1 (12 kDa). Exposure to high glucose increased the amount of TGF-β1 receptor complex in all three types (Fig. 4). This binding was specific because the addition of unlabeled TGF-β eliminated measurable labeled TGF-β1 binding.

Next, quantitative changes in binding among these receptors, both in response to increased glucose and/or to cyclic stretch, were investigated. The amount of ^{125}I-TGF-β1 receptor complex in all three types was increased by either high glucose or 48 hours of cyclic strain, but did not demonstrate an additional increase when exposed to both stimuli (Fig. 5). The increase mediated by stretch was moderate (25 to 50%) as compared with that of glucose (100 to 250%), and the observed response was equivalent in all three receptor types. Additional experiments produced similar results after 24 and 72 hours of stretch (not shown).

Effect of high glucose and cyclic stretching on βRI and βRII mRNA levels

The cross-linking studies demonstrated that the enhanced binding in response to high glucose and stretch involved multiple TGF-β receptors. To determine if this correlated to the message levels for these receptors, MCs grown in either 8 or 35 mM glucose-containing media and exposed to 48 hours of cyclic stretching, or static conditions, were subjected to Northern analysis and were probed for βRI or βRII. MCs contained transcripts for both of these receptors (Fig. 6). High glucose, as well as mechanical strain, induced a 30 to 50% increase in the level of βRI mRNA (Fig. 6A). As observed in cross-linking studies, the two stimuli applied in combination failed to produce an additive or synergistic effect. Message levels of βRII were also increased by high glucose or stretching with changes mirroring those of βRI (Fig. 6B).

Modulation of βRI and βRII protein expression determined by immunocytochemistry

The results discussed earlier in this article indicated that increased expression of multiple TGF-β receptor types, rather than altered binding affinity, accounts for the increase in ligand binding induced by high glucose and stretch. If this were the case, increased quantities of cell-associated receptor protein in response to the stimuli should be present and might therefore be visualized by immunofluorescent labeling. Laser confocal microscopy showed that antibodies to βRI bound MCs, producing a punctate, but homogeneous distribution of fluorescence throughout the cell (Fig. 7A). High-glucose exposure increased the overall number of antibody-labeled foci, but did not alter their distribution (Fig. 7B). The pattern for βRII labeling was similar but was more diffuse than that for βRI (Fig. 7C). As was the case for βRI, βRII staining was similarly augmented by high-glucose exposure (Fig. 7D). This effect was confirmed by quantitative image analysis, with an 83 and 62% elevation over control in βRI and βRII labeling, respectively (Fig. 8).

Cells grown in low-glucose–containing media were also subjected to various intervals of mechanical strain and were then fixed and studied by immunocytochemistry. Because in this system the stretching force is not uniform, but increases are toward the periphery of the well, we examined cells in the same outermost zone of culture after 0 to 96 hours of stretching. βRI expression was substantially up-regulated by 48 hours of cyclic stretch and then appeared to diminish with longer periods of stretch (Fig. 9) but still remained significantly elevated above control levels even after 96 hours. In the same experiments, βRII labeling was also increased by 48 hours

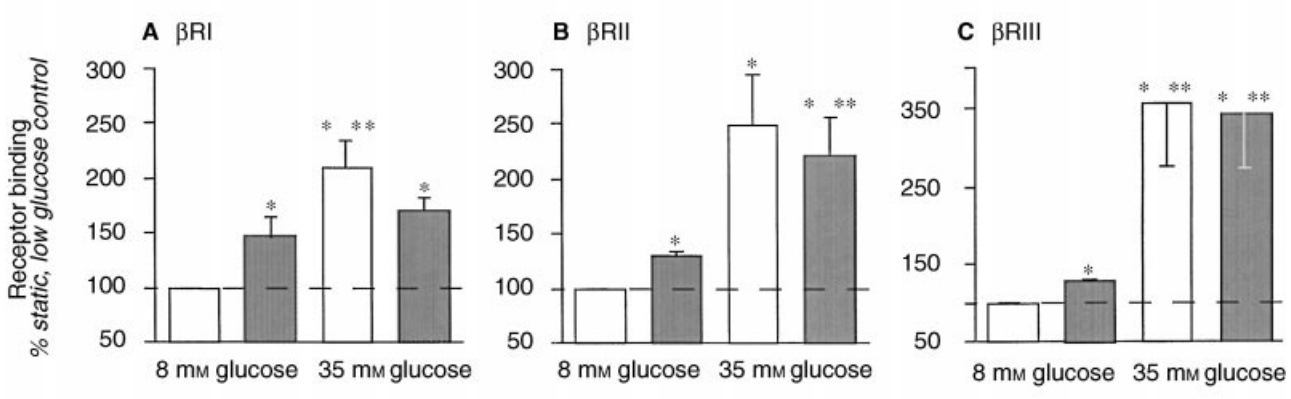

Fig. 5. Effect of high glucose or mechanical strain on transforming growth factor-β1 (TGF-β1) binding to specific receptors. The intensities of bands corresponding to βRI, βRII, and βRIII from five to seven cross-linking experiments were quantitated following optical scanning. Mesangial cells (MCs) were grown in normal or high glucose medium and were subjected to stretch (■) or static (□) conditions. *$P < 0.05$ vs. static, 8 mM control. **$P < 0.05$ vs. stretch, 8 mM.

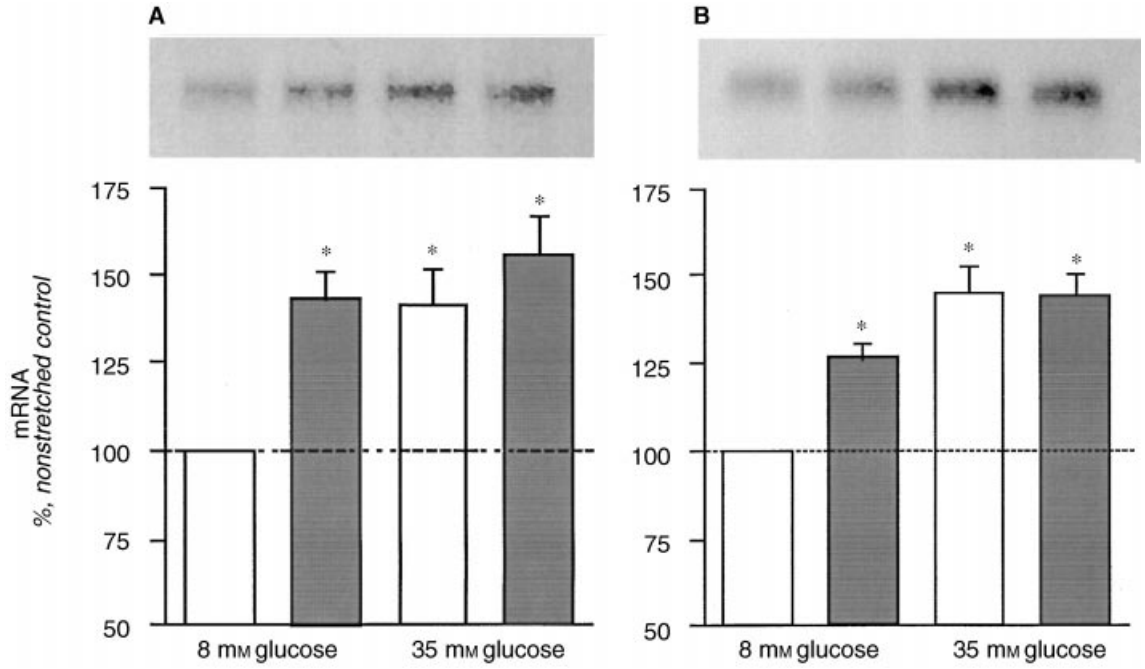

Fig. 6. Northern blot analysis of mRNA for βRI and βRII after exposure to cyclic stretch and/or high-glucose medium. A single band was detected corresponding to (*A*) βRI (6.0 kb) or (*B*) βRII (5.5 kb), shown from a representative experiment (upper panel). Quantitative image analysis of band intensities from four (βRI) or seven (βRII) experiments (lower panel); static (□) or stretched (■) cultures. Results shown were normalized to the sum of 18S and 28S ribosomal RNAs. *$P < 0.002$ vs. static, 8 mM control.

of stretching. The magnitude of the change was lower than for βRI, yet it remained stable over time. As with βRI, although the intensity of staining increased with stretching, there was no discernible change in the cellular pattern or distribution of the immunoreactive protein (data not shown). This induction of receptor protein appeared primarily localized to the cells experiencing the greatest force, because cells present in the center of the well increased TGF-βRI staining 35% ($P < 0.05$) by the end of this period, whereas RII protein in the center of the well decreased 49% ($P < 0.05$) over the same time.

DISCUSSION

The primary goal of this study was to determine the effects of high glucose and cyclic mechanical strain on modulation of TGF-β receptor activities. First we showed that TGF-β1 binding was time- and concentration-

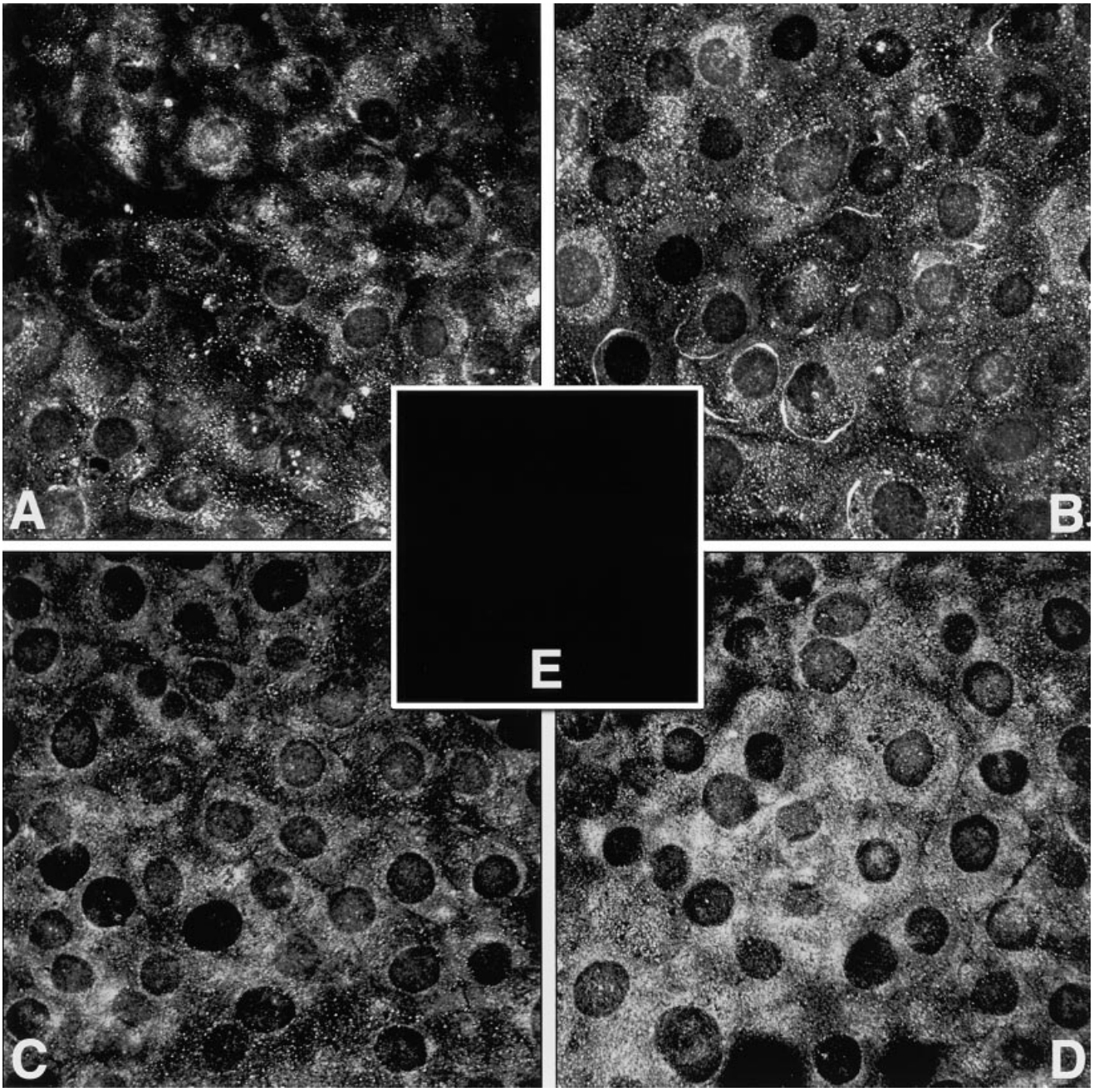

Fig. 7. Immunofluorescent localization of βRI and βRII protein in mesangial cells (MCs). Following growth in 8 mM (*A, C*) or 35 mM (*B, D, E*) glucose, cells were fixed and examined by laser scanning confocal microscopy for localization of βRI (A, B) or βRII (C, D). A negative control consisted of substitution of the primary antibody with rabbit nonimmune IgG (inset E). Representative photos are shown.

dependent with MCs expressing approximately 6750 high-affinity (apparent K_d = 25 pM) receptors per cell when grown to near confluency. Our findings are comparable to those of Wakefield et al, who tested a wide variety of cell types [36], and of MacKay et al, who examined mouse MCs [37]. The former reported a range of 600 to 40,000 high-affinity receptors per cell, with K_d values ranging from 1 to 60 pM, whereas the latter reported 3900 receptors per MC with a K_d of 5 pM. We observed, as did Wakefield et al, that cell density was an important determinant of receptor binding [36]. Therefore, our studies of receptor modulation by high glucose and mechanical strain were run under conditions that were established to produce equivalent final cell densities in the control and treated groups. Our cross-linking studies demonstrated that the observed specific binding of TGF-β was to three major proteins, corresponding to βRI, βRII, and βRIII. In addition, MCs

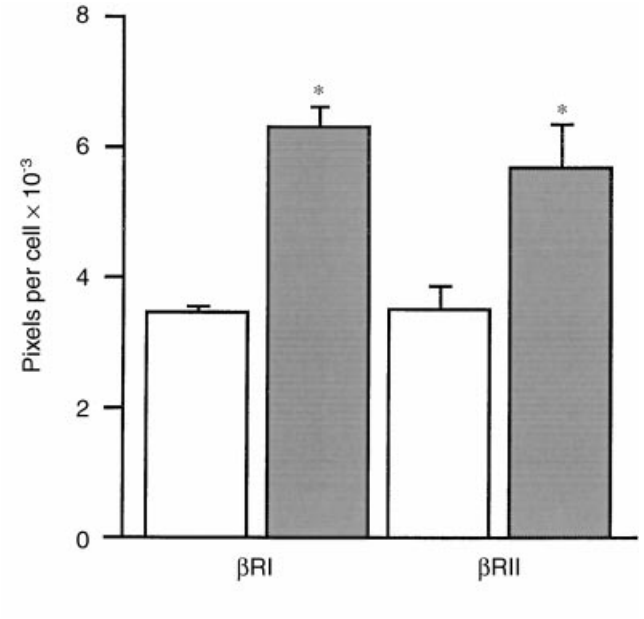

Fig. 8. Effect of increased glucose on the expression of βRI and βRII protein. Quantitative image analysis measurements were obtained from the same set of experiments represented in Figure 7. Images used for quantitation were constructed from all of the 0.5 μm serial sections contained in the cell layer with fluorescent signal. Bars indicate the mean of measurements in eight separate areas, each containing 10 to 20 cells. $*P < 0.05$ vs. 8 mM control.

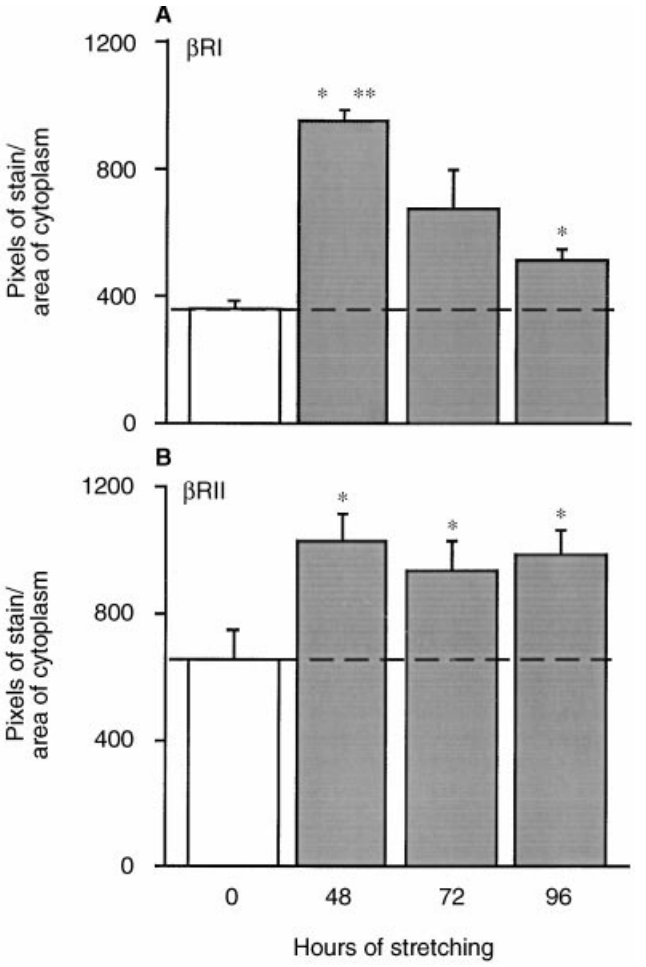

Fig. 9. Effect of cyclic stretching on the expression of βRI and βRII protein. Mesangial cells (MCs) grown in 8 mM glucose-containing media were subjected to static or stretch conditions for the times indicated. Cell layers were then subjected to immunocytochemical analysis and measurements of receptor expression determined as in as in Figure 8. $*P < 0.05$ vs. static control. $**P < 0.05$ vs. 96 hours of stretch.

expressed mRNAs for βRI and βRII and demonstrated a homogenous cytoplasmic distribution of the corresponding protein as determined by immunocytochemistry. The expression of mRNA and protein for βRIII was not examined in this study because of the scarcity of specific reagents. Others have reported the presence, in rat glomeruli, of two TGF-β–binding proteins equivalent to βRII and βRIII (85 and 200 kDa) [38].

The exposure of MCs to high-glucose concentrations for a total of 10 to 12 days increased overall specific binding, a change caused by a 50% increase in receptor number. Although not statistically significant, we also observed a 63% increase in the mean apparent dissociation constant, suggesting that a simultaneous reduction in the binding affinity cannot be totally ruled out. However, it is likely that this difference can be attributed to the interexperimental variability in the calculated affinities. Nevertheless, protein cross-linking analysis of ^{125}I-TGF-β bound to RI, RII, and RIII indicated that, at the concentration of ligand used, the net result is increased binding. The finding that high-glucose treatment increased to similar levels mRNA transcripts for βRI and βRII as well as the immunoreactive receptor proteins indicates that the observed increase in binding may result from elevated synthesis of the receptors.

Cyclic mechanical force also increased receptor binding, a change that was qualitatively similar to that induced by high-glucose exposure. As was the case for glucose, stretch also increased the amount of ligand associated with the three receptors types and was accompanied by a corresponding elevation of receptor mRNA and protein. A somewhat greater induction by stretch of message and protein expression was observed for βRI, in comparison to βRII. This difference may have been related to the period studied, because our observations of protein expression showed a decrease in the magnitude of induction with stretching time. By 96 hours, the degree of elevation of βRI protein was nearly equivalent to βRII. A differential response of these two receptors was not observed following exposure to high-glucose concentrations. Another difference in the response to the two stimuli was in the magnitude of the increase. The greater induction of binding by high glucose, as compared with stretch, may also be time dependent; however, this issue was not examined in these studies.

In no case was there an additive or synergistic effect when both stimuli were applied. This finding is in contrast to our previous observations on TGF-β production and collagen synthesis in response to these same treatments [13, 15]. In those studies, although stretching or high-glucose exposure increased TGF-β1 secretion twofold and the corresponding mRNA levels by 30 to 45%, the combination of both stimuli enhanced TGF-β1 secretion greater than fivefold. The significance of this synergistic effect on TGF-β1 secretion was indicated by the greater stretch-induced collagen accumulation that occurred when high glucose was also present [13, 15]. This increase was totally blocked by the neutralization of all mammalian forms of TGF-β with specific antibodies. In the absence of high-glucose concentrations, collagen synthesis was increased by stretch, but due to a corresponding increase in breakdown, collagen accumulation was not enhanced. Taken together, these studies suggest that the mechanisms for induction of TGF-β1 production are unlike those for induction of TGF-β receptors. It may be that increased numbers of TGF-β1 receptors, although important in the degree of the metabolic response to the ligand, particularly when the amount of TGF-β is overabundant, may not be responsible for the combined effects of stretch and high glucose on collagen accumulation that was previously observed.

The precise mechanism(s) for the increase in TGF-β receptor activity is not yet understood. Although it is possible that the increase in active TGF-β, occurring as a response to high glucose or stretch, could be a direct modulator of TGF-β receptors, published studies suggest that this is not likely the case [39]. For example, in human lung carcinoma cells [36] and in rat kidney fibroblasts [32], short-term incubation with exogenous TGF-β down-regulates, rather than increases, the number of TGF-β receptors. There are little data available concerning the long-term effects of TGF-β on the modulation of receptor expression. However, Wakefield et al, in a comparison of 12 different human and rodent cell lines, found no relationship between the amount of TGF-β secreted and the number of TGF-β receptors expressed [36]. Our findings, in this study, of an additive response to mechanical strain and high-glucose levels of increased TGF-β, without similar additive changes in TGF-β receptors, also support this idea. Verification of this lack of relationship between the cytokine and the expression of its receptor may be difficult, as cultured MCs produce and activate abundant amounts of TGF-β, even in the absence of stimulation by high levels of glucose or stretching. A blockade of TGF-β over this period would alter a large number of cellular events, including cell replication, adhesion, and production of matrix, which may be expected to be associated with altered TGF-β binding.

Angiotensin II appears to stimulate ECM synthesis through the induction of TGF-β [40] and may also be a factor in the up-regulation TGF-β receptors. However, this is not a likely mechanism for the observed effects of high glucose and stretch in this study. Although there are rare reports of renin production in MCs [41, 42], it is questionable whether this results in the generation of angiotensin II in tissue culture. We have observed that treatment of MCs with the angiotensin-converting enzyme inhibitor ramipril failed to alter either the baseline collagen synthesis in static, control cells or the collagen production stimulated by stretch (unpublished observation).

Given our understanding that a ternary complex of βRI, βRII, and the ligand is required for signaling, an up-regulation of both receptors may be required for increased responsiveness [16]. However, there is evidence suggesting otherwise. We have successfully transfected MCs with a dominant negative mutant βRII that lacks the cytoplasmic serine/threonine kinase domain but retains the ability to bind TGF-β. Overexpression of this mutant βRII blocked the growth inhibitory effects of TGF-β, but failed to block the induction of collagen type I and fibronectin mRNA (abstract; Choi et al, *J Am Soc Nephrol* 8:493A, 1997). This differential induction indicates alternative signaling pathways for different responses and suggests that increased activity of one of the two signaling receptors may be sufficient to alter specific responses. The function of βRIII is not clear, but it appears to present TGF-β to βRII, which then allows increased interaction of βRII and the ligand with the signaling receptor [18]. The observed increase in βRIII with high glucose or stretching suggests that it too may be involved in the induction of ECM synthesis.

The findings from these *in vitro* experiments may be relevant *in vivo.* Hyperglycemia is an established causal factor in diabetic glomerulosclerosis [43, 44] and may exert its actions, at least in part, by its direct effects on the MCs [11]. This is supported by extensive evidence showing that increased concentrations of glucose, similar to those employed in this study, stimulate ECM accumulation by MCs in culture [7–9, 45]. Glomerular hypertension is a second major causal factor in glomerulosclerosis [46, 47]. The observations made in this study using cyclic mechanical strain in tissue culture are relevant to pathophysiological forces that occur *in vivo* as a result of increased glomerular pressure. Our studies on the elasticity of isolated perfused glomeruli have demonstrated that the degree of stretching used in these studies may be similar to that in the glomerular mesangium during experimental diabetes [14, 44]. In normal glomeruli, effective autoregulation at the afferent arteriole protects against exposure to significant variations in systemic blood pressure and the resulting distention [48, 49]. However, in experimental diabetes [50] and in remnant glomeruli after extensive renal ablation [51], the loss of autoregulation results in the transferal to the glomerular capillaries of the large (up to 55 mm Hg) moment-to-moment variations in systemic blood pressure [52]. As

calculated from our direct measurements of glomerular compliance, these oscillations in intraglomerular pressure may result in the repeated distention and contraction of up to 7.3% of mean glomerular volume and compare with an estimated change of 0.4% under normal circumstances [43]. To what extent the observed increased levels of TGF-β receptor expression and binding contribute to this pathological alteration is undetermined. However, in *in vivo* conditions, including the progressive sclerosis of remnant glomeruli and in diabetic glomerulosclerosis, months or years of evolution of the disease are required for the appearance of advanced glomerular injury. This suggests that even small changes in TGF-β receptor expression may be, in the long term, of pathological significance.

Work in at least two experimental models of glomerulosclerosis support our findings on receptor overexpression. Sharma et al reported that βRII mRNA levels in mice kidney cortices increase up to fourfold following the induction of diabetes by streptozotocin and occur concomitantly with an increase in mRNA encoding for TGF-β1, type IV collagen, and fibronectin [4]. They also observed that, although administration of an antibody that neutralizes TGF-βs 1, 2, and 3 completely blocked the increase in TGF-β1, this treatment did not fully reverse either the diabetic-induced kidney hypertrophy or the elevated mRNA levels for matrix genes and had no effect on increased βRII message. Tamaki et al, in a study on the role of TGF-β1 in adriamycin-induced progressive focal glomerulosclerosis in the same species, observed an increase in the mRNA levels for βRII and βRIII in the renal cortex that paralleled changes in fibronectin, TGF-β1, and renal histological alterations [25].

In summary, there are multiple sites for the regulation of TGF-β action that include synthesis, secretion, and activation of the latent molecule, binding, and signal transduction. We have previously shown that mechanical strain and high glucose are factors that influence TGF-β1 activity by a combined mechanism, altering mRNA levels and secretion and activation of the molecule, to promote ECM production. The findings presented here extend these effects to receptor modulation and suggest that effective therapy to mitigate the effects of TGF-β1 action in diabetic kidney disease may require intervention at multiple levels, or alternatively, downstream of TGF-β binding.

ACKNOWLEDGMENTS

This work was supported by a Juvenile Diabetes Foundation International Grant #1921461 (to Dr. Riser), a Kidney Foundation of Michigan Grant (Dr. Ladson-Wofford), and donations from the John Kontich Renal Research Fund and the Robert T. and Marjorie Herndon Fund (Dr. Guerin). This work was presented in part at the annual meeting of the American Society of Nephrology on November, 1997, in San Antonio, TX, USA. We thank Janet M. Grondin, K.S. Sury Sastry, and Clare Hasset for the expert technical assistance.

Reprint requests to Bruce L. Riser, Ph.D., Henry Ford Hospital, Division of Nephrology and Hypertension, CPF-509, 2799 West Grand Boulevard, Detroit, Michigan 48202, USA.
E-mail: riserb@usa.net

REFERENCES

1. Border WA, Ruoslahti E: Transforming growth factor-β in disease: The dark side of tissue repair. *J Clin Invest* 90:1–7, 1992
2. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by transforming growth factor-β. *Nature* 346:371–374, 1990
3. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E: Glomerulosclerosis induced by in vivo transfection of transforming growth factor-β or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92:2597–2601, 1993
4. 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
5. 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
6. Sharma K, Ziyadeh F, Alzahabi B, McGowan TA, Kapoor S, Kurnik BRC, Kurnik PB, Weisberg LS: Increased renal production of transforming growth factor-β1 in patients with type II diabetes. *Diabetes* 46:854–859, 1997
7. 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
8. Haneda M, Kikkawa 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
9. Nahman NS, Leonhart KL, Cosio FG, Heber CL: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int* 41:396–402, 1992
10. 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
11. Davies M, Martin J, Tomas GJ, Lovett DH: Proteinases and glomerular matrix turnover. *Kidney Int* 41:671–678, 1992
12. Mauer SM, Steffes MW, Ellis EN, Sutherland DER, Brown DM, Goetz FC: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
13. 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
14. Riser BL, Cortes P, Zhao X, Berstein J, Dumler F, Narins RG: Intraglomerular pressure and mesangial stretching stimulate extracellular matrix formation in the rat. *J Clin Invest* 90:1932–1943, 1992
15. Riser BL, Cortes P, Yee J, Sharba AK, Asano K, Rodriguez-Barbero A, Narins RG: Mechanical strain- and high glucose-induced alterations in mesangial cell collagen metabolism: Role of TGF-β. *J Am Soc Nephrol* 9:827–836, 1998
16. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massague J: TGFβ signals through a heteromeric protein kinase receptor complex. *Cell* 71:1003–1014, 1992
17. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J: Mechanism of activation of the TGF-β receptor. *Nature* 370:341–347, 1994
18. Lopez-Casillas F, Wrana JL, Massague J: Betaglycan presents ligand to the TGFβ signaling receptor. *Cell* 73:1435–1444, 1993
19. Rizzino A, Kazakoff P, Ruff E, Kuszynski C, Nebelsick J: Regulatory effects of cell density on the binding of transforming growth factor β, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. *Cancer Res* 48:4266–4271, 1988
20. 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
21. Brandes ME, Wakefield LM, Wahl SM: Modulation of monocyte type I transforming growth factor-β receptors by inflammatory stimuli. *J Biol Chem* 266:19697–19703, 1991
22. Fine A, Panchenko MP, Smith BD, Yu Q, Goldstein RH: Discordant regulation of transforming growth factor-beta receptors by prostaglandin E2. *Biochim Biophys Acta* 126:19–24, 1995
23. Choi ME, Kim EG, Huang Q, Ballerman BJ: Rat mesangial cell hypertrophy in response to transforming growth factor-β1. *Kidney Int* 44:948–958, 1993
24. 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
25. Tamaki K, Okuda S, Ando T, Iwamoto T, Nakayama M, Fujishima M: TGF-β1 in glomerulosclerosis and interstitial fibrosis of adriamycin nephropathy. *Kidney Int* 45:525–536, 1994
26. Dumler F, Cortes P: Uracil ribonucleotide metabolism in rat and human glomerular epithelial and mesangial cells. *Am J Physiol* 255:C712–C718, 1988
27. 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
28. 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
29. Banes AJ, Link GW, Gilbert JW, Tay RTS, Monbureau O: Culturing cells in a mechanically active environment. *Am Biotechnol Lab* 8:12–22, 1990
30. 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, Cold Spring Harbor Press, 1989, p 7.37–7.42
31. Choi ME, Liu A, Ballerman BJ: Differential expression of transforming growth factor-β receptors in rat kidney development. *Am J Physiol* 273(3 Pt 2):F386–F395, 1997
32. Frolik CA, Wakefield LM, Smith DM, Sporn MB: Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. *J Biol Chem* 259:10995–11000, 1984
33. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
34. Holm S: A simple sequentially rejective multiple test procedure. *Scan J Stat* 6:65–70, 1979
35. Scatchard G: Attraction of proteins for small molecules and ions. *Ann NY Acad Sci* 51:660–672, 1949
36. Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MS: Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J Cell Biol* 105:965–975, 1987
37. MacKay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE: Transforming growth factor-β: Murine glomerular receptors and responses of isolated glomerular cells. *J Clin Invest* 83:1160–1167, 1989
38. MacKay K, Robbins AR, Bruce MD, Danielpour D: Identification of disulfide-linked transforming growth factor-β1-specific binding proteins in rat glomeruli. *J Biol Chem* 265:9351–9356, 1990
39. Choi ME, Kim E-G, Haung Q, Ballerman BJ: Rat mesangial cell hypertrophy in response to transforming growth factor-β1. *Kidney Int* 44:948–958, 1993
40. Kagami S, Border WA, Miller DE, Noble NA: Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-β expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431–2437, 1994
41. Dzau VJ, Kreisberg J: Cultured glomerular mesangial cells contain renin: Influence of calcium and isoproterenol. *J Cardiovasc Dis* 10(Suppl):S6–S10, 1986
42. Chansel D, Dussaule J-C, Ardaillou N, Ardaillu R: Identification and regulation of renin in human cultured mesangial cells. *Am J Physiol* 252:F32–F38, 1987
43. 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
44. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
45. Cortes P, Zhao X, Riser BL, Narins RG: Role of mechanical strain in the pathogenesis of diabetic nephropathy. *Kidney Int* 51:57–68, 1997
46. Hostetter TH, Troy JL, Brenner BM: Glomerular hemodynamics in experimental diabetes mellitus. *Kidney Int* 19:410–415, 1981
47. 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
48. Casellas D, Moore LC: Autoregulation of intravascular pressure in preglomerular juxtamedullary vessels. *Am J Physiol* 264:F315–F324, 1993
49. Brenner BM, Griffin KA, Picken M, Daugharty DM: The dynamics of glomerular ultrafiltration in the rat. *J Clin Invest* 50:1776–1780, 1971
50. Hayashi K, Epstein M, Loutzenhiser R, Forster H: Impaired myogenic responsiveness of the afferent arterole in streptozotocin-induced diabetic rats: Role of eicosanoid derangements. *J Am Soc Nephrol* 2:1578–1586, 1992
51. Pelayo J, Wescott JY: Impaired regulation of glomerular capillary hydrostatic pressure in the rat remnant nephron. *J Clin Invest* 88:101–105, 1991
52. 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