Regulation of Glomerular Epithelial Cell Production of Fibronectin and Transforming Growth Factor-β by High Glucose, Not by Angiotensin II

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Accumulation of matrix proteins is a prominent feature of diabetic nephropathy. Glomerular visceral epithelial cells (GVECs) are important contributors to extracellular matrix (ECM) production in the glomerulus. Factors involved with increased accumulation of ECM proteins are high glucose, angiotensin II (ANG II), and transforming growth factor (TGF)-β. Therefore, we investigated the effects of high glucose and ANG II on fibronectin and TGF-β production by human GVECs in vitro. We found that ANG II had no effect on the production of fibronectin and TGF-β by GVECs. Using reverse transcriptase–polymerase chain reaction analysis, no ANG II receptor could be detected on these cells. However, high glucose induced a twofold increase in fibronectin ($P < 0.01$) and a three- to sixfold increase in TGF-β ($P < 0.001$) production. Similar results were obtained by analyzing the mRNA levels of fibronectin (increased 2.7-fold) and TGF-β (increased 3.5-fold). Addition of increasing concentrations of rTGF-β to control cells resulted in increased fibronectin production. Neutralizing antibodies against TGF-β significantly reversed the increase in fibronectin protein and mRNA caused by high glucose back to control levels. We conclude that high glucose concentrations stimulate the synthesis of fibronectin and that this effect is mediated by induction of TGF-β. These results suggest that in diabetic nephropathy, high glucose levels play a role in changing the matrix composition of the glomerular basement membrane through induction of TGF-β. Our results indicate that a contribution to this process by an effect of ANG II on GVECs seems unlikely. *Diabetes* **46:834–840, 1997**

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ATCC, American Type Culture Collection; ATR1, ANG receptor type 1; ATR2, ANG receptor type 2; BSA, bovine serum albumin; D FCS, heat-inactivated fetal calf serum; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GBM, glomerular basement membrane; GVEC, glomerular visceral epithelial cell; MC, mesangial cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcriptase; TGF, transforming growth factor.

An important hallmark of diabetic nephropathy is matrix accumulation in the glomerulus, which is represented morphologically as thickening and expansion of the glomerular basement membrane (GBM) and the mesangium (1). These two types of glomerular matrices are each composed of heparin sulfate/chondroitin/dermatan proteoglycans, laminin, fibronectin, and type IV collagen (2). The mesangial cells (MCs), glomerular visceral epithelial cells (GVECs), and endothelial cells are most likely to be responsible for the biosynthesis and maintenance of the mesangial matrix and GBM (3). An important regulator for the biosynthesis of these matrix molecules is TGF-β. We found that in human MCs, TGF-β induced the synthesis of heparan sulfate proteoglycan and that the production of this matrix molecule was completely blocked after addition of neutralizing anti–TGF-β antibodies (4). In rat MCs, it was found that TGF-β induced the production of biglycan and decorin. The synthesis of other matrix molecules by these cells was unaffected (5). Since TGF-β production in the glomerulus is also likely to affect the epithelial cells, the same authors investigated the effect of TGF-β on matrix production in rat epithelial cells and found that it enhanced the synthesis of both proteoglycans and type IV collagen, laminin, and fibronectin (6). The fact that TGF-β specifically induced an increase in nonproteoglycan components of the extracellular matrix (ECM) (fibronectin, laminin, type IV collagen) in epithelial cells suggests that these cells may be responsible in part for the TGF-β–induced increase of these matrix components in glomerular diseases, including diabetic nephropathy (7–11).

It was previously shown that proximal tubular cells and MCs cultured in high glucose express modest increases in TGF-β1 mRNA and bioactivity. Neutralizing TGF-β bioactivity with specific antibodies reversed the effects of high glucose on the stimulation of collagen (12). In this study, we investigated whether high glucose concentrations had an effect on fibronectin production in human GVECs and what the role of TGF-β was in this respect.

Besides the obvious increase in glucose concentration, there is evidence to suggest a role for ANG II in the pathogenesis of diabetic nephropathy. Inhibition of the generation of ANG II by ACE inhibitors or ANG II receptor antagonists was found to attenuate the progression of glomerulosclerosis in several disease models (13–15) and to slow progression of diabetic nephropathy in humans (16). Studies performed with

MCs, which are known to express ANG II receptors on their surface, have shown that ANG II induces synthesis of several matrix proteins (4,17–20). These effects of ANG II were found to be mediated by induction of TGF-β (4,18). Some authors have suggested a role for ANG II in regulating the matrix production by GVECs, although evidence for an ANG II receptor on these cells is controversial (21–23). Therefore, we studied the effect of ANG II on matrix production by GVECs, the role of TGF-β in this respect, and whether the ANG II receptor could be detected on these cells.

RESEARCH DESIGN AND METHODS

Cell culture. GVEC's were cultured from glomeruli obtained from normal human adult kidneys (n = 5) that could not be used for transplantation because of anatomical reasons. The use of human kidneys (adult and fetal) was approved by the Medical Ethical Committee, University Hospital Leiden. Methods used to culture GVEC's have been published previously (24,25). The cells were characterized by their morphology and immunofluorescence staining. In brief, immediately after outgrowing from the glomeruli (1 week), GVEC's were passaged using phosphate-buffered saline (PBS)–20 mmol/l EDTA into a T25 or T75 flask and grown in Dulbecco's modified Eagle's medium (DMEM) with 5% heat-inactivated fetal calf serum (D FCS; Hyclone Laboratories, Logan, UT), trypsinized, and seeded into T25 or T75 flasks. Characterization of GVEC's was done on the basis of *1*) cell morphology (confluent monolayer of polygonal cells); *2*) positive staining with monoclonal antibodies TN10, anti-cytokeratin (RGE3) (Eurodiagnostics, Apeldoorn, The Netherlands), and anti-CALLA (Dakopatt, Denmark); and *3*) absence of staining using monoclonal antibodies against TN9, anti–von Willebrand factor, and anti-desmin. The monoclonal antibodies TN9 and TN10 specifically recognize proximal tubular epithelial cells and GVECs (26), respectively, and were a gift from Drs. G. Müller and M. Nesper (Medizinische Klinik, Tübingen, Germany). For experimental purposes, cells were grown in 12-well plates in DMEM with 5% D FCS for 24 h and subsequently starved for 24 h in DMEM with 0.5% D FCS. Some wells were washed with PBS, trypsinized, and counted in the Coulter counter to determine the number of cells at the beginning and end of each experiment. The rest of the wells were cultured for a 3-day period in the same medium and assessed for fibronectin and TGF-β production in medium alone or in the presence of 15 or 25 mmol/l glucose or ANG II (Sigma, St. Louis, MO) alone (10^{-6} mol/l or 2×10^{-8} mol/l). Additional experiments were performed using neutralizing anti-human TGF-β1,2,3 monoclonal antibodies (2G7) (27) or human recombinant TGF-β1 (R&D Systems, Abingdon, U.K.).

For RT-PCR analysis, we also used mesangial cells grown out of glomeruli isolated from human kidneys. Methods used to culture these cells are described in detail elsewhere (25). Kidney cortex was prepared from human kidneys by mechanical dissociation.

Immunofluorescence. Cells were grown on sterile glass cover slips (10,000 cells/well) in DMEM containing 5% D FCS. The cells were washed three times with PBS, fixed in acetone for 5 min at 0°C, and air-dried; and immunofluorescent staining was performed by incubating with goat anti-human fibronectin antibodies (Sigma) and rabbit anti-EHS laminin (E-Y Laboratories, San Mateo, CA) antibodies for 30 min. After extensive washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (RAG IgG-FITC) or FITC-conjugated goat anti-rabbit IgG (GAR-FITC) (Nordic) for 30 min in the dark. FITC-conjugated rabbit anti-goat IgG and FITC-conjugated goat anti-rabbit IgG were used as controls. After being washed three times with PBS, the slides were mounted in 1,4 diazobicyclo(2,2,2)octane-glycerol (DABCO)-glycerol), assessed for fluorescence at 340–380 nm (FITC), and photographed through a Leitz microscope (Wetzlar, Germany).

Fibronectin enzyme-linked immunosorbent assay (ELISA). Secreted fibronectin in the medium was measured using an inhibition ELISA as described previously by Nahman et al. (28) with some minor modifications.

TGF-β bioassay and ELISA. GVEC's were cultured in 12-well plates and cultured as described above. The cell media were analyzed for latent and active TGF-β. Latent and active TGF-β were measured by the inhibition of growth of the mink lung cell line CCL-64 (American Type Culture Collection [ATCC], Rockville, MD). Growth inhibition was measured as the decreased uptake of neutral red (29). TGF-β was activated by heating in a water bath for 10 min at 80°C, followed by immediate cooling in ice water. Specificity for TGF-β was demonstrated by the full reversal of any inhibitory activity by the addition of a monoclonal antibody that specifically recognizes human TGF-β1,2,3 (2G7) (27). Latent and active TGF-β in the medium was also measured by sandwich ELISA, using 10 ng/ml human rTGF-β1 as standard for every plate. TGF-β was activated by pH. In brief, mouse anti-human TGF-β (2G7) (2 μg/ml in PBS) was allowed to adhere to 96-well plates for 1 h at 37°C and overnight at 4°C. After extensive washing and a 1-h blocking step with PBS, 0.2% Tween-20, 2% bovine serum albumin (BSA), the samples were added in duplicate to the wells for 2 h at room temperature. After washing, chicken anti-human TGF-β coupled to digoxigenin (DIG) antibodies were incubated for 1 h at room temperature. Plates were washed and anti-DIG F(ab)'$_2$ conjugated to horse radish peroxidase was allowed to bind for 1 h at room temperature. Peroxidase reactivity was visualized by addition of the peroxidase substrate ABTS (2,2-azino-bis-3-ethylbenzthiazolin; Sigma) prepared in 100 mmol/l citrate, 100 mmol/l phosphate buffer, pH 4.2. Optical density was measured at 415 nm.

RT-PCR analysis of the ANG II receptor. Total RNA was isolated from human GVECs, MCs, and kidney cortex, and these isolates were subsequently reverse transcribed into cDNA as described previously (25). Deoxy-oligonucleotide primers were constructed from the published cDNA sequences of ANG receptor type 1 (ATR1) (30), ANG receptor type 2 (ATR2) (31), and β-actin (32). The sequence of the ATR1 was 5′-GCCGTGTCCACAATATCTGC-3′ for the 5 primer and 5′-TGTAAGATTGCTTCAGCCAGA-3′ for the 3 primer. The sequence of the ATR2 was 5′-CCTGAGAAATATGCCCAATGG-3′ for the 5 primer and 5′-CCTTGGAGCCAAGTAATTGG-3′ for the 3 primer. The primers were prepared with a DNA synthesizer. RT-PCR of human MCs and kidney cortex using the ATR1 primers revealed a band of 506 bp. To exclude any mistakes in the PCR reaction, the ANG II PCR 506-bp amplicon was subsequently cloned in p*CR II* according to the manufacturer's instructions. One clone was selected, and sequence analysis identified the clone fragment sequence as identical to ATR1 derived sequence (data not shown).

Northern blot analysis. Total RNA was isolated with RNAzol from GVECs grown in T25 flasks in medium alone or supplemented with 25 mmol/l glucose, 25 mmol/l glucose plus anti–TGF-β (25 ng/ml), or 25 mmol/l glucose plus rTGF-β (10 ng/ml). Fifteen micrograms of total RNA was separated on a 1% (wt/vol) agarose gel containing 2.2 mol/l formaldehyde, pH 4.0, and MOPS buffer (0.02 mol/l MOPS, pH 7.0, 8 mmol/l sodium acetate, 1 mmol/l EDTA, pH 8.0) and blotted to reinforced nitrocellulose (Schleicher & Schuell, Keene, NH), as described by Maniatis et al. (33). Prehybridization and hybridization were done in a hybridization mix consisting of 0.5 mol/l sodium phosphate buffer, pH 7.2, 7% (wt/vol) SDS, 1% (wt/vol) BSA (Sigma), and 1 mmol/l EDTA, 100 μg/ml single-stranded herring sperm DNA as described by Church and Gilbert (34). After 2 h of prehybridization at 65°C, a cDNA probe specific for TGF-β1 (X02812) or a cDNA probe specific for fibronectin (61326) (both from ATCC) was radiolabeled with [α-^{32}P]dCTP by random primed labeling (35) and added to the blot for hybridization overnight at 65°C. After hybridization, the blots were washed for 30 min with three buffers with decreasing molarity of sodium phosphate buffer (0.5, 0.25, and 0.1 mol/l, respectively [pH 7.2]), 1% SDS, and 1 mmol/l EDTA. In addition to ethidium bromide staining, control hybridization for equal loading was performed using 0.5-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (78105; ATCC). The intensities and areas of the bands on the autoradiograms were determined with an Ultroscan XL (LKB, Woerden, The Netherlands).

Statistical analysis. The data for control and experimental groups are expressed as means ± SD.

Statistical analysis was performed using Student's t test for unpaired samples and an analysis of variance test for multiple group comparisons. P values of <0.05 were used to determine significance.

RESULTS

GVECs were characterized according to the described methods. A phase-contrast picture of these cells is given in Fig. 1*A*. Immunofluorescence staining of GVECs with polyclonal antibodies that specifically recognize fibronectin resulted in primarily extracellular staining. Fibronectin was layered as a network on top of the cells (Fig. 1*B*). Fibronectin production was measured using a specific fibronectin ELISA. GVECs cultured in different concentrations of glucose (15 and 25 mmol/l) resulted in a 1.7-fold (15 mmol/l) and 2.3-fold (25 mmol/l) increase in fibronectin production as compared with control levels (5 mmol/l) (Fig. 2, left panel). This moderate increase was also observed in the mRNA levels (Fig. 5, *lane 1* [control] vs. *lane 2* [25 mmol/l]). The addition of different concentrations of ANG II (10^{-6} or 2×10^{-8} mol/l) did not result in any changes in fibronectin production compared with controls (9.9 ± 1.4, 11.1 ± 2.3, and 10.9 ± 2.8 μg/10^5 cells, respectively). Therefore, we analyzed with RT-PCR whether ATR1 or

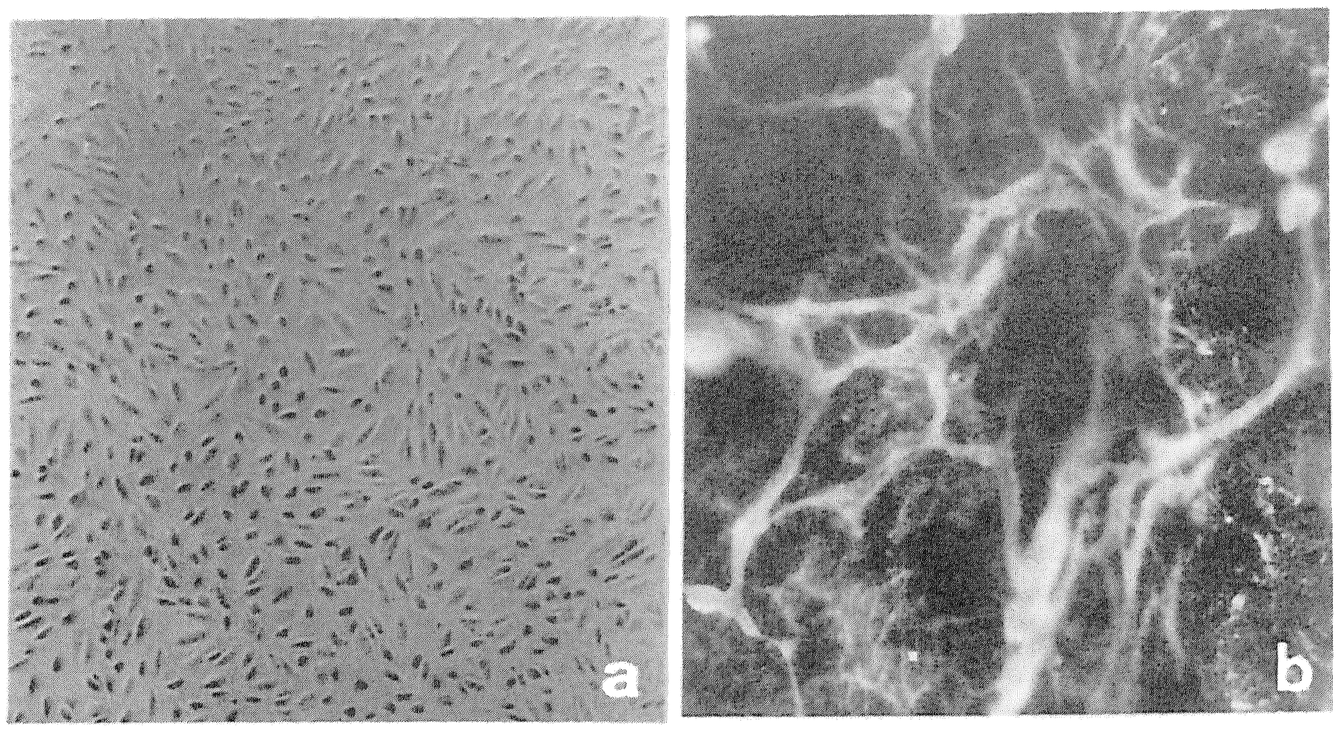

FIG. 1. Phase-contrast microscopy of GVECs in culture (*a*; magnification ×100) and immunofluorescence microscopy of GVECs in culture stained with goat anti-human fibronectin (*b*; ×400). Rabbit anti-goat FITC and goat anti-rabbit used as controls were both negative (data not shown).

ATR2 is present on GVECs. RT-PCR analyses of MCs and adult whole kidney cortex were used as controls for expression of ATR1 (17,20,36,37). Whole fetal kidney was used as a control for ATR2 expression (37–38). Aliquots of GVECs, MCs, and kidney cortex cDNA that yielded 540-bp β-actin PCR products with similar intensities (Fig. 3) were used for ATR1 and ATR2 amplification. Amplification with the specific ATR1 primer set revealed the presence of transcript for the 506-bp ATR1 product in the cDNA obtained from MCs (*lane 1*) and kidney cortex (*lane 4*). Amplifications of cDNA from other MC lines were all positive (n = 4; data not shown). However, amplification of cDNA from four different GVEC lines obtained from different donors (Fig. 3, *lanes 2* and *3* are shown as a example) did not result in an ATR1 product. Cultured GVECs are known to undergo phenotypic modulation and obtain characteristics that have been described for fetal renal tissues (CALLA positive) (24). Since the ATR2 receptor is found to be present predominantly on fetal kidneys (37,38), we also performed amplification for the ATR2 product to determine whether GVECs express this type of receptor. Only amplification of human fetal kidney resulted in a 426-bp ATR2 product (Fig 3, *lane 4*). cDNA of different lines of MCs (n = 4) (*lane 1* as example) and GVECs (n = 4) (*lane 2* as example) did not result in an ATR2 product. Amplification of human adult kidney cortex resulted in a vague band (*lane 3*).

To establish that the rise in fibronectin production caused by high glucose was accompanied by the induction of TGF-β, active TGF-β was measured using the standard mink lung cell line bioassay and specific TGF-β ELISA. Using the bioassay, the mean active TGF-β in four control cultures was 0.17 ng · ml^{-1} · 10^5 $cells^{-1}$ (Fig. 4, left panel), whereas we could hardly detect any active TGF-β using the ELISA (Fig. 4, right panel). When cocultured with 15 mmol/l glucose, an 8-fold increase was seen (Fig. 4; $P < 0.01$), and with 25 mmol/l glucose, the mean active TGF-β was increased 33-fold (Fig. 4; $P < 0.001$). This increase in TGF-β activity was completely abrogated by neutralizing anti–TGF-β antibody, whereas mouse IgG had no effect (Fig. 4, middle panel), indicating the specificity of the bioassay. Similar results were seen when active TGF-β was measured with the specific TGF-β ELISA. Northern blot analysis revealed that there was a low basal expression of TGF-β message (Fig. 5, *lane 1*). On stimulation

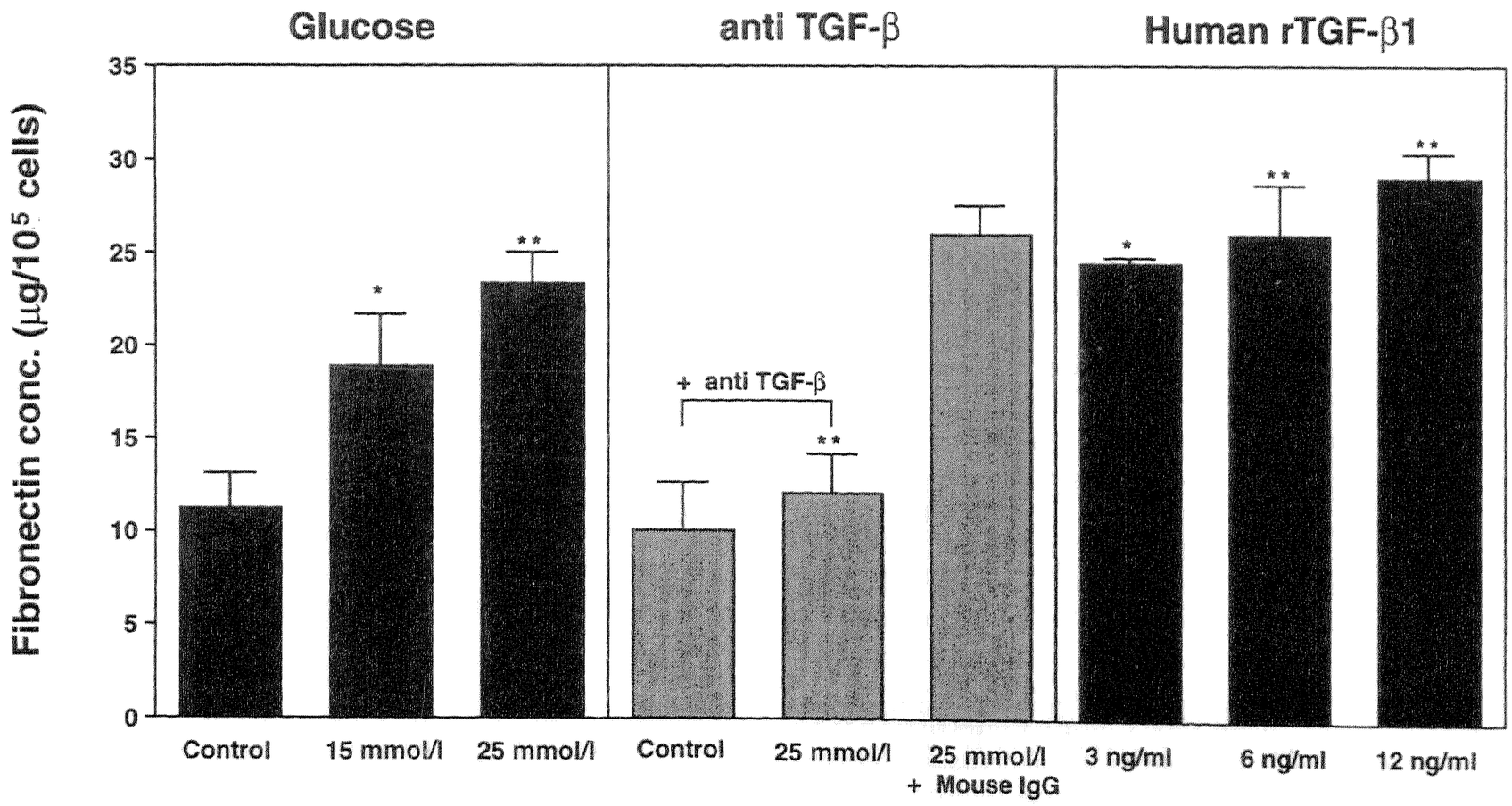

FIG. 2. The effect of increasing concentrations of glucose on the production of fibronectin by GVECs in vitro. GVECs were cultured for three days in the presence of normal (5 mmol/l) or high (15 and 25 mmol/l) glucose concentrations. Excreted fibronectin was measured using a specific inhibition ELISA (left panel). The role of TGF-β in the induction of fibronectin by high glucose was determined by treatment of high-glucose–stimulated cells with anti–TGF-β antibodies (25 µg/ml). Mouse IgG (25 µg/ml) was used as control (middle panel). The right panel represents the effect of human recombinant TGF-β1 (3–12 ng/ml) on fibronectin production. Fibronectin is expressed as grams per 10^5 cells. *$P < 0.01$, **$P < 0.001$; n = 4.

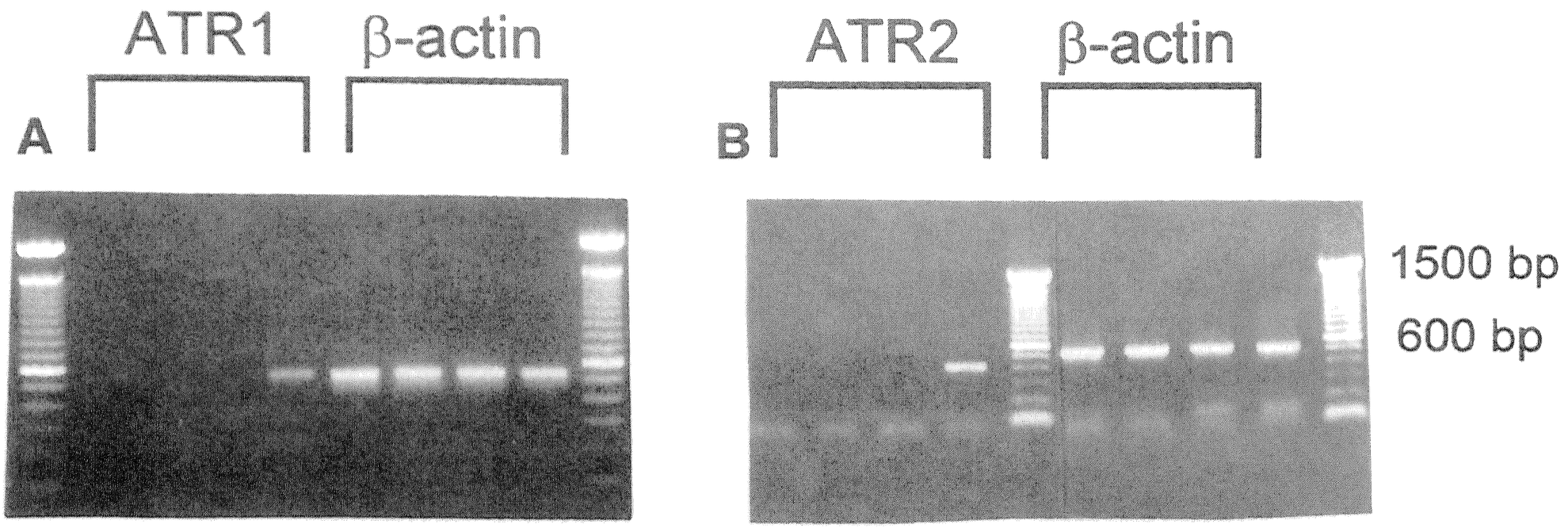

FIG. 3. *A:* **RT-PCR for ATR1 expression on human MCs (*lane 1*), human GVECs (*lanes 2* and *3*; representative of four different cell lines), and human adult kidney cortex (*lane 4*). *B:* RT-PCR for ATR2 expression on human MCs (*lane 1*), human GVECs (*lanes 2* and *3*; representative of four different cell lines), and human fetal kidney cortex (*lane 4*). Amplification of the cDNA obtained from human MCs, human GVECs, and human adult and fetal kidney cortex yielded a 540-bp β-actin PCR product with similar intensity. Amplification with the ATR1 primer set revealed the presence of transcripts for the ATR1 product of 506 bp in both human kidney and human MCs. No transcript could be detected for any GVEC line ($n = 4$). Amplification with the ATR2 primer set revealed the presence of transcripts for the ATR2 product of 426 bp in both human fetal kidney and human MCs. No transcript could be detected for any GVEC line ($n = 4$). Numbers on the right are markers in base pairs.**

with high glucose (25 mmol/l), this basal mRNA was increased 3.5-fold (Fig. 5, *lane 2*).

To assess whether the increase in active TGF-β was associated with the increase in fibronectin, high-glucose–treated cells (25 mmol/l) were co-incubated with neutralizing anti–TGF-β antibodies or control mouse IgG (Fig. 2, middle panel). Treatment of high-glucose–stimulated cells with anti–TGF-β antibodies almost completely prevented the increase in fibronectin production. This was not seen with the control mouse IgG. However, no change in basal level of fibronectin was observed in control cells after addition of neutralizing TGF-β antibodies, indicating that this basal fibronectin secretion by GVECs is independent of the TGF-β concentration. The fact that sufficient neutralizing TGF-β antibodies had been added was confirmed by the finding that in this conditioned medium, control levels of TGF-β were measured (<0.17 ng · ml^{-1} · 10^5 cells^{-1}) (Fig. 4). Northern blot analysis revealed a reverse in fibronectin mRNA levels when high-glucose–stimulated cells were treated with anti–TGF-β antibodies (25 mmol/l plus anti–TGF-β antibodies, 1.5-fold increase compared with 25 mmol/l, 2.7-fold increase) (Fig. 5, *lane 3*). It has been previously shown by immunoprecipitation that rat GVECs exposed to rTGF-β1 increase their fibronectin secretion around threefold (6). The role of TGF-β in the production of fibronectin by GVECs was investigated by addition of different concentrations of rTGF-β1. For this we used concentrations of TGF-β that were in the range of those observed after stimulation of GVECs with high glucose (3–12 ng/ml). Using the quantitative fibronectin ELISA, we found that rTGF-β increased fibronectin production between 2.5-

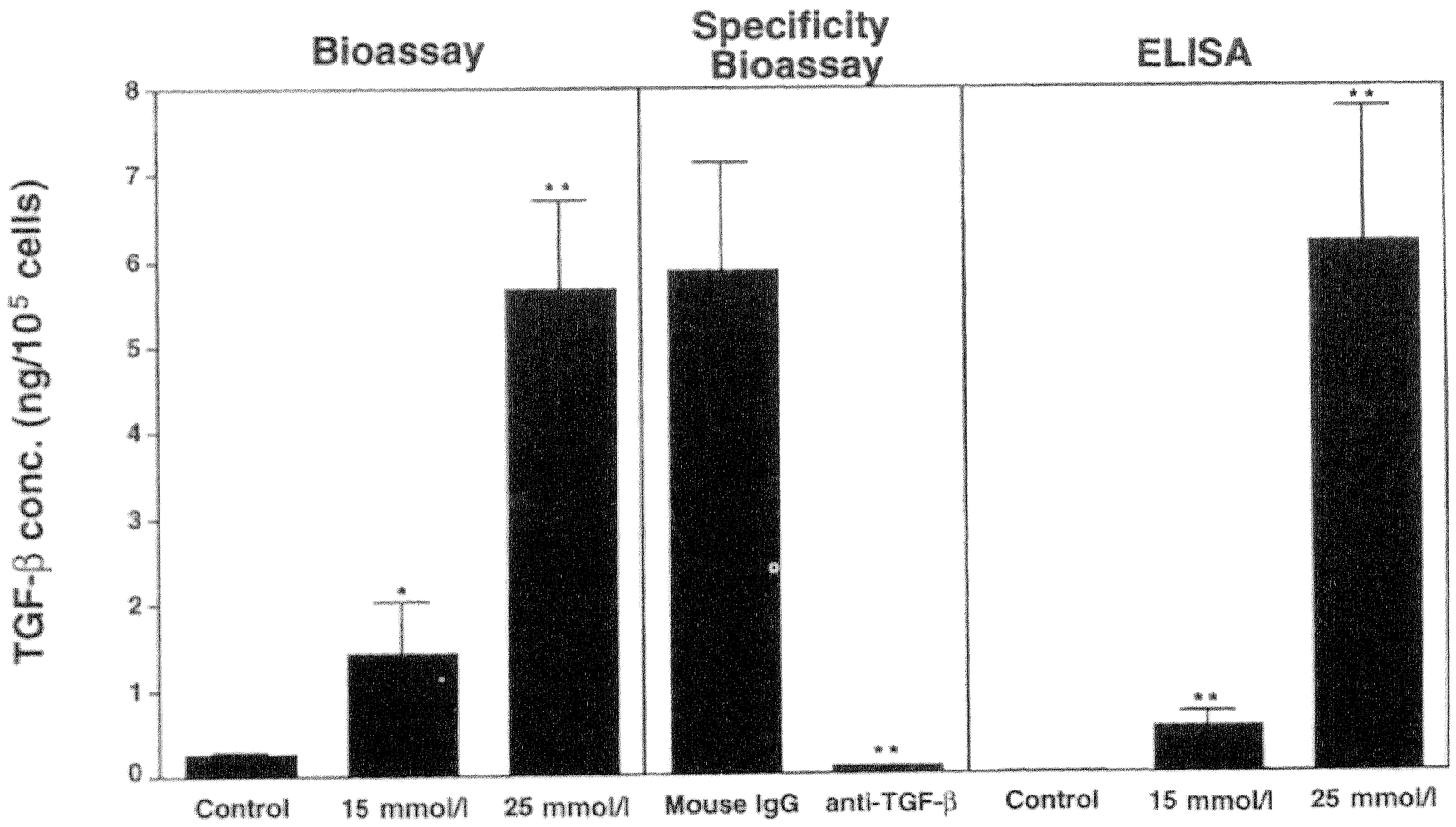

FIG. 4. Effect of increasing concentrations of glucose on the production of TGF-β by GVECs in vitro. GVECs were cultured for 3 days in the presence of normal (5 mmol/l) or high (15 and 25 mmol/l) glucose concentrations. Excreted TGF-β was activated and subsequently measured using a bioassay and an ELISA. Specificity for the bioassay was checked using neutralizing anti–TGF-β antibodies with mouse IgG as control. Active TGF-β is expressed as nanograms per 10^5 cells. *$P < 0.01$, **$P < 0.001$; $n = 4$.

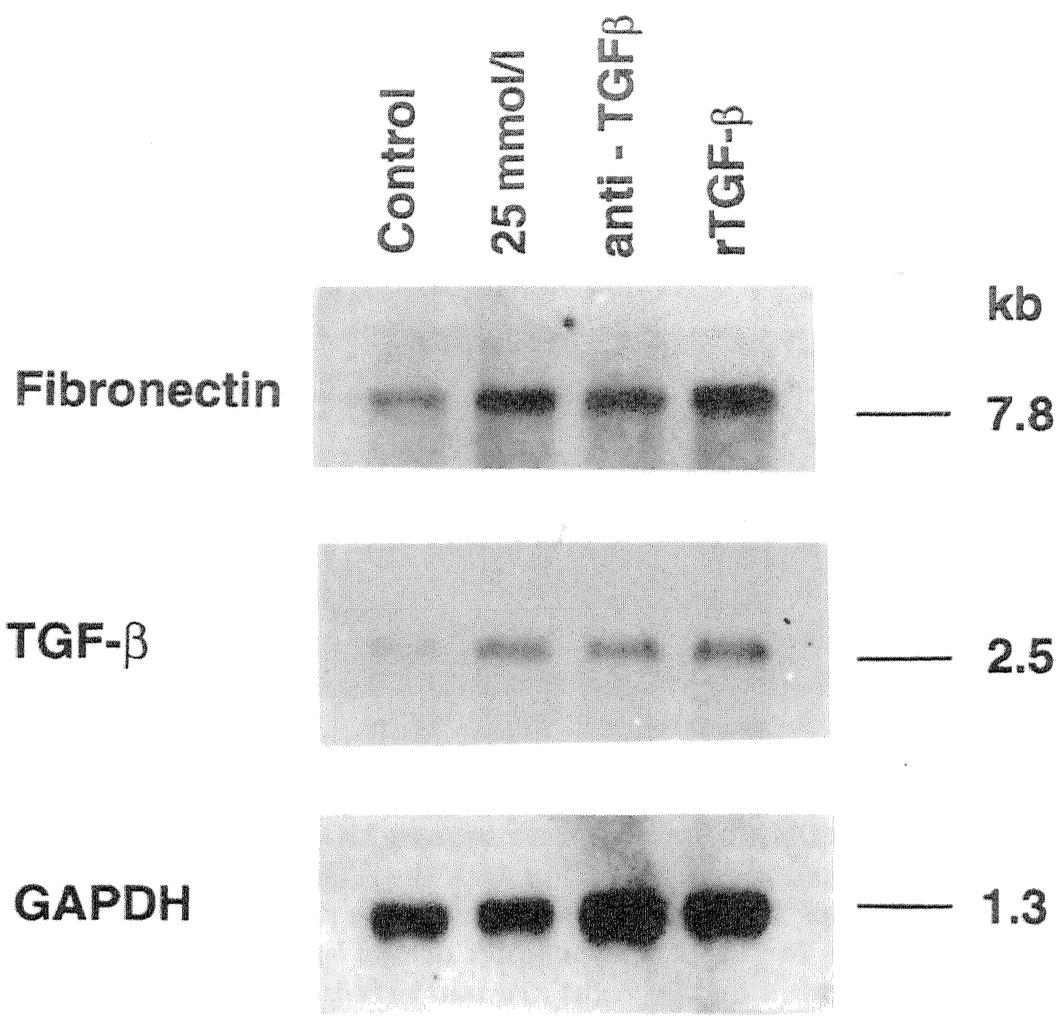

FIG. 5. Effect of high glucose (25 mmol/l) (*lane 2*), 25 mmol/l plus anti–TGF-β (25 μg/ml) (*lane 3*), and human rTGF-β1 (10 ng/ml) (*lane 4*) on mRNA levels of fibronectin (7.8 kb) and TGF-β (2.5 kb) in GVECs compared with control (*lane 1*). Northern analysis of 10 μg total RNA isolated from GVECs grown in T25 flasks was performed. Control hybridization was performed using GAPDH (1.3 kb).

and 3-fold (Fig 2, right panel). This effect on fibronectin protein also occurred at the mRNA level (2.3-fold increase) (Fig. 5, *lane 4*). Northern blot analysis showed that rTGF-β1 also induced its own mRNA by threefold (Fig. 5, *lane 4*).

DISCUSSION

Diabetic nephropathy encompasses structural alterations that are characterized by early hypertrophy of the glomerulus, thickening of the GBM, progressive accumulation of ECM components, and proteinuria (40–44). The important role of hyperglycemia in the initiation of diabetic nephropathy has been substantiated by examining the effects of high glucose levels in renal cell cultures. Studies growing rat and human cultured MCs in high glucose found stimulated type IV collagen, laminin, and fibronectin protein synthesis and mRNA levels (12,28,45–49). Two studies (12,49) have found the high-glucose–induced stimulation of matrix production to be mediated by TGF-β. In fact, TGF-β was found to induce matrix production in both rat MCs and rat GVECs. Another factor that has recently emerged as a potent regulator of matrix production is ANG II. In light of the findings that ACE inhibitors, which block the generation of ANG II, may protect against deterioration of renal function in diabetes and that this therapy-decreased proteinuria is independent of hemodynamic alterations, several investigators have studied the specific role of ANG II in that respect. Using rat MCs, it was recently found that ANG II specifically increases biglycan and decorin production, and this effect was found to be mediated by TGF-β (18).

GVECs are thought to be the major producers of the matrix proteins within the GBM. We hypothesized that GVECs could play a important role in the alteration of these matrix components in the GBM and thereby have an effect on proteinuria in glomerular diseases such as diabetic nephropathy. We studied the effect of two potential parameters that can change matrix production in the glomerulus, high glucose and ANG II, on the fibronectin production by human GVECs and found that high glucose plays a decisive role in increasing the fibronectin production, since this was unaffected by ANG II. Also, no synergistic effect was found when GVECs were cocultured with high glucose and ANG II. Furthermore, since no effect of ANG II on the fibronectin production was found we analyzed whether a specific ANG II receptor was present on these cells. Yamada et al. (21) and Bianchi et al. (22) have reported binding of ^{125}I-labeled ANG II to the visceral epithelium in vivo. Although these earlier studies have speculated on the possible presence of an ANG II receptor on GVECs, convincing evidence is still lacking. Using specific primers, we could not detect the ATR1 or ATR2 on four different human GVEC lines. Control experiments verified that these primers amplify the correct products. van Son et al. (23) reported recently that the ATR1 could not be detected on mouse GVECs.

We examined the possibility that ECM produced by human GVECs is a biochemical target for TGF-β, as was recently described for rat GVECs (6). First, we examined endogenous production of TGF-β, which to our knowledge had not been described earlier. Similar to findings in proximal tubule cells (50) and MCs (51), we found using the bioassay that human GVECs produced low levels of endogenous active TGF-β. No endogenous TGF-β (latent or active) could be detected using the TGF-β ELISA. The difference in this respect between the results obtained from ELISA and those from bioassay can possibly be explained by the differences in sensitivity and methods used to detect TGF-β (bioassay, inhibition of cell proliferation caused by TGF-β; ELISA, recognition of the TGF-β epitope). Using both methods, we found that addition of 15 or 25 mmol/l glucose to GVECs significantly increased the active TGF-β produced. Furthermore, an increase in TGF-β mRNA level was also observed after coculture with high glucose, indicative of regulation at a transcriptional level.

TGF-β itself was found to upregulate the production of proteoglycans in rat MCs without any effect on fibronectin, laminin, or type IV collagen. However, in rat GVECs, TGF-β resulted in an increase of these nonproteoglycan matrix proteins. It has been previously shown by immunoprecipitation that rat GVECs exposed to rTGF-β1 increase their fibronectin secretion around threefold (6). Similar to the findings in rat GVECs, we found that rTGF-β1 stimulated fibronectin production by human GVECs. This was also observed at the mRNA level. In addition, our findings clearly show that for GVECs, the increase in fibronectin production induced by high glucose is mediated via TGF-β, since neutralizing this with anti–TGF-β antibodies reversed the effect. This was observed at both the protein and mRNA levels. In human umbilical vein endothelial cells (HUVECs), high glucose was found to increase matrix synthesis, and this was associated with increases in TGF-β (52). However, contrary to our results obtained in human GVECs, upregulation of fibronectin by high glucose was not mediated by TGF-β.

The mechanisms by which elevated glucose increases TGF-β levels are unknown, but several hypotheses have been put forward. First, increased glucose levels affect the polyol pathway, with subsequent alterations in *myo*-inositol metab-

olism in a number of target tissues, including kidneys of diabetic rats (53). In a mouse cortical tubule cell line, it was found that addition of *myo*-inositol reduced the glucose-induced increase in type I and IV collagen (54). A second mechanism involves the nonenzymatic glycation of extracellular or intracellular proteins (52,55–56). This could lead to alterations in the structure of receptors and/or regulatory proteins and thus alter TGF-β activity or synthesis. A third mechanism could involve TGF-β itself, since it is known that TGF-β can change its production and mRNA levels by self-induction (57). Indeed, the mRNA of TGF-β was increased when GVECs were cocultured with rTGF-β1. A fourth mechanism could involve a high-glucose–induced change in decorin metabolism, since this chondroitin/dermatan sulfate proteoglycan is known to bind and thereby inactivate TGF-β (58). Finally, protein kinase C could have an important effect on mediating TGF-β increases in diabetes. Protein kinase C activity is highly upregulated in the glomeruli of animal models of diabetes (59,60). Several activators of protein kinase C, such as high glucose, ANG II, phorbol ester (59–62), and LDL, increase TGF-β bioactivity and mRNA and thereby increase ECM production.

In conclusion, we have shown that high glucose stimulates the synthesis of fibronectin and that these effects are mediated by induction of TGF-β. No effect of ANG II was found and no ANG II receptor could be detected on GVECs. These results suggest that in diabetic nephropathy, the high glucose levels may play a role in changing the matrix composition of the GBM.

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