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

Nicole F. van Det, Nicole A.M. Verhagen, Jouke T. Tamsma, Jo H.M. Berden, Jan A. Brujin, Mohamed R. Daha, and Fokko J. van der Woude

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)-ß. Therefore, we investigated the effects of high glucose and ANG II on fibronectin and TGF-ß production by human GVECs in vitro. We found that ANG II had no effect on the production of fibronectin and TGF-ß by GVECs.

Using reverse transcriptase–polymerase chain reaction (RT-PCR) 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-ß (P < 0.001) production. Similar results were obtained by analyzing the mRNA levels of fibronectin (increased 2.7-fold) and TGF-ß (increased 3.5-fold). Addition of increasing concentrations of rTGF-ß to control cells resulted in increased fibronectin production. Neutralizing antibodies against TGF-ß 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-ß.

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-ß. Our results indicate that a contribution to this process by an effect of ANG II on GVECs seems unlikely.

A n 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 heparan 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-ß. We found that in human MCs, TGF-ß induced the synthesis of heparan sulfate proteoglycan and that the production of this matrix molecule was completely blocked after addition of neutralizing anti–TGF-ß antibodies (4). In rat MCs, it was found that TGF-ß induced the production of biglycan and decorin. The synthesis of other matrix molecules by these cells was unaffected (5). Since TGF-ß production in the glomerulus is also likely to affect the epithelial cells, the same authors investigated the effect of TGF-ß 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-ß 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-ß–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-ß1 mRNA and bioactivity. Neutralizing TGF-ß 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-ß 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. GVECs 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 Ethics Committee, University Hospital Leiden. Methods used to culture GVECs 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), GVECs were passaged using phosphate-buffered saline (PBS)-20 mmol/l EDTA into T25 or T75 flasks as a monolayer of polygonal cells; J L > J positive staining with monoclonal antibodies (Sigma) and anti-desmin (Boehringer, Germany). The monoclonal antibodies TN10, anti-vitronectin (R&D) (Euroulgoostics, Apeldoorn, The Netherlands), and anti-CALB (Dako, Denmark) and J L absence of staining using monoclonal antibodies against TN10, anti-vitronectin, and anti-desmin. The monoclonal antibodies TN10 and TN10 specifically recognize proximal tubular epithelial cells and GVECs (20), respectively, and were a gift from Drs. G. Müller and M. Nescper (Medizinische Klinik, Tübingen, Germany). For experimental purposes, cells were grown in 12-well plates in DMEM (with 5% heat-inactivated fetal calf serum (DPCS; Ilyclone Diabetics, Vol. 49, May 1997

Characterization of GVKCs was done on the basis of L cell morphology (confluent monolayer of polygonal cells); J L positive staining with monoclonal antibodies (Sigma) and rabbit anti-ECS integrin (E-Y laboratories, San Mateo, CA) antibodies for MO min. After extensive washing with PBS, the cells were prepared with a DNA synthesizer. RT-PCR of human MCs mid kidney cortex, and these isolates were subsequently cloned in pcR 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 DMEM (26) mmol/l glucose, 25 mmol/l glucose plus anti-TGF-β (25 ng/ml), or 25 mmol/l glucose plus TGF-β (10 ng/ml). Fifteen micrograms of total RNA was separated on a 1% (w/v) agarose gel containing 2.2 mol/l formaldehyde, pH 4.0, and MOPS buffer (0.25 mol/l MOPS, pH 7.0, 0.9 mmol/l sodium acetate, 1 mmol/l EDTA, pH 8.0) and blotted to nitrocellulose (Schleicher & Schuell, Keene, NH), as described by Maniatis et al. (39). Hybridization and hybridization were done in a hybridization mix consisting of 0.5 mol/l sodium phosphate buffer, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA (Sigma), and 1 mmol/l EDTA, 100 ng/ml single-stranded herring sperm DNA as described by Church and Gilbert (34). After 2 h prehybridization at 65°C in a CNA probe specific for TGF-β1 (X02812) or a CNA probe specific for fibronectin (013260) (both from ATCC) was radiolabeled with [32P]dCTP by random priming labeling (35) and added to the blot for hybridization overnight at 0°C. After hybridization, the blots were washed for 30 min with three buffers with decreasing stringency 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 with 0.5 kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (78105; ATCC). The intensities and areas of bands on the autoradiograms were determined with an Ultrilum XL (LAS, 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. 1A. 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. IB). 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 2.5-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 glucose]). The addition of different concentrations of ANG II (10-6 or 2 × 10-5 mol/l) did not result in any changes in fibronectin production compared with controls (3.0 ± 1.4, 11.1 ± 2.3, and 10.9 ± 2.8 μg/106 cells, respectively). Therefore, we analyzed with RT-PCR whether ATR1 or
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) 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 - 10⁵ 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.
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 fibroblast, 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 fibroblasts production. This was not seen with the control mouse IgG. However, no change in basal level of fibroblasts was observed in control cells after addition of neutralizing TGF-β antibodies, indicating that this basal fibroblasts secreting 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 · 10⁶ cells⁻¹) (Fig. 4). Northern blot analysis revealed a reverse in fibroblasts 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 fibroblasts secretion around threefold (6). The role of TGF-β in the production of fibroblasts 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 fibroblasts ELISA, we found that rTGF-β increased fibroblasts production between 2.5-

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.

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 10⁵ cells. *P < 0.01, **P < 0.001; n = 4.
**DISCUSSION**

Controlled production of recombinant human c-Fos was performed using GAdPl (7.1.3).

The expression of c-Fos is detected by immunochemistry with the antibody to c-Fos. The expression of c-Fos is further enhanced by the addition of TGFβ.

In the presence of TGFβ, the expression of c-Fos is increased, and the production of TGFβ is also increased. This suggests that TGFβ acts as a positive regulator of c-Fos expression.

**Figure 1**

- **TGFβ**
- **c-Fos**
- **Control**

The expression of c-Fos is detected by immunochemistry with the antibody to c-Fos. The expression of c-Fos is further enhanced by the addition of TGFβ.

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**Figure 2**

- **anti-TGFβ**
- **TGFβ**
- **Control**

The expression of c-Fos is detected by immunochemistry with the antibody to c-Fos. The expression of c-Fos is further enhanced by the addition of TGFβ.

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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 co-cultured with RTG-2 β. 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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