Sequential effects of high glucose on mesangial cell transforming growth factor-β1 and fibronectin synthesis

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Background. Transforming growth factor (TGF)-β is recognized as the final common mediator of the principal lesions of diabetic nephropathy such as renal hypertrophy and mesangial expansion. To gain better understanding of the temporal relationships between high glucose (HG) and mesangial cell (MC) TGF-β1 synthesis and between TGF-β1 and extracellular matrix (ECM) synthesis, the present study examined early and sequential effects of HG on TGF-β1 and fibronectin (FN) mRNA expression and protein synthesis.

Methods. Confluent primary rat MC was stimulated with 5.6 (control) or 30 (high) mM glucose after synchronizing the growth by incubation with serum-free media for 48 hours.

Results. Mesangial cell TGF-β1 mRNA expression increased significantly in six hours and continued to increase until 48 hours in response to HG. The level of TGF-β1 mRNA was 1.5-fold higher than that of control glucose at six hours and 1.8-fold at 48 hours. TGF-β activity in heat-activated conditioned media under HG increased 1.5- and 1.6-fold at 24 and 48 hours, respectively, compared to control glucose. FN mRNA increased significantly at 24 and 48 hours and 1.4-fold that of control glucose at both time points. FN protein also increased 1.5-fold that of control glucose at 48 hours. Anti-TGF-β antibody completely abolished HG-induced FN synthesis.

Conclusions. The present findings demonstrate that HG stimulates TGF-β1 very early and prior to FN production and that HG-induced FN production is mediated by TGF-β. This finding is consistent with the view that TGF-β mediates increased ECM accumulation by MC under high glucose conditions.

Excessive deposition of extracellular matrix (ECM) and subsequent mesangial expansion is the principal structural lesion of diabetic kidney [1, 2]. Transforming growth factor (TGF)-β stimulates the deposition of ECM through (i) directly up-regulating the genes of matrix proteins leading to their increased synthesis, (ii) suppressing the production of proteases that normally degrade the matrix and increasing synthesis of the inhibitors of the same proteases, (iii) modulating the expression of integrins on the cell’s surface in a manner that facilitates attachment to the newly synthesized matrix, and (iv) autoinducing its own production, which greatly amplifies its action [3–6]. Increased expression of TGF-β and ECM mRNAs and proteins in the kidneys of diabetic animals [7–15] and diabetic patients [9, 16, 17] implicates TGF-β in the pathogenesis of diabetic nephropathy.

Mesangial cells cultured under high glucose exhibit increased expression of ECM mRNAs and proteins [18, 19] and is an accepted in vitro model of diabetic nephropathy. Expression of TGF-β mRNA and bioactivity are also increased in this in vitro model [20–22].

Recent studies have demonstrated that neutralizing antibody against TGF-β significantly reduced high glucose-induced collagen production in mesangial cells [21] and fibronectin production in glomerular epithelial cells [23]. TGF-β1 antisense oligonucleotides [24] attenuated porcine mesangial fibronectin and heparan sulfate proteoglycan production stimulated by high glucose. Anti-TGF-β antibody also attenuated renal hypertrophy and ECM gene expression in diabetic mice [25]. All these studies suggest that TGF-β is the final common mediator of the principal lesions of diabetic nephropathy [6].

Consistent with this hypothesis, increased TGF-β expression was observed in renal cortex very early after the onset of diabetes and before manifestation of ECM expansion. Streptozotocin-induced diabetic rats showed an increased TGF-β expression at 24 hours after the induction of diabetes as measured by polymerase chain reaction (PCR) analysis [11]. A recent study utilizing in situ hybridization demonstrated increased glomerular TGF-β1 mRNA expression at three days after injection of streptozotocin [15]. Spontaneous diabetic BB rat and NOD mouse [12] and streptozotocin-induced diabetic mice [25] also exhibited increased TGF-β1 mRNA and protein levels in the renal cortex a few days after the appearance of glycosuria.


HORMONES - CYTOKINES - SIGNALING

Key words: diabetic nephropathy, TGF-β1, anti-TGF-β antibody, renal hypertrophy, extracellular matrix, protein synthesis.

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However, the temporal relations between high glucose and mesangial cell TGF-β1 mRNA expression and protein synthesis and between TGF-β1 and fibronectin mRNA expression and protein synthesis have not been established. The present study examined early and sequential effects of high glucose concentration on mesangial cell TGF-β1 and fibronectin mRNA expression and protein synthesis. In addition, the effect of neutralizing antibody to TGF-β on fibronectin synthesis was determined to examine the role of TGF-β in high glucose-induced fibronectin synthesis by mesangial cells.

**METHODS**

**Mesangial cell culture**

Mesangial cells were obtained by culturing glomeruli isolated from kidneys of 100 to 150 g male Sprague-Dawley rats by conventional sieving methods as previously described [26, 27]. Mesangial cells were identified by phase contrast microscopy according to the morphological criteria and by immunofluorescence technique. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone, 44 mM NaHCO₃, and 14 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES). Near confluent mesangial cells grown in 100 mm culture dish were incubated with 5.6 and 30 mM glucose media in the presence of 25 μg/ml neutralizing mouse anti-bovine TGF-β antibody (Ig G fraction, neutralizes TGF-β1, -β2, and -β3; Genzyme Corporation, Cambridge, MA, USA). After additional incubation, the media were collected, centrifuged to remove cell debris, and used for bioassay of TGF-β1 and immunoblot analysis of fibronectin. After removing the media, the mesangial cells were washed with phosphate buffered saline (PBS), then lysed using 1 ml of a mixture consisting of 4 m guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 mM 2-mercaptoethanol for RNA isolation. All experiments were performed using cells between the 6th and 8th passages.

**Northern blot analysis**

Standard Northern blot was performed as previously described [28] after isolation of total RNA using the method of Chomczynski and Sacchi [29]. In brief, cells were homogenized with 4 M guanidium thiocyanate. Total protein and DNA were extracted with acid phenol, and RNA precipitated with isopropanol. After washing with ethanol, the amount of RNA was quantitated by measuring the absorbance at 260 nm using a spectrophotometer (Ultraspec 3000; Pharmacia BioTech, England, UK). Twenty micrograms of total RNA were electrophoresed through a 1.2% agarose gel with 2.2 M formaldehyde. The RNA from the gel was transferred onto nylon membranes using a capillary transfer and covalently cross-linked to the membrane with UV light using a gene-linker (Bio-Rad, Richmond, CA, USA).

Prehybridization was performed for five hours at 42°C using a commercial prehybridization buffer (GIBCO BRL, Gaithersburg, MD, USA). Hybridization was conducted for 20 hours at 42°C using excised cDNA inserts as probes after [32P]dCTP-labeling by a random primer extension method (Pharmacia Biotech, Uppsala, Sweden). Rat cDNA probes for TGF-β1 and fibronectin were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was constructed based on the previously published sequence using the PCR [30]. The membranes were washed three times for 30 minutes: first in 2× sodium chloride and sodium citrate (SSC; 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) with 0.1% sodium dodecyl sulfate (SDS) at room temperature; the second in 0.2× SSC with 0.1% SDS at room temperature; and the third in 0.2× SSC with 0.1% SDS at 55°C. Autoradiography was performed by exposing the blots to Kodak X-Omat K XK-1 X-ray film with intensifying screens at −70°C for one to five days. The blots were then rehybridized with a 32P-labeled human GAPDH cDNA probe as an internal control to assess RNA quantity and integrity. Quantification of mRNA signals was performed by densitometry using MCID (Imaging Research Inc., St. Catharines, Ontario, Canada) and normalized with GAPDH mRNA signals.

**Bioassay for TGF-β activity**

The activity of TGF-β present in the conditioned media was measured using the mink lung epithelial cell (MLEC) growth inhibition assay, where human recombinant TGF-β1 (Genzyme; 0.64 to 10,000 pg/ml) was used to generate a standard curve [31]. Preliminary study revealed undetectable TGF-β activity in the conditioned media under our experimental condition. The activation of latent TGF-β was performed by heating conditioned media at 80°C for 15 minutes. In brief, CCL-64 cells (ATCC) were grown in 96-well culture plates with RPMI containing 5% FBS and conditioned media for 96 hours. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue) was then added and cells were incubated for another eight hours. Elution buffer was added and cells were incubated for additional 48 hours and optical density was measured at 562 nm. Under our experimental condition, half-maximally effective concentrations of TGF-β1 inhibiting MLEC growth (EC50) was around 200 ng/liter. Unconditioned media containing 30 mM glucose did not exhibit discernible effect on MLEC growth inhibition assay.
Immunoblot analysis of fibronectin protein

Immunoblot analysis was performed to determine the production and secretion of fibronectin into the mesangial cell conditioned media [32]. After measuring the concentrations of protein by the Bradford method [33] using the Bio-Rad assay, aliquots of conditioned media were mixed with sample buffer containing SDS and β-mercaptoethanol and heated at 95°C for 15 minutes. Samples were applied to 5% polyacrylamide gel and electrophoresed. A prestained SDS-PAGE standard (broad range, Bio-Rad) was also electrophoresed as a molecular weight marker. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris and heated at 95°C for 2 hours at room temperature. After washing, the membranes were incubated with a color reagent (50 mg 3,3'-diaminobenzidine, 0.1 ml H₂O₂ in 100 ml PBS) for 15 minutes or ECL detection reagents (Amersham Life Science, Little Chalfont, England, UK) for precisely one minute followed by autoradiography. Positive immunoreactive bands were quantitated densitometrically and compared to controls.

Analysis of data

All results are expressed as mean ± standard error (SE). Analysis of variance was used to assess the differences between multiple groups. If the F statistic was significant, the mean values obtained from each group were then compared by Fisher’s least significant difference method. A P value < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

Time sequence of high glucose-induced TGF-β1 and fibronectin mRNAs in mesangial cells

Quiescent mesangial cells grown in serum-free media for two days expressed both TGF-β1 and fibronectin mRNAs (Fig. 1). The steady state levels of TGF-β1 and GAPDH mRNAs remained constant for an additional 48 hours incubation in serum-free control glucose media. Fibronectin mRNA expression tended to decrease, although the difference between 0 and 48 hours did not reach statistical significance (P > 0.05). The mRNA level in response to high glucose was compared with that in response to control glucose at a given time point. As summarized in Table 1, the time sequence of high glucose-induced TGF-β1 mRNA was different from that of fibronectin. Mesangial cell TGF-β1 mRNA expression increased significantly at six hours (P < 0.05) and continued to increase until up to 48 hours in response to high glucose. The level of TGF-β1 mRNA was 1.5× that of control glucose at six hours and 1.8× at 48 hours. Compared to TGF-β1, the increase in fibronectin mRNA in response to high glucose was delayed, significant only at 24 and 48 hours.

Effects of high glucose on mesangial cell TGF-β bioactivity

MLEC growth inhibition assay of mesangial cell conditioned media was performed to determine the translation of TGF-β1 mRNA into protein synthesis and secretion. Heat-activated conditioned media showed a time-dependent increase in TGF-β1 activity in both control and high glucose (P < 0.05; Fig. 2) in the face of little change in total protein concentration (Table 2). High glucose significantly
increased TGF-β1 secretion by mesangial cells when compared to control glucose at 24 and 48 hours, with the respective total TGF-β1 at 0.77 ± 0.22 and 0.80 ± 0.10 ng/ml in control glucose and 1.20 ± 0.22 and 1.34 ± 0.06 ng/ml in high glucose (P < 0.05). Conditioned media without heat activation exhibited undetectable inhibition of MLEC growth, suggesting that rat mesangial cells secreted TGF-β1 protein mostly in inactive or latent forms.

Effects of high glucose and anti-TGF-β antibody on fibronectin protein production by mesangial cells

To confirm the existence of fibronectin protein in conditioned media, we performed immunoblot analysis. Cumulative production of fibronectin by MC cultured in both control and high glucose increased progressively with time as summarized in Figure 3. Fibronectin was always detectable at six hours after the addition of serum-free control glucose media and was assigned a relative value of 1. Relative increase in fibronectin at 6, 12, 24 and 48 hours was 1, 2.3 ± 0.9, 3.1 ± 0.2 and 4.7 ± 0.3 under control glucose, and 1.4 ± 0.4, 2.6 ± 0.8, 4.5 ± 0.9 and 7.2 ± 1.4 under high glucose, respectively. Thus, fibronectin protein production in high glucose at 6, 12, 24, and 48 hours was 1.40×, 1.13×, 1.45×, and 1.53× that of control glucose, respectively. The difference between control and high glucose was significant only at 48 hours (P < 0.05).

Studies with neutralizing antibody to TGF-β were performed in order to examine the involvement of TGF-β on increased fibronectin production by mesangial cells cultured under high glucose conditions. Figure 4 demonstrates that anti-TGF-β antibody at 25 μg/ml completely abolished high glucose-induced fibronectin synthesis without a significant effect in basal production of fibronectin in control glucose.

DISCUSSION

Increased glomerular TGF-β expression in experimental diabetic animals [7–15] and diabetic patients [9, 16, 17], attenuation of renal hypertrophy and enhanced ECM gene expression in diabetic animals with anti-TGF-β antibody [25], and inhibition of high glucose-induced mesangial cell fibronectin and heparan sulfate proteoglycan synthesis by TGF-β antisense oligonucleotides [24] suggest that TGF-β is the first common mediator of diabetic nephropathy. High glucose-induced collagen production by mesangial cells [21] and fibronectin production by glomerular epithelial cells [23] have been effectively inhibited by anti-TGF-β antibody. However, the role of TGF-β in high glucose-induced mesangial fibronectin production and the temporal relations among high glucose, TGF-β1, and fibronectin in mesangial cells have not been studied. The present study examined effects of high glucose concentration on mesangial cell TGF-β1 and fibronectin mRNA expression and protein synthesis from 3 to 48 hours after stimulation. Mesangial cells cultured under high glucose is an accepted in vitro model of diabetic nephropathy and has been used to investigate pathogenetic mechanisms of diabetic nephropathy at molecular and cellular levels.

In agreement with previous studies [34, 35], there was constitutive expression of TGF-β1 mRNA in quiescent mesangial cells. In response to high glucose, TGF-β1 mRNA increased 1.5-fold above control at six hours and 1.8-fold at 48 hours. The extent of high glucose-induced TGF-β1 mRNA expression was comparable to the findings of Wolf et al [20], who reported a 50% increase at 48 hours after stimulation, and Mogyorosi et al [22], who reported a 100% increase at 72 hours after stimulation. In contrast, when synchronized mesangial cells were stimulated with serum or phorbol ester, TGF-β1 mRNA began to increase as early as one hour and reached the maximum at nine hours to several-fold that of pre-stimulation values [34]. Mesangial cells used in the present study also exhibited a fivefold increase in TGF-β1 mRNA in response to phorbol ester (data not shown), comparable to the findings of Kaname et al [34]. Angiotensin II caused a threefold increase in mesangial TGF-β1 mRNA expression at four hours, a sixfold at eight hours, and a threefold increase at 48 hours compared to control values [35].

Our study demonstrates that high glucose induces TGF-β1 mRNA very early and significantly at six hours. Wolf et al reported that high glucose increased TGF-β1 mRNA expression at 48 hours but not at 24 hours [20]. The differences in mesangial cells used in the two studies, primary rat mesangial cells in the present study versus

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**Figure 2. Effects of high glucose on mesangial cell TGF-β1 bioactivity.**

After incubation of quiescent mesangial cells with control (5 mM; □) or high (30 mM; ■) glucose for the given period, aliquots of mesangial cell conditioned media were analyzed by a mink lung epithelial cell (MLEC) growth inhibition assay. Values are expressed as mean ± SE from three separate experiments. *P < 0.05 compared to control glucose. Heat-activated conditioned media showed time-dependent increase in TGF-β1 protein in both control and high glucose conditions. High glucose significantly increased TGF-β1 secretion at 24 and 48 hours when compared to control glucose.
transformed mouse mesangial cell line in the previous study, and the presence versus absence of serum deprivation period to synchronize the cell cycle before stimulation may explain the apparent difference.

Compared to TGF-β1, the increase in fibronectin mRNA expression in response to high glucose was delayed, that is, it was significant only at 24 and 48 hours, which is consistent with the view that high glucose-induced fibronectin synthesis is mediated by TGF-β1. Ayo et al reported that high glucose increased mRNAs of ECM including fibronectin at three to seven days but not at one and two days [19]. In their study, cells between the 20th and 40th passages were used, and this may give a different time course in high glucose-induced fibronectin mRNA expression [36]. The significance of the present study is that high glucose induced TGF-β1 mRNA expression prior to fibronectin in the same cell. The rates of TGF-β1 and fibronectin mRNA degradation following actinomycin D (5 μg/ml) were similar between mesangial cells cultured under control glucose and under high glucose (data not shown), suggesting that high glucose did not alter the stabilities of TGF-β1 and fibronectin mRNAs in rat mesangial cells.

Elevation of TGF-β1 mRNA at six hours in response to high glucose was followed by increased protein synthesis at 24 and 48 hours. Elevated fibronectin mRNA at 24 hours was followed by a significant increase in fibronectin protein at 48 hours. In addition, the amount of TGF-β protein secreted by mesangial cells grown under control and high glucose conditions during the first 24 hours were comparable to the reported concentrations of TGF-β1 stimulating synthesis of fibronectin [24] and collagen [21]. Further, neutralizing antibody to TGF-β completely abolished the

### Table 2. Concentration of total protein in mesangial cell conditioned media

<table>
<thead>
<tr>
<th>Time after stimulation (hours)</th>
<th>Control glucose μg/ml</th>
<th>High glucose μg/ml</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>36.0 ± 6.6</td>
<td>26.0 ± 5.0</td>
</tr>
<tr>
<td>3</td>
<td>31.1 ± 7.9</td>
<td>27.6 ± 4.2</td>
</tr>
<tr>
<td>6</td>
<td>30.5 ± 7.5</td>
<td>32.2 ± 4.2</td>
</tr>
<tr>
<td>12</td>
<td>35.1 ± 7.4</td>
<td>32.0 ± 2.1</td>
</tr>
<tr>
<td>24</td>
<td>33.9 ± 7.9</td>
<td>30.9 ± 5.4</td>
</tr>
<tr>
<td>48</td>
<td>32.5 ± 8.5</td>
<td>36.0 ± 2.1</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE of three separate experiments.
high glucose-induced mesangial cell fibronectin production without a significant change in basal production of fibronectin in control glucose, indicating that high glucose-induced but not basal production of fibronectin by mesangial cells is due to an increase in TGF-β bioactivity. The present data are thus consistent with the previous studies [21, 23–25] demonstrating that increased ECM synthesis associated with diabetes is mediated by TGF-β.

In conclusion, our data demonstrate that high glucose induces TGF-β1 mRNA and protein synthesis very early and prior to fibronectin production by mesangial cell. TGF-β1 mRNA is induced as early as six hours after stimulation with high glucose, while fibronectin mRNA is induced at 24 hours. TGF-β1 protein synthesis was significantly increased at 24 hours and fibronectin protein at 48 hours. These findings along with complete inhibition by anti-TGF-β antibody of high glucose-induced fibronectin production support the view that high glucose-induced fibronectin synthesis is mediated by TGF-β.

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APPENDIX

Abbreviations used in this article are: DMEM, Dulbecco’s modified eagle’s medium; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPES, N-2-hydroxy-ethylpiperazine-N’-2-ethane sulfonic acid; HG, high glucose; MC, mesangial cell; MLEC, mink lung epithelial cell; MT, 3-[4, 5-dimethyl-2-y]-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, sodium chloride and sodium citrate; TGF, transforming growth factor.

REFERENCES


