Kidney International, Vol. 41 (1992), pp. 107–114

Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule

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Elevated glucose stimulates TGF-β gene expression and bioactivity in proximal tubule. Our previous studies have demonstrated that raising ambient glucose from 100 to 450 mg/dl significantly inhibited the proliferation of mouse renal proximal tubule cells in culture. This effect was demonstrated after a latent period of 24 to 48 hours. Because transforming growth factor-beta (TGF-β) inhibits cell proliferation in most epithelial cell lines, we hypothesized that the inhibitory effect of high glucose levels on cell proliferation may be mediated by TGF-β. The present studies were performed to test the hypothesis that TGF-β is an autocrine cytokine whose activity can be modulated by ambient glucose. Exogenous TGF-β inhibited [^{3}H]-thymidine incorporation in a dose-dependent fashion and with high affinity (picomolar range), but with slightly lower potency in high versus normal glucose media. Northern analysis of mRNA demonstrated that proximal tubule cells constitutively express TGF-β_1 transcripts, and that the steady state level of TGF-β_1 mRNA was, on average, 63% higher in the cells grown for 48 hours in high versus normal glucose media. Furthermore, the conditioned media of cells exposed to 450 mg/dl glucose exhibited endogenous TGF-β bioactivity as measured by inhibition of cell proliferation. The addition of a rabbit antiporcine TGF-β neutralizing antibody significantly increased basal thymidine incorporation in high glucose media to levels approaching those of cells grown in normal glucose media. In contrast, the anti-TGF-β antibody did not have a significant effect on the growth of cells in the normal glucose media. Control rabbit IgG did not stimulate growth. We conclude that TGF-β functions as an autocrine cytokine in proximal tubule cells and that expression of TGF-β is stimulated by high glucose concentration. The growth inhibitory effect of elevated glucose levels may be largely mediated by increased endogenous TGF-β production and/or activity.

Transforming growth factor-beta (TGF-β) is a highly conserved cytokine that is ubiquitous in living organisms. TGF-β and its receptors are located in virtually all organ systems studied, including the kidney [1–4], and TGF-β can act in either an autocrine or paracrine fashion to influence a multitude of cellular functions [5].

TGF-β has a particularly crucial role in regulating cell growth as it inhibits the proliferation of most epithelial cell types in culture, as well as of mesenchymally derived lymphocytes and vascular endothelial cells [5]. In fibroblasts, TGF-β can be growth stimulatory or inhibitory, depending upon the presence of other specific growth factors or on the developmental stage of the cell [6]. TGF-β inhibits cell proliferation in most renal cell lines examined, including glomerular mesangial, epithelial and endothelial cells [5], in proximal tubule cells [7] and African green monkey kidney epithelial (BSC-1) cells [8].

TGF-β also stimulates matrix synthesis, inhibits matrix degradation and stimulates the synthesis of receptors for matrix proteins [9], and can induce increased synthesis of the proteoglycans biglycan and decorin [10, 11], and of collagen IV, laminin and fibronectin in glomerular cells [11–13]. Because of these effects on cell matrix, recent studies have focused on the potential role of TGF-β in renal fibrogenesis. In experimental models of glomerular injury, TGF-β has been implicated in the genesis of renal fibrosis caused by the administration of anti-glomerular basement membrane antibody [14] and of glomerulonephritis induced by antithymocyte serum [15].

In an effort to explore some aspects of the pathobiology of the renal tubulointerstitium in diabetes mellitus, we have recently developed an in vitro model of diabetic renal disease using murine proximal tubule cells in culture. We have demonstrated that high glucose levels induce cell hypertrophy and collagen transcription and secretion [16] while concurrently inhibiting proliferation of proximal tubule cells. These effects of high glucose levels are reminiscent of those induced by TGF-β on epithelial cells. We therefore postulated that at least some of the effects of high glucose media may be caused by the endogenous production of TGF-β by the proximal tubule cells. In this study, we demonstrate that TGF-β_1 mRNA is constitutively expressed in proximal tubule cells and we provide evidence that the reduction of proximal tubule cell proliferation, which is induced by elevated glucose levels, is largely mediated by increased endogenous TGF-β production and/or activity.

Methods

Cell culture

The cell line designated MCT (mouse cortical tubule) was derived from kidneys of normal SJL mice. Proximal tubule cells were selected by immunodissection using specific antibodies against the 3M-1 glycoprotein, a target antigen for autoimmune interstitial nephritis [17]. These cells were virally transformed with a non-replicating, non-capsid forming strain of SV-40. By microscopy, the cells demonstrate many characteristics of proximal tubule cells including positive staining for alkaline phosphatase and cytokeratin, and surface projections on electron microscopy consistent with brush border villi. Details of

Received for publication June 13, 1991
and in revised form August 14, 1991
Accepted for publication August 19, 1991

the isolation and characterization of these cells have been previously described [18].

Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The cells were passaged every 48 to 72 hours and were carried in RPMI 1640 media with a D-glucose concentration of 200 mg/dl and supplemented with 10% inactivated fetal calf serum, 5×10^{-7} M hydrocortisone, 10 mM HEPES buffer, 24 mM sodium bicarbonate, 5 μg/ml insulin, 5 μg/ml transferrin, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Thymidine incorporation

Ten thousand MCT cells per well were subcultured in flat-bottomed 96-well plastic plates (Nunclon, Denmark) each containing 200 μl of a 1:1 mixture of serum-free Ham's F12 nutrient medium and Dulbecco's modified Eagle medium (DMEM) to give a final glucose concentration of 315 mg/dl. After 24 to 48 hours of quiescence, the media were removed and 200 μl of fresh serum-free DMEM with a D-glucose concentration of either 100 mg/dl (5.6 mM) or 450 mg/dl (25 mM) were added to each well. The DMEM was supplemented with penicillin and streptomycin (as above) and with 2 μg/ml transferrin and 2 mM glutamine. Some wells also received TGF-β_1 (from porcine platelets, R & D Systems, Minneapolis, Minnesota, USA), neutralizing rabbit antiporcine TGF-β antibody (IgG fraction; binds to the active forms of both TGF-β_1 and TGF-β_2; R & D Systems), normal rabbit IgG (Sigma Chemical Co., St. Louis, Missouri, USA) or rabbit anti-sheep IgG (Zymed, San Francisco, California, USA). The cells were grown for an additional 24 to 72 hour period (40 to 80% subconfluence), and during the last six hours of culture, they were pulsed with 1 μCi/well of [^{3}H]-thymidine (5 Ci/mmol; Amersham, Arlington Heights, Illinois, USA). The cells were collected with a cell harvester (M-24S, Brandel, Gaithersburg, Maryland, USA) onto glass-microfiber filter paper (934-AH, Whatman, UK). The radioactivity was assayed by counting filters in scintillation fluid for β-emissions. [^{3}H]-thymidine incorporation into cell DNA, expressed in counts per minute, was used as an index of cell proliferation. Each experimental condition was tested in four to eight replicate wells and the mean was taken to represent an individual experiment.

Northern blot analysis

For each condition, 2×10^6 MCT cells in serum-free culture medium were plated into a 75-cm^2 plastic flask (Falcon, Lincoln Park, New Jersey, USA). After 48 hours, the media were replaced with fresh serum-free DMEM containing either 100 mg/dl or 450 mg/dl of D-glucose. The cells were grown for an additional 24, 48 or 72 hours and then were lysed and denatured by adding to the flasks 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. Total RNA was then extracted by phenol-chloroform according to Chomczynski and Sacchi [19]. In some experiments, poly $(A)^+$-enriched RNA from MCT cells was extracted directly using the Fast Track mRNA isolation kit (Invitrogen, San Diego, California, USA). Ten to 20 μg of total RNA or 2 to 8 μg of poly$(A)^+$-RNA, as determined by UV spectrophotometry, was electrophoresed through a 1.0% agarose gel with 2.2 M formaldehyde.

RNA transfer to positively charged nylon membranes (Zetabind, CUNO Laboratory Products, Meriden, Connecticut, USA) was performed by electroblotting (Horizontal Blotting System, IBI, New Haven, Connecticut, USA) followed by short wave UV crosslinking (UV Stratalinker, Stratagene, La Jolla, California, USA). Prehybridization was performed for two hours at 42°C using a prehybridization buffer composed of 5× SSPE (1× contains 149 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5× Denhardt's solution (1× contains 0.1 g Ficoll, 0.1 g polyvinylpyrrolidone, 0.1 g bovine serum albumin and H_2O to 500 ml), 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml of denatured, sheared salmon sperm DNA, and 50% (vol/vol) formamide. Hybridization was conducted for 20 hours at 42°C using excised cDNA inserts as probes labeled with [^{32}P]-dCTP (>3000 Ci/mmol) by the random hexamer priming technique [20]. The hybridization buffer was similar to that used for prehybridization except for the use of 2× Denhardt's solution. The cDNA probes used were a 1.04 kb EcoR1 fragment encoding the human TGF-β_1 gene from pSP64 (from Dr. A. Singh, Genentech, San Francisco, California, USA) and a 1.3 kb PstI fragment encoding the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. After hybridization, the membranes were washed in 2× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, adjusted to pH 7.0) with 0.1% SDS for 30 minutes at room temperature, followed by 0.1× SSC with 0.1% SDS at 62°C for 15 minutes twice. The membranes were then exposed to X-ray film (Kodak XAR-S film) between two intensifying screens at −70°C for three to seven days (TGF-β) or one to three days (GAPDH). Relative mRNA levels were determined by densitometric scanning (Hoefer Scientific Instruments, San Francisco, California, USA) followed by area integration. The filters were first probed with the TGF-β cDNA. To account for variations in RNA loading, the filters were subsequently stripped for one to two hours at 65°C with a solution containing 5 mM Tris, 0.2 mM EDTA (pH 8.0) and 5% sodium pyrophosphate in order to remove the signal. The filters were then rehybridized with the GAPDH cDNA probe.

Dot-blot hybridization

For dot-blot hybridization, MCT cells were cultured in flasks as described above. The cells were grown for 24 to 72 hours in media containing either 100 mg/dl or 450 mg/dl of D-glucose, then were released with a trypsin-EDTA mixture, washed twice in PBS and counted in a Coulter cell Counter (Coulter Electronics, Hialeah, Florida, USA). The cells were lysed with 0.5% NP-40 in Tris-EDTA buffer. Four times 10^5 cell equivalents of cytoplasmic RNA were denatured with 40% formalin in 12× SSC at 65°C for 15 minutes. The samples were then centrifuged and the supernatants spotted onto Zetabind filters using a hybridot apparatus (Bethesda Research Laboratories, Bethesda, Maryland, USA). In parallel experiments, additional blots were obtained by using serial dilutions of measured RNA (2.5 to 10 μg per dot) which had been extracted as described above [19]. The blots were dried, then prehybridized at 42°C for 16 hours in fluid containing 0.5 M phosphate buffer at pH 7.2, 150 μg/ml Poly A, 150 μg/ml denatured, sheared salmon sperm DNA, 7% SDS, 1% bovine serum albumin and 1 mM EDTA. Hybridization was performed in fluid containing [^{32}P]-labeled cDNA probes for TGF-β and, after stripping, for GAPDH. The filters were washed twice with 3× SSC, 0.5% SDS at 55°C for 15

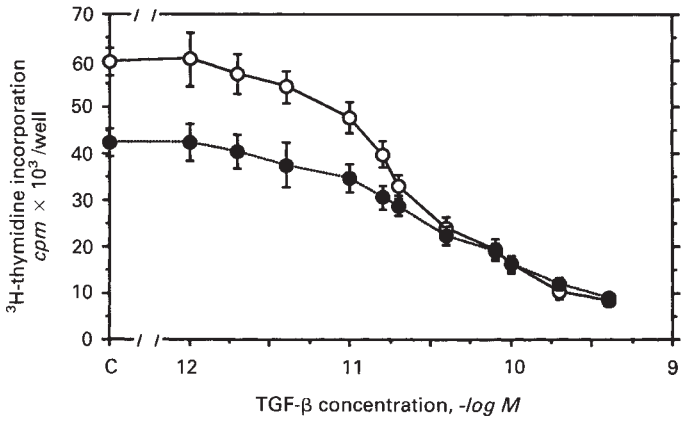

Fig. 1. *Effect of exogenous TGF-β on mouse proximal tubule (MCT) cell proliferation as measured by tritiated thymidine incorporation.* MCT cells were exposed to serum-free DMEM containing either 100 (open symbols) or 450 mg/dl D-glucose (closed symbols) for a total of 72 hours. In the absence of TGF-β (C), thymidine incorporation in 450 mg/dl glucose was significantly lower ($P < 0.001$) than in 100 mg/dl glucose. TGF-β was added at the indicated doses for the last 24 hours of culture. Values are expressed as the mean ± SE; $N = 8$ replicates.

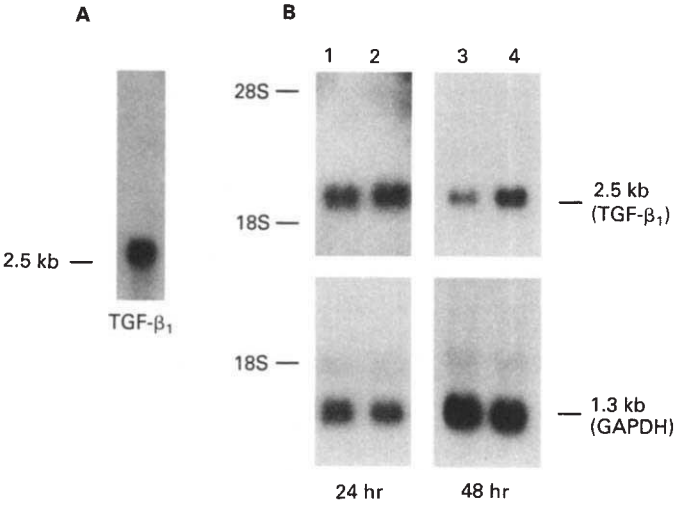

Fig. 2. *Northern blot analysis of MCT mRNA hybridized with a human cDNA probe for TGF-β$_1$.* **A.** Representative blot demonstrating constitutive expression of TGF-β$_1$; 4 μg of poly(A)$^+$-RNA was isolated from cells cultured for 72 hours in serum-free DMEM containing 100 mg/dl D-glucose. The single band corresponds to the 2.5-kb TGF-β$_1$ mRNA. **B.** For each condition, 10 μg of total RNA was extracted from proximal tubule cells cultured for 24 or 48 hours in serum-free medium containing 100 mg/dl or 450 mg/dl of D-glucose. The RNA was loaded onto an agarose gel, electrophoresed and transferred by electroblotting to a nylon membrane. The band depicted in the upper panels is of the predicted size for the TGF-β$_1$ transcript. The band depicted in the lower panels was obtained by stripping the filter and reprobing with the GAPDH probe. Arrows indicate the location of the 18 S and 28 S ribosomal RNA. Conditions were as follows: lane 1: 24 hours, 100 mg/dl D-glucose; lane 2: 24 hours, 450 mg/dl D-glucose, lane 3: 48 hours, 100 mg/dl D-glucose; lane 4: 48 hours, 450 mg/dl D-glucose.

minutes, and once with 0.1× SSC, 0.1% SDS at 62°C for 15 minutes, then autoradiographed as described above. Relative mRNA levels for TGF-β$_1$ were determined as the ratio of the densitometric signal for TGF-β$_1$ mRNA to the corresponding signal for GAPDH mRNA. For cells cultured in 100 mg/dl of D-glucose this ratio was assigned a value of unity.

Statistics

The mean for the replicates of each experiment was determined. The data are presented as the mean ± SE with N indicating the number of experiments. Comparison between two groups was performed using the paired or unpaired t-test, as appropriate. Statistical significance was defined as a $P < 0.05$.

Results

Effects of glucose on cell proliferation

In our previous study [16], we demonstrated that the concentration of glucose in serum-free culture medium induces a time and dose dependent inhibition of proliferation of proximal tubule cells. Thymidine incorporation of cells grown in 450 mg/dl glucose was reduced, on average, by 14%, 26% and 33% after 24, 48 and 72 hours, respectively. Inhibition of cell proliferation was confirmed when cell number was also determined. Moreover, growth inhibition was specific for D-glucose and was not related to an effect of high osmolarity per se; the addition of either L-glucose or D-mannitol did not have any significant effect on cell proliferation [16]. In the current study, a glucose-induced inhibition of cellular proliferation was confirmed. For example, Figure 1 demonstrates that the basal level of thymidine incorporation of cells cultured in 450 mg/dl glucose for 72 hours was reduced by 29% in the high versus the normal glucose medium ($P < 0.001$, $N = 8$).

Effect of TGF-β on cell proliferation

When TGF-β$_1$ was added in the final 24 hours of culture to cells exposed for a total of 72 hours to either 100 or 450 mg/dl glucose, it produced a dose dependent inhibition of tritiated thymidine incorporation (Fig. 1). The half maximal inhibition of proliferation by TGF-β was approximately 20 pM and 30 pM in the normal and high glucose media, respectively. Note that with increasing concentrations of TGF-β, the difference in thymidine incorporation between the two cell populations became progressively smaller. In other experiments (not shown), the inhibitory effect of TGF-β on proliferation persisted following its addition for 48 or 72 hours. This inhibition also could be prevented with cotreatment with either 2 μg/ml insulin or 2 ng/ml epidermal growth factor (EGF) (data not shown). These peptides are known mitogens in proximal tubule cells [7, 21].

TGF-β expression in proximal tubule cells

By Northern blot analysis, using a human cDNA probe for TGF-β, it was found that proximal tubule cells constitutively express the predicted 2.5 kb mRNA for TGF-β (Fig. 2A). This result raised the possibility that TGF-β$_1$ may serve as an autocrine factor in proximal tubule cells. Furthermore, we postulated that the observed decrease in cell proliferation in cultures containing 450 mg/dl D-glucose may be attributed to the increased production of TGF-β by the cells. To test this hypothesis, we first examined whether high glucose levels

stimulated endogenous TGF-β production by MCT cells. Relative TGF-β_1 mRNA levels were thus determined in MCT cells grown in media with a D-glucose concentration of either 100 mg/dl or 450 mg/dl. Figure 2B demonstrates that cells grown for 48 hours in high glucose media expressed levels of TGF-β_1 mRNA, standardized to GAPDH mRNA levels, that were approximately twice as high as those in cells grown in normal glucose concentration. This response to high glucose medium became apparent at 48 hours of culture (Fig. 2B), and was less pronounced at 72 hours (data not shown). For further relative quantification of mRNA levels, a series of dot-blot RNA hybridizations were performed (Figure 3). The glucose-induced stimulation in transcript levels at 48 hours varied between 36% and 104% among different experiments ($N = 4$). The average increase in TGF-β mRNA in high glucose medium was 63% above the level in normal glucose medium. This conclusion was valid whether comparisons were made on blots of equal quantities of RNA (Fig. 3) or on RNA extracted from equal numbers of cells (data not shown).

Effect of TGF-β neutralizing antibody on cell proliferation

A TGF-β neutralizing antibody was used as a functional assay for endogenous TGF-β-like activity in the conditioned media. We initially confirmed that this antibody (IgG fraction of antiserum) exhibited a neutralizing effect on TGF-β action. As shown in Figure 4, the reduction in thymidine incorporation produced by exogenous TGF-β (40 pM) can be largely prevented by the addition of 30 μg/ml anti-TGF-β antibody. In this set of experiments, "control" cells were cultured in 450 mg/dl glucose without the addition of TGF-β. Rabbit anti-sheep IgG added as a control had no effect on thymidine incorporation. In other experiments (not shown) the neutralizing property of anti-TGF-β antibody to exogenously added TGF-β was also demonstrable when the cells were grown in 100 mg/dl glucose.

The addition of 30 μg/ml of anti-TGF-β antibody in high glucose media increased basal thymidine incorporation of these cells to levels which approximate the basal incorporation of thymidine in cells grown in normal glucose media (Fig. 5). The addition of anti-TGF-β antibody to cells raised in normal glucose media, however, had no significant effect on [^{3}H]-thymidine incorporation. As a control, normal rabbit non-immune IgG (30 μg/ml) had no significant effect on cell proliferation in either high or normal glucose media (Fig. 5). Similar results were obtained when a lower dose of anti-TGF-β antibody (10 μg/ml) was employed in an additional set of six different experiments (Fig. 6). As a control, a rabbit anti-sheep IgG was used in these studies. In normal glucose media, the addition of either anti-TGF-β antibody or rabbit anti-sheep IgG (10 μg/ml) had no significant effect on basal [^{3}H]-thymidine incorporation. In high glucose media, however, the addition of anti-TGF-β antibody, but not the control rabbit anti-sheep IgG, resulted in a 30% increase in thymidine incorporation above basal levels.

The response to anti-TGF-β antibody in the experiments given in Figure 5 suggest that the growth inhibitory effect of high glucose is predominantly, but not totally, attributable to endogenous TGF-β bioactivity. It is possible that doses higher than 30 μg/ml of the antibody may be required for total neutralization.

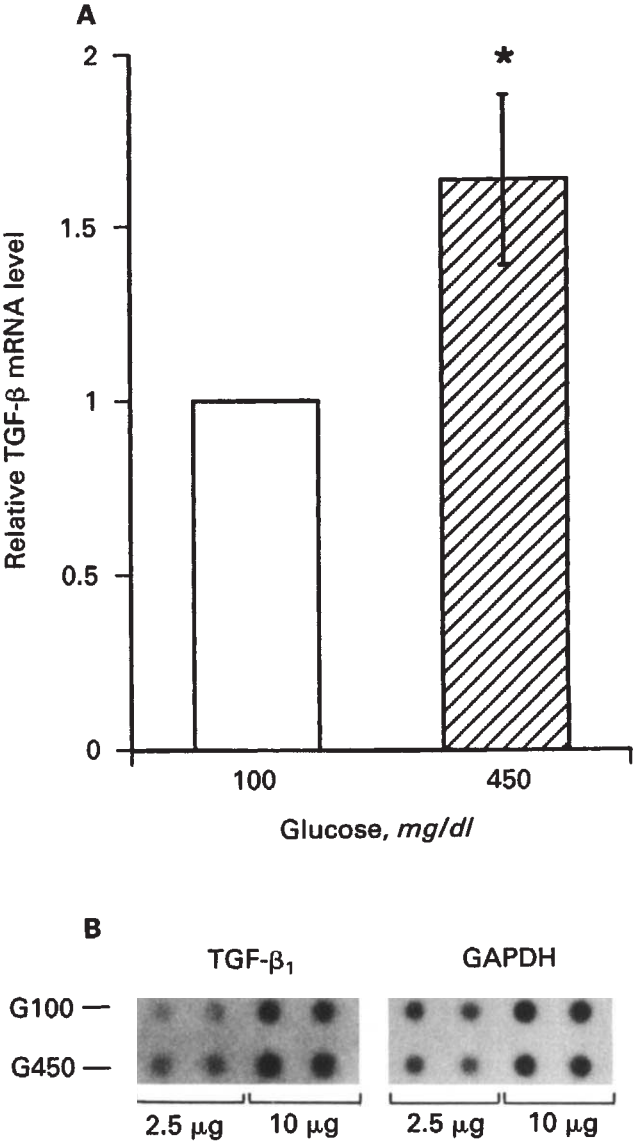

Fig. 3. *Effect of media glucose concentration on TGF-β_1 mRNA levels.* **A.** Mouse proximal tubule cells were cultured for 48 hours in media containing 100 mg/dl glucose (open bar) or 450 mg/dl glucose (hatched bar). RNA dot-blot hybridization with the TGF-β_1 cDNA probe was then performed, followed by stripping the filter and reprobing with the control GAPDH probe. The ratio of the densitometric signal for TGF-β_1 mRNA to that of GAPDH was determined. For cells cultured in 100 mg/dl glucose, this ratio was assigned a value of unity. The relative TGF-β_1 mRNA level (normalized for GAPDH mRNA level) was significantly higher (* $P < 0.02$, $N = 4$) in cells grown in high versus normal glucose media. **B.** Representative autoradiographs from a single experiment of dot-blots hybridized with TGF-β_1 cDNA and, after stripping, with GAPDH cDNA. The upper dots are for cells cultured in 100 mg/dl glucose (G100) and the lower dots for cells cultured in 450 mg/dl glucose (G450). As indicated, each dot was loaded in duplicate with either 2.5 or 10 μg of total RNA for each culture condition.

Discussion

Our studies suggest that the inhibition in cell proliferation induced by high glucose concentration is largely mediated by the increased TGF-β activity in proximal tubule cells and a

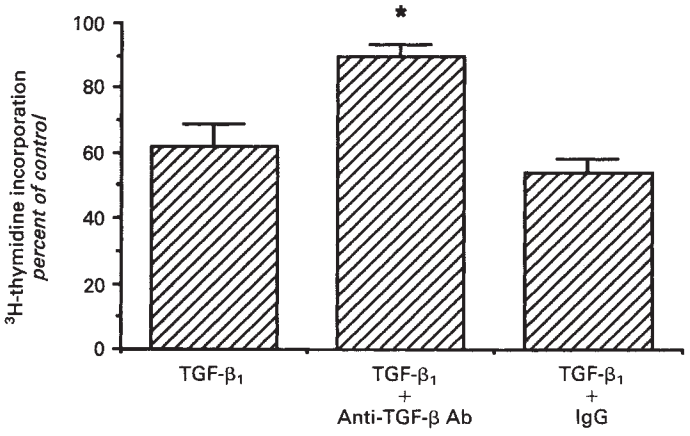

Fig. 4. *Demonstration of the neutralizing effect of anti-TGF-β antibody against exogenously administered TGF-β.* Mouse proximal tubule cells were cultured for 72 hours in media containing 450 mg/dl glucose with 40 pM of exogenous TGF-β alone (first bar), or with the addition of anti-TGF-β neutralizing antibody (second bar) or control rabbit anti-sheep IgG (third bar). The medium containing 450 mg/dl glucose without any supplements is taken as control in this set of experiments. Note that anti-TGF-β antibody partially restores the antiproliferative effect of TGF-β, but that the control rabbit anti-sheep IgG had no effect.

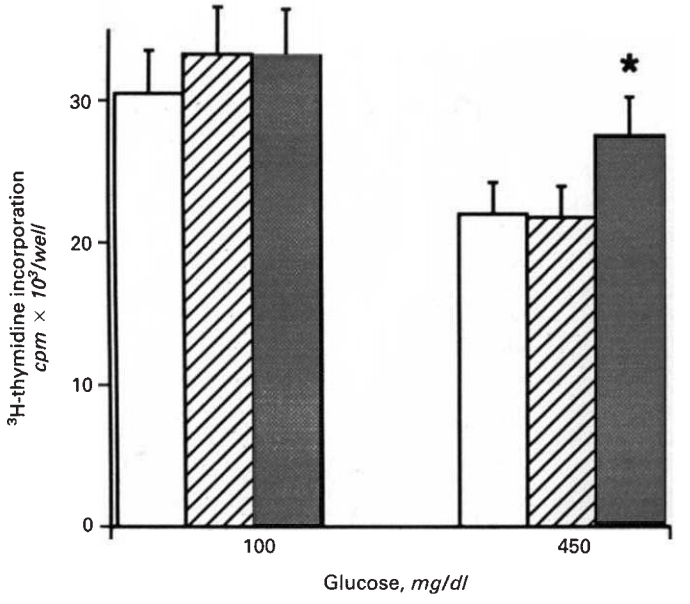

Fig. 5. *Effect of anti-TGF-β antibody on mouse proximal tubule cell proliferation.* Cells were cultured for 48 hours in serum-free media containing 100 or 450 mg/dl of D-glucose with the addition of anti-TGF-β antibody at 30 μg/ml (solid bars), control normal rabbit IgG at 30 μg/ml (hatched bars) or vehicle (open bars). Results are expressed as the mean absolute cpm of [^{3}H]-thymidine incorporation ± SE for $N = 6$ replicates.

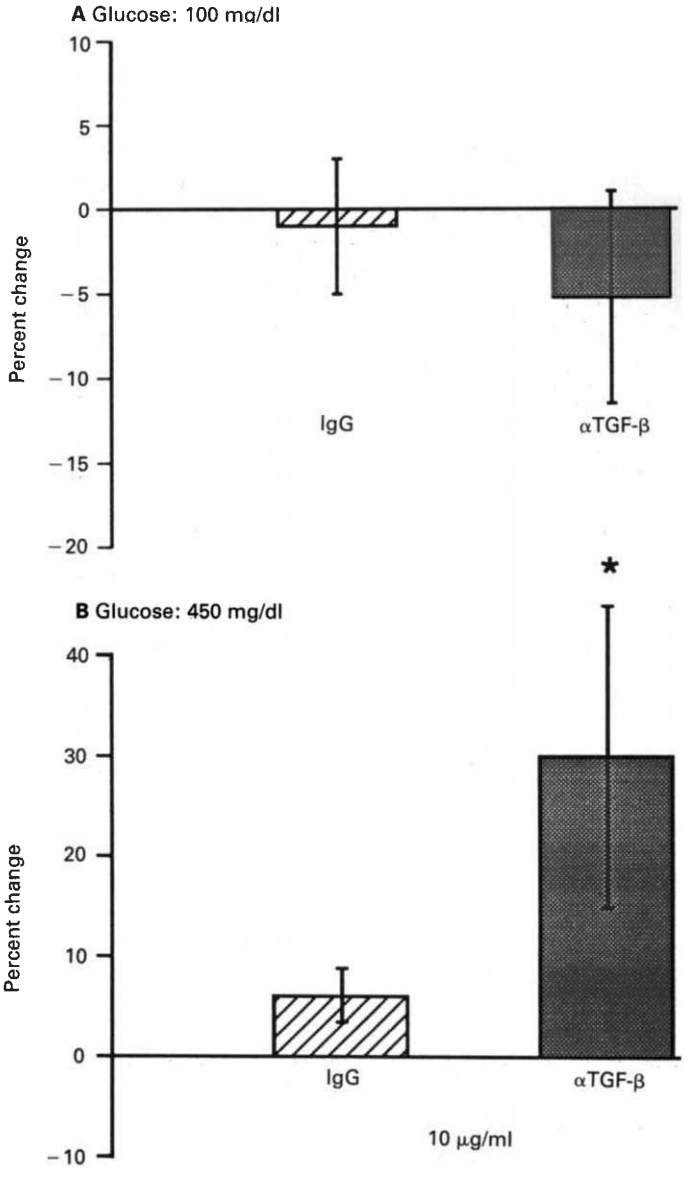

Fig. 6. *Effect of anti-TGF-β antibody on mouse proximal tubule cell proliferation.* Cells were cultured for 48 hours in serum-free media containing 100 mg/dl or 450 mg/dl of D-glucose with the addition of 10 μg/ml of anti-TGF-β antibody (αTGF-β) (solid bars) or control rabbit anti-sheep IgG (hatched bars). Results are expressed as percent proliferation compared to cells grown without the addition of antibody. **A.** Cells cultured in serum-free media with a D-glucose concentration of 100 mg/dl ($N = 6$). No significant effect on proliferation was seen with either antibody. **B.** Cells cultured in serum-free media with a D-glucose concentration of 450 mg/dl. TGF-β antibody, but not control rabbit anti-sheep IgG, increased cell proliferation. Values are expressed as the mean ± SE for $N = 6$ experiments. * $P < 0.05$ comparing the effect of anti-TGF-β antibody to control IgG for cells cultured in 450 mg/dl of D-glucose.

concurrent increase in the steady state levels of TGF-β_1 mRNA. These observations raise the possibility that TGF-β may mediate in an autocrine fashion some of the effects of high glucose concentrations on proximal tubule cells.

The growth inhibitory effects of TGF-β have been confirmed in a wide variety of epithelial cells as well as in most renal cell lines examined [5]. In the glomerulus, TGF-β inhibits the proliferation of endothelial, epithelial [13] and mesangial cells [22, 23], although in mesangial cells, one group has reported that inhibition occurs if the cells are plated at low density, and stimulation if the cells are plated at high density [13]. In renal tubular epithelial cells, our results are similar to those found in

rabbit proximal tubule cells [7] and in a mouse collecting tubule cell line (Ziyadeh, unpublished data).

The nuclear mechanisms responsible for the inhibitory effect of TGF-β_1 on cell proliferation are not fully characterized, but are believed to be partly related to changes in the expression of the *c-myc* proto-oncogene or to the state of phosphorylation of the protein product of the retinoblastoma gene (pRB) [24, 25]. These two actions may be related. In Mv1Lu lung epithelial cells, TGF-β_1 prevents phosphorylation of pRB leading to the arrest of the cells in the late G1 phase of the cell cycle [26]; under-phosphorylation of the RB gene product prevents progression to later phases of the cell cycle. Also, virally transformed skin keratinocytes which produce specific DNA tumor virus oncoproteins that can bind to the pRB are resistant to the inhibitory effects of TGF-β on growth inhibition and *c-myc* levels [27]. Keratinocytes that produce mutant viral proteins that are unable to bind to the pRB, however, demonstrate inhibition of proliferation when exposed to TGF-β [27].

Previous studies have shown that epithelial cells derived from the African green monkey kidney (BSC-1) produce both TGF-β_1 and TGF-β_2 [8]. Also, in the glomerulus of the rat kidney, both TGF-β_1 and TGF-β_2 are produced, with protein levels several-fold higher than those found in whole kidney [28]. We confirmed the presence of TGF-β_1 mRNA in nonglomerular tissue by demonstrating the 2.5-kb TGF-β_1 transcript by Northern analysis in mouse proximal tubule cells (Fig. 2). In addition, receptors for TGF-β have been located in most organ systems examined [3, 4], although the number of receptors per cell can vary widely [4]. In the kidney, receptors have been identified on glomerular endothelial, mesangial and epithelial cells [13] as well as on kidney fibroblasts and tubular epithelial cells [4]. The concentration of TGF-β which yields a half maximal inhibition of cellular proliferation (IC_{50} = 20 to 30 pM; Fig. 1) implies that the response of proximal tubule cells to TGF-β likely involves the interaction with high affinity receptors. In other cell systems studied, the dissociation constant (Kd) for the binding of TGF-β to its receptors is usually within the same picomolar range [4].

Northern analysis and dot-blot hybridization of mRNA isolated from MCT cells grown in normal and high glucose media demonstrated that the steady state level of TGF-β_1 mRNA is increased in the presence of high glucose media. Furthermore, by using an antibody which neutralizes the activity of TGF-β_1 and TGF-β_2 [29], we reversed the inhibition of proliferation caused by high glucose media. Both these results support the hypothesis of the interaction between the high glucose effect and TGF-β_1 (or TGF-β_2) expression. The reduction in basal levels of cellular proliferation which is observed in the high glucose medium becomes prominent at 48 hours in culture. This lag period appears to correspond to the time which is required to stimulate TGF-β mRNA levels.

The increased levels of TGF-β_1 mRNA induced by high glucose concentration could be due to an increased rate of gene transcription and/or due to decreased mRNA degradation or message stabilization. We did not attempt to determine which of these mechanisms may be applicable in our system. However, evidence suggests that both mechanisms may operate concurrently as high glucose levels were found to stimulate the transcription rate of collagen genes in MCT cells [16] as well as stabilize the message of the insulin gene in β-islets of the pancreas [30].

The conclusion that the reduction in basal levels of thymidine incorporation in high glucose media could be attributed to increased endogenous TGF-β activity is indirectly supported by data in Figure 1. As the concentration of exogenous TGF-β was increased, the difference in thymidine incorporation between high and normal glucose media became progressively smaller. This observation suggests the presence of a higher endogenous level of growth-inhibitory activity in the high glucose media as further dosing of exogenous TGF-β produced little additional inhibition of proliferation. The profile of these dose-response curves (Fig. 1) thus provides indirect evidence that the cells cultured in high glucose media elaborate higher basal levels of TGF-β-like inhibitory activity.

The studies involving the anti-TGF-β antibody provide strong evidence that the conditioned medium of MCT cells grown in 450 mg/dl glucose contains quantities of bioactive TGF-β which are sufficient to alter basal levels of DNA synthesis as determined by tritiated thymidine incorporation. It should be noted here that the antibody only binds to the active rather than the latent form of TGF-β (R & D, manufacturer's data file). However, since the antibody binds to at least two isoforms of the cytokine, TGF-β_1 and TGF-β_2 [29], its neutralizing properties in the conditioned media may be attributed to the binding of one or more TGF-β isoforms. Moreover, while the observations on the antibody action support the conclusion that high glucose concentrations induce increased TGF-β bioactivity, they do not point to a specific mode of activation. The increased levels of bioactive TGF-β may reflect increased synthesis, decreased degradation or an increased conversion of latent to active forms. The finding of increased mRNA levels implies an increased rate of cytokine synthesis, but other mechanisms of TGF-β activation cannot be excluded. Additionally, our studies do not exclude the possibility that a minor component of the growth inhibitory effect of high glucose may be attributed to induction of inhibitory factors other than TGF-β and/or inhibition of stimulatory factors.

Cellular or nuclear mechanisms by which elevated ambient glucose increases TGF-β levels are unknown but several possibilities exist. First, high glucose levels increase the activity of the polyol pathway with subsequent alterations in cellular *myo*-inositol metabolism in a number of target tissues [31], including kidneys of diabetic rats [32] and in MCT cells [33]. For example, we have found that the addition of 800 μM *myo*-inositol to MCT cells grown in high glucose media restores proliferation to levels seen in normal glucose media [34]. These changes in proliferation are associated with parallel effects on collagen gene expression as *myo*-inositol supplementation reduces the glucose-stimulated secretion and mRNA levels of type I and type IV collagen [34]. A second mechanism of action by which elevated glucose levels may act involves the non-enzymatic glycation of extracellular or intracellular proteins [31, 35]. Non-enzymatic glycation could alter the structure of receptors and/or cellular regulatory proteins and thus influence TGF-β synthesis or bioactivity.

Other factors can also increase TGF-β mRNA levels including TGF-β_1 itself through self-induction [36]. Moreover, an inhibitory system regulating TGF-β activity has been described. Decorin, a chondroitin/dermatan sulphate proteoglycan, is produced by many cell lines and its synthesis can be induced by TGF-β [10, 11, 37]. In turn, decorin can bind to TGF-β, thus

neutralizing TGF-β activity [38]. It is possible that ambient glucose could interfere with decorin production or alter the decorin molecule through nonenzymatic glycation, thereby modulating TGF-β action.

The relevance of these in vitro findings to the problem of diabetic nephropathy remains to be elucidated. In early diabetes, the kidney enlarges predominantly through cellular hypertrophy [39]; previous studies have shown that TGF-β induces hypertrophy in renal proximal tubule cells in vitro [40]. Whether the hypertrophy seen in vivo is, at least in part, the result of TGF-β action in response to hyperglycemia remains to be tested. In MCT cell cultures, high ambient glucose levels appear to be a sufficient stimulus to promote cellular hypertrophy [16]. TGF-β may also transform a mitogenic stimulus into a hypertrophic one [40]. After various forms of renal injury, renal tubular cell proliferation becomes an important component of the regenerative process. It is tempting to speculate that in diabetic nephropathy, the activation of intra-renal TGF-β may impair this regenerative process and thus delay the recovery phase of acute renal failure.

In experimental models of renal injury, TGF-β has been implicated in the genesis of fibrotic lesions caused by the administration of antiglomerular basement membrane antibody [14] or antithymocyte serum [15]. A role for TGF-β in renal fibrogenesis of various disease states including diabetes also deserves additional studies. In the diabetic kidney, the pathologic changes in the glomerulus [31] and tubulointerstitium [41] may be mediated by increased synthesis and decreased degradation of extracellular matrix (ECM) components; these changes, particularly in the glomerular mesangium, are correlated with a decline in glomerular filtration rate [31, 42, 43]. Also, interstitial fibrotic changes are common in advanced diabetic nephropathy [41], and the degree of fibrosis is directly related to the serum creatinine level [43, 44]. This increase in ECM components could be due, at least in part, to increased expression of TGF-β. For example, in normal rat kidney (NRK) cells, TGF-β stimulates collagen and fibronectin synthesis and the level of mRNA for collagen types I, III and V and fibronectin. TGF-β also inhibits the breakdown of ECM components by stimulating the synthesis of protease inhibitors and decreasing the synthesis of several proteases, including transin/stromeolysin, serine and thiol protease and plasminogen activator [9]. Thus, it is possible that hyperglycemia leads to increased TGF-β expression, which participates in the increased synthesis of ECM components in tubules and glomeruli of the diabetic kidney.

In summary, elevated glucose levels in cultured medium of proximal tubule cells modulate the expression and the responsiveness to specific autocrine or paracrine growth-regulatory factors. In a previous study, we demonstrated that high glucose potentiated the cellular hypertrophogenic response to exogenously added angiotensin II [45]. In the present study, we demonstrate that the growth inhibitory effect of elevated glucose levels in culture medium of mouse proximal tubule cells appears to be mediated by the increased production of endogenous TGF-β. This autocrine function of TGF-β provides for a novel mechanism of action through which ambient glucose influences certain target cells.

Acknowledgments

This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-39727, DK-39565, and DK-07006) and by a grant from the American Diabetes Association (Philadelphia). M.R. was supported by a fellowship from the National Kidney Foundation. The human TGF-β_1 cDNA used in this study was a gift from Dr. A. Singh, Genetech, San Francisco, California, USA. Portions of this work were presented at the 23rd Annual Meeting of the American Society of Nephrology (1990) and appeared in abstract form (*J Am Soc Nephrol* 1:448, 1990).

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