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High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β

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High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-β. We investigated the effects of glucose concentration in serum-free media on the proliferative growth response of a cultured murine mesangial cell line. Raising the ambient D-glucose concentration from 100 mg/dl to 450 mg/dl stimulated cell proliferation after 24 to 48 hours but had a growth inhibitory effect after 72 to 96 hours of incubation. This biphasic proliferative response to high glucose concentration was not mediated by the elevated osmolarity of the medium and did not occur when L-glucose was used. The early phase of glucose-induced proliferation was associated with increased expression of the immediate early genes c-*myc* and *egr*-1 as well as with induction of the S-phase related proliferating nuclear cell antigen (PCNA). Several lines of evidence indicated that the late phase of glucose-induced growth inhibition was mediated by the bioactivation of endogenous transforming growth factor beta (TGF-β). Neutralizing antibody against TGF-β prevented the late inhibitory effects of glucose on proliferation. On the other hand, exogenous TGF-β (1 ng/ml) significantly inhibited basal proliferation in mesangial cells. Furthermore, Northern blot analysis revealed that TGF-β_1 mRNA was induced by 450 mg/dl glucose in the medium after 48 to 72 hours, but not after 24 hours. Cell cycle analysis demonstrated that mesangial cells incubated in high glucose for 24 hours have a higher percentage of cells in the S-G_2 phase of the cell cycle compared with cells grown in normal glucose concentration. After 48 hours of culture in elevated glucose concentration, the percentage of cells in S-G_2 phase was decreased, and became comparable to that of cells in normal glucose concentration. However, the addition of neutralizing anti-TGF-β antibody stimulated the progression of cells towards S-G_2 in high glucose medium after 48 hours. The findings of this study demonstrate a biphasic growth response of mesangial cells when they were cultured in high glucose concentration; initially there was a transient stimulation of replication for 24 to 48 hours followed by a sustained inhibition after longer incubation periods. This inhibition may be mediated by the glucose-induced synthesis and/or bioactivation of TGF-β which can inhibit proliferation of mesangial cells in an autocrine fashion.

Diabetic glomerulopathy is generally believed to be the major cause for the development of chronic renal failure in diabetes mellitus [1]. Glomerular hypertrophy, mesangial expansion and a thickened glomerular basement membrane, all leading to glomerulosclerosis with associated proteinuria and decreased glomerular filtration, are prominent characteristics of glomerular pathology in this disease [2, 3]. The pathogenesis of the glomerular lesion is not completely understood. Functional abnormalities such as glomerular hyperfiltration and hypertension as well as disturbances caused by the diabetic milieu may contribute to the glomerulopathy [4, 5]. Additionally, alterations in metabolic pathways and disturbances in the synthesis or action of various eicosanoids, cytokines and polypeptide growth factors have all been hypothesized to be involved in the pathogenesis of diabetic glomerulopathy [5, 6]. Hyperglycemia *per se* also seems to be an important factor in the development of renal hypertrophy in diabetes [5, 6]. Furthermore, elevated glucose can cause an increase in extracellular matrix proteins in cultured rat glomerular cells [7, 8]. Whether elevated glucose levels can also alter the proliferation of the different types of resident glomerular cells remains to be studied.

Autoradiographic studies and direct cell counts have demonstrated that prominent proliferation of mesangial cells is not a feature of diabetic glomerulopathy [9, 10]. Some investigators have reported modest increases in the number of podocytes [10]. Cell culture studies revealed that non-enzymatic glycosylation of extracellular matrix proteins inhibits mesangial cell proliferation [11]. However, the profile of mesangial cell proliferation may vary during the time-course of the disease and may be influenced by many factors in the diabetic environment. Furthermore, small initial changes in mesangial cell number with subsequent inhibition of proliferation might not be detected by autoradiography or cell counting in vivo.

In the present study we investigated whether elevated ambient glucose concentrations influenced the proliferation of cultured murine mesangial cells. To define the isolated influence of ambient glucose on cell growth we developed a mesangial cell line which retains differentiated characteristics in serum-free medium. Our studies demonstrate that raising ambient glucose concentration results in transient stimulation of growth at 24 to 48 hours, and in growth inhibition after longer (≥72 hr) periods. This time-related down-regulation of growth appears to be mediated through increased endogenous expression and synthesis of bioactive transforming-growth factor-beta (TGF-β). Additional studies on non-transformed mesangial cells yielded similar results. Thus, some of the latent effects of increased glucose concentration on mesangial cell growth are mediated by bioactivation of endogenous TGF-β. Additionally, it is postulated that TGF-β might, in turn, influence extracellular matrix production with the subsequent development of glomerulosclerosis.

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Methods

Cell culture

Murine mesangial cells (MMCs) were isolated from kidneys harvested from 8- to 10-week-old naive SJL/J (H-2^S) mice according to standard procedures [12]. MMCs, cloned from glomerular outgrowths, were grown for 72 hours in the presence of 50 mM D-valine (Sigma Chemical Co., St. Louis, Missouri, USA), replacing L-valine in the medium to exclude fibroblasts. Subconfluent MMCs were transformed with non-capsid forming SV-40 virus to establish a permanent cell line [13]. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL, Gaithersburg, Maryland, USA) containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The doubling time was approximately 36 to 48 hours. MMCs were passaged every 72 hours by trypsinization.

Multiple morphological and functional criteria demonstrate that this cell line represents differentiated mesangial cells: MMCs have the typical spindle-like appearance, stain positive for vimentin and desmin, but fail to bind an antibody against a proximal tubular antigen [13]. In contrast to syngeneic renal fibroblasts, MMCs grow in the presence of D-valine [13]. Furthermore, MMCs express angiotensin II-receptors of the AT-1 type and respond to angiotensin II stimulation by contraction [13].

To corroborate our observations in the transformed mesangial cell line, we undertook a limited series of experiments for the measurement of cellular proliferation and TGF-β_1 mRNA levels in three different populations of non-transformed cells: primary cultures of mesangial cells derived from the same pool of MMCs (10 to 11 passages) which did not undergo viral transformation; non-transformed mesangial cells harvested from a 6-week-old Sprague-Dawley rat, which have been propagated in long-term culture (32 to 33 passages); and mesangial cells derived from 10-week-old MRL-*lpr/lpr* mice (14 to 17 passages) prior to the development of lupus-like autoimmune nephritis (gift of Drs. Borut Cizman and Michael P. Madaio, University of Pennsylvania, Philadelphia, Pennsylvania, USA).

Cell proliferation assays

10^4 MMCs were transferred to each well of 96-well microtiter plate (Nunclon, Denmark) and were rested for 24 to 48 hours in DMEM with a D-glucose concentration of 100 mg/dl (5.6 mM) without any additional factors. Fresh serum-free DMEM with a D-glucose concentration ranging between 100 to 600 mg/dl (5.6 to 33 mM) was then added. For some experiments, the osmolarity of the medium containing lower glucose concentrations was adjusted with D-mannitol so that the final osmolarity of all media were equal. Furthermore, additional MMCs were incubated with DMEM containing supplemental L-glucose. Some cells were also treated with transforming growth factor-beta 1 (TGF-β_1 from porcine platelets; R & D Systems, Minneapolis, Minnesota, USA), neutralizing rabbit anti-porcine TGF-β antibody (IgG fractions, neutralizes TGF-β_1 and TGF-β_2; R & D Systems), or normal rabbit IgG (Sigma). Cells were grown for an additional 24 to 96 hour period, and 1 μCi ^{3}[H]thymidine (5 Ci/mmol, Amersham, Arlington Heights, Illinois, USA) was included in all media for the last six hours of growth [14–16]. Cells were lysed and harvested on glass-fiber paper and counted for scintillations as previously described [14–16].

For cell counts, 5×10^4 MMCs were rested in serum-free DMEM with 100 mg/ml D-glucose in a 24-well plate and the media were subsequently modulated as described above. At the end of the experiment, cells were harvested from the plate and counted with an automated cell counter (Coulter Electronics, Hialeah, Florida, USA).

Cell cycle analysis

Cell cycle analysis was done as previously described [16]. Briefly, 10^7 MMCs incubated for 24 to 72 hours in different culture conditions as described above, were washed three times in ice-cold phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde for five minutes. Cells were then permeabilized for three minutes in 1% Triton X-100 (Sigma), and treated for 30 minutes at 37°C with 150 U DNAse-free RNAse I-AS (Sigma) to digest all cellular RNA. Remaining cellular DNA was stained with propidium iodide (Sigma, 50 μg/ml in PBS). After extensive washing in PBS, 10^4 MMCs were analyzed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, California, USA) with forward cell scatter (FSC, E00/1.00), side cell scatter (SCC, 270/1.00), and fluorescence channel 2: red (FL2, 420/1.00). Cells were gated to remove dead cells. Data were analyzed with LYSYS II software (Version 1.0, Becton-Dickinson) and are represented as histograms with relative cell number on the Y-axis and relative DNA content (FL2 intensity) on the X-axis [16]. Percentages of cells in the G_1 and S-G_2 phase of the cell cycle were determined from the histograms by computer analysis (LYSYS II software).

Northern hybridizations

A total of 10^7 MMCs were cultured for different time periods in serum-free DMEM containing either 100 mg/dl or 450 mg/dl D-glucose. Cells were then washed in RNAse-free PBS (pH 7.2), and total RNA was isolated as described [16, 17]. For Northern blots, 20 μg total RNA was electrophoresed through a 1.2% agarose gel with 2.2 M formaldehyde. The RNA was electroblotted onto Zetabind membranes (Cuno, Meriden, Connecticut, USA), UV-cross-linked, and the membrane was prehybridized overnight at 42°C in a buffer containing 5 × SSPE (1 × = 149 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 × Denhardt's (50 × Denhardt's = 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml denatured salmon sperm DNA, and 50% (vol/vol) formamide. cDNA inserts were separated from their plasmids in low-melt agarose and labelled with 5 μCi ^{32}[P]deoxycytidine 5′-triphosphate (3,000 Ci/mmol, Amersham) using hexamer primers. The probes used were an EcoR1 fragment encoding the human TGF-β_1 gene (gift of Dr. A. Singh, Genentech, San Francisco, California, USA), a PstI fragment from the c-*myc* clone p*myc*41 [18], a PstI insert of the mouse *egr*-1 gene ([19] gift of Dr. Vikas P. Sukhatme, University of Chicago, Chicago, Illinois, USA), and a PstI fragment encoding the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [20]. The labelled probes were separated from unincorporated nucleotides by Sephadex G-50 spin columns (Boehringer Mannheim, Indianapolis, Indiana, USA), and blots were hybridized with 1×10^6 cpm/ml probe in hybridization buffer (same as prehybridization buffer except that 2 × Denhardt's was used)

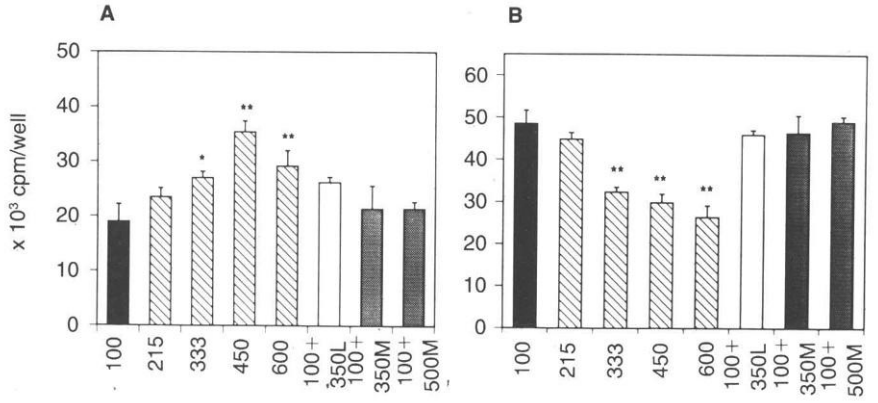

Fig. 1. *Thymidine incorporation into mesangial cells (MMCs) incubated for 24 hours (A) or 72 hours (B) in the presence of various medium D-glucose concentrations.* **A.** After 24 hr, thymidine incorporation was significantly stimulated by 333 to 600 mg/dl D-glucose containing medium compared to normal glucose medium (100 mg/dl). **B.** However, after 72 hr of incubation, proliferation was significantly inhibited by the high glucose concentrations. This effect was specific for D-glucose and was not observed when 350 mg/dl L-glucose (L) was added to the control medium containing 100 mg/dl D-glucose. Furthermore, the proliferation was independent of the medium osmolarity since raising the osmolarity of the medium containing 100 mg/dl D-glucose by the addition of 350 mg/dl or 500 mg/dl D-mannitol (M) had no significant effect on thymidine incorporation ($N = 8$, $^*P < 0.05$ vs. cells incubated in 100 mg/dl D-glucose, $^{**}P < 0.01$ vs. cells incubated in 100 mg/dl D-glucose).

for 24 hours at 42°C. Membranes were washed for 30 minutes in 2 × SSC (20 × SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) with 0.1% SDS at room temperature, followed by two 15-minute high stringency washes in 0.1 × SSC, 0.1% SDS at 62°C. The membranes were then autoradiographed with intensifying screens at −70°C for three to four days (TGF-β, c-*myc*, *egr*-1). Blots were stripped and subsequently rehybridized with the probe encoding the housekeeping gene GAPDH to account for small loading the transfer variations. Exposed films were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, California, USA) and relative RNA levels were calculated as described.

Quantitative cDNA amplification

Relative levels of proliferating cell nuclear antigen (PCNA), which is only expressed during DNA-replication [21], was assessed by quantitative cDNA amplification. cDNA was synthesized from 10 μg of total RNA harvested from MMCs grown for 24 hours in serum-free DMEM with 100 mg/dl or 450 mg/dl D-glucose. For some experiments, RNA was also harvested from MCT cells, a syngeneic proximal tubular cell line [14–17], grown either in 100 mg/dl or 450 mg/dl glucose for 24 hours. Reverse transcriptions were primed with 0.7 μg of poly-d(T) primer (Pharmacia-LKB, Piscataway, New Jersey, USA) in the presence of 500 units of maloney murine leukemia virus reverse transcriptase diluted in 50 μl of buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 500 μM dNTPs [22, 23]. Reaction products were precipitated with 7.5 M ammonium acetate and isopropanol, spun, washed in 70% ethanol, air dried, resuspended in distilled water, and the concentration of cDNA was determined by UV spectrophotometry [23].

Polymerase chain reactions (PCR) for quantitative comparison of PCNA transcripts were normalized for the presence of the housekeeping transcript GAPDH as previously described [22, 23]. Five hundred ng of cDNA was used in the amplification reactions. A total of 150 ng of each of the following primers was used:

$$\text{PCNA: } \frac{5'\text{GCCATCTGGTGAATTTGCAGG3'},}{5'\text{GCAATTTTATACTCTACAACAAGG3'}}$$

$$\text{GAPDH: } \frac{5'\text{AATGCATCCTGCACCACCAA3'},}{5'\text{GTAGCCATATTCATTGTCATA3'}}$$

The complete amplification mix without the primers was equivalently distributed to separate tubes containing either PCNA or GAPDH primers. Reactions were performed using the GeneAmp kit (Perkin Elmer Cetus, Norwalk, Connecticut, USA) with 200 μM of each dNTP, 0.1 μCi ^{32}[P]-dCTP, 2.5 units of AmpliTaq polymerase in PCR-buffer (20 mM Tris-HCl, pH 8.3; 25 mM KCl, 2.0 mM $MgCl_2$, and 0.05% Tween 20). Forty cycles with an annealing temperature of 55°C, an extension step at 72°C, and the denaturation step at 92°C were performed. We have previously shown [22, 23] that using 40 cycles and a template concentration of 500 ng is in the linear range of the amplification curve so that the amount of product is not likely limited by the concentration of primers, dNTPs or polymerase (data not shown). Ten microliter of the reaction product were run out on a 1.6% agarose gel containing 0.5 μg/ml ethidium bromide. Southern blots performed with internal primers revealed the identity of the amplification products [23] (data not shown). For quantification, the bands representing the reaction product were excised from the gel, transferred into scintillation fluid and counted for β-emission. A normalization quotient between the cpms for PCNA and GAPDH was calculated as previously described [23]. The quotients from cells grown in 100 mg/dl glucose were arbitrarily assigned a relative value of one.

Results

Effects of glucose on cellular proliferation

We have been interested in whether increasing the glucose concentration in serum-free media has an effect on proliferation in a murine mesangial cell line. As shown in Figure 1A, raising the ambient glucose concentration for 24 hours significantly increases thymidine incorporation in MMCs. This increase in

Table 1. Effect of high glucose concentration on thymidine incorporation in non-transformed rat mesangial cells

	Thymidine incorporation ($\times 10^3$ cpm/well)			
	24 hr	48 hr	72 hr	96 hr
Glucose *mg/dl*				
100	16.0 ± 1.11	70.0 ± 3.2	16.0 ± 1.55	13.3 ± 4.49
450	19.7 ± 0.78[a]	92.1 ± 4.8[b]	11.2 ± 0.59[b]	10.3 ± 1.4[a]

A non-transformed rat mesangial cell line propagated in long-term culture was used. Cells from the 32nd passage were plated in DMEM containing 100 mg/dl glucose and 10% fetal calf serum; after 24 hr the cells were rested for another 24 hr in serum-free DMEM containing 100 or 450 mg/dl glucose. Cell proliferation assay was as described for MMCs in **Methods**. Values are mean ± SE for 10 to 12 replicate wells. [a] $P < 0.05$ and [b] $P < 0.01$ for comparison between the two glucose media at each time period. Another experiment yielded similar results.

thymidine incorporation was dose-dependent for the glucose concentration, with an optimal response achieved at a glucose concentration of 450 mg/dl. Therefore, this glucose concentration was included in all subsequent experiments. The stimulatory effect was not mediated by an increase in the osmolarity of the medium since raising the osmolarity of medium containing 100 mg/dl glucose to 450 mg/dl or 600 mg/dl through the addition of D-mannitol did not significantly stimulate thymidine incorporation (Fig. 1A). Furthermore, supplementing the low D-glucose medium with 350 mg/dl L-glucose was without effect. The observed stimulatory effects of high glucose were still evident, albeit to a lesser degree, after 48 hours of incubation (data not shown). However, as demonstrated in Figure 1B, thymidine incorporation was significantly inhibited, in a dose-dependent fashion, by elevated glucose concentrations after 72 hours of incubation. Again these effects were due to D-glucose in the medium and were not mimicked by L-glucose or D-mannitol. A similar inhibition of proliferation by high glucose was observed after 96 hours of incubation (data not shown).

The biphasic response with early stimulation (24 to 48 hr) and subsequent inhibition (≥72 hr) of thymidine incorporation into MMCs by high glucose concentration was reflected in similar changes in cell number. Raising the ambient glucose concentration for 24 hours from 100 mg/dl to 450 mg/dl significantly increased total cell number whereas incubation for 72 hours was not associated with a significant increase in cell number (100 mg/dl glucose for 24 hr: $6.4 \pm 0.2 \times 10^5$ cells/well, 450 mg/dl glucose for 24 hr: $8.0 \pm 0.4 \times 10^5$ cells/well, $P < 0.02$; 100 mg/dl glucose for 72 hr: $8.3 \pm 0.2 \times 10^5$ cells/well, 450 mg/dl glucose for 72 hr: $8.4 \pm 0.18 \times 10^5$ cells/well, not significant, $N = 6$).

Table 1 demonstrates a similar biphasic effect of elevated glucose concentration on thymidine incorporation in non-transformed rat mesangial cells which have been propagated in long-term culture. Compared with the serum-free medium containing 100 mg/dl glucose, the serum-free medium containing 450 mg/dl glucose stimulates thymidine incorporation at 24 and 48 hours (by 23% and 32%, respectively), but it inhibits the incorporation at 72 and 96 hours (by 30% and 23%, respectively). These findings suggest that the modulation of cellular proliferation by high glucose concentration may not depend on viral transformation of the cells.

Effects of glucose on proto-oncogene and proliferating cell nuclear antigen expression

To further evaluate the biphasic proliferative response of MMCs to the glucose concentration in the medium, the expression of two immediate early genes and the proliferating cell nuclear antigen (PCNA) was examined. The expression of c-*myc* and *egr*-1 was evaluated by Northern blot analysis. Both are immediate early genes encoding transcriptional factors which are expressed in the G_1-phase of the cell cycle [24, 25]. As shown in Figure 2A, exposure of quiescent MMCs for 24 hours to 450 mg/dl glucose resulted in induction of c-*myc* expression (standardized to GAPDH mRNA levels) when compared to medium with 100 mg/dl glucose. However, this response to high-glucose medium did not persist after 48 to 72 hours. A similar temporal response to high glucose levels was observed for the transient expression of the immediate early gene *egr*-1 (Fig. 2B). In one of three experiments, the transient induction of the two immediate early genes was evident at 48 hours rather than at 24 hours.

We next evaluated the expression of PCNA by semi-quantitative cDNA amplification. PCNA is a nuclear protein [21] which acts as a co-factor of DNA polymerase delta [26]. Its maximal expression takes place in the S-phase of the cell cycle [21, 26]. We relied on quantitative cDNA amplification to assess PCNA transcripts because of the increased sensitivity and reliability of this method compared to Northern blots [23]. Figure 3 shows that incubation of MMCs for 24 hours in medium with 450 mg/dl glucose significantly increased cDNA amplification products for PCNA whereas the expression of the housekeeping gene GAPDH was not affected. Although the size of the reaction products for PCNA and GAPDH were predicted from the primer distances, the identity was established by Southern hybridization using end-labelled internal oligonucleotides (data not shown). As an additional control, PCNA expression was evaluated in MCT cells, a syngeneic proximal tubular cell line, grown in 100 mg/dl or 450 mg/dl glucose. We have previously demonstrated that proliferation in MCT cells, in contrast with MMCs, is inhibited by medium containing 450 mg/dl glucose after 24 hours [14, 15]. As shown in Figure 3, in accordance with our previous findings, PCNA expression in MCT cells is significantly inhibited by 450 mg/dl glucose compared to 100 mg/dl glucose.

Effect of glucose on TGF-β RNA expression

We have previously shown that high glucose levels increase the expression of steady-state RNA levels for TGF-β in syngeneic proximal tubular cells [27]. We hypothesized that, after a lag period, similar mechanisms may be operating in MMCs: the early proliferation stimulated by high glucose may be subsequently abolished by the autocrine induction of the growth-inhibitory effect of TGF-β. We evaluated the expression of TGF-β_1 with a human cDNA probe, detecting a transcript of 2.5 kb. Figure 4 is a representative autoradiograph showing the effect of glucose on TGF-β gene expression. Raising the glucose concentration from 100 mg/dl to 450 mg/dl increased TGF-β steady-state mRNA levels by 51 ± 6% ($N = 4$, $P < 0.02$) after 48 hours, and this response persisted for 72 hours; however, this effect was not present after an incubation time of only 24 hours.

Similar results were obtained in non-transformed mouse mesangial cells. Because of a low RNA yield in two experiments using relatively small numbers of cells, we resorted to a semi-quantitative assay of TGF-β_1 message using cDNA amplification by the polymerase chain reaction of reverse transcribed

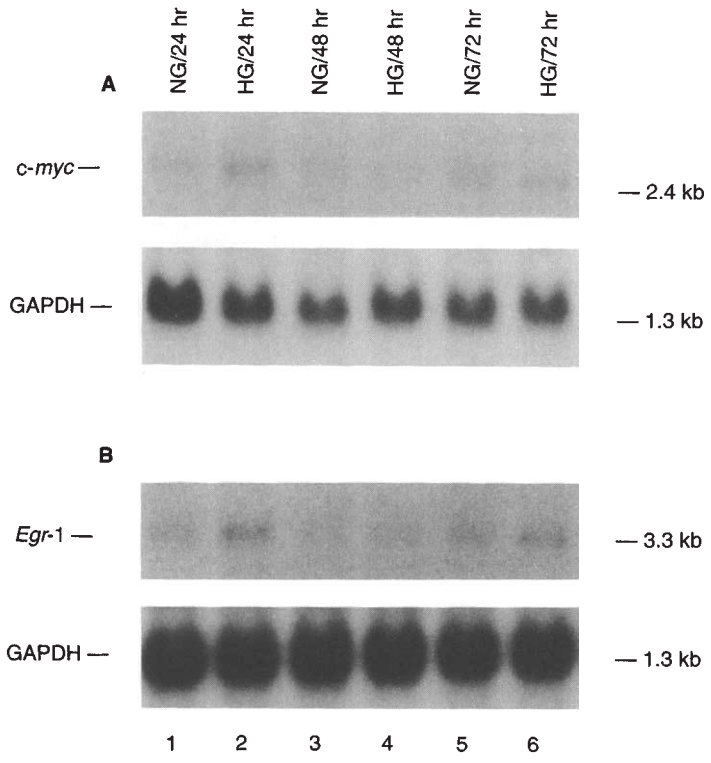

Fig. 2. *Northern blots of mRNA isolated from mesangial cells (MMCs) grown for different times in media containing either 100 mg/dl or 450 mg/dl D-glucose (NG or HG, respectively) hybridized with cDNA probes for* c-myc *(A) and (B)* egr-1. Cells incubated for 24 hr in high-glucose containing medium expressed more RNA for c-*myc* and for *egr*-1 as compared with normal-glucose containing medium. This induced expression was down-regulated after 48 to 72 hr of incubation in 450 mg/dl glucose medium. The blots were stripped and rehybridized with a probe for the housekeeping gene GAPDH to adjust for small variations in RNA loading and transfer.

total RNA [22]. At 72 and 96 hours in culture, there was slightly more than twofold increase in TGF-β_1 mRNA levels, relative to the GAPDH signal, in the cells exposed to 450 mg/dl glucose compared with 100 mg/dl glucose (data not shown).

Northern analysis of RNA isolated from non-transformed mesangial cells of young MRL-*lpr/lpr* mice demonstrated that high glucose concentration in the culture medium stimulated TGF-β_1 message levels after a lag period of at least 48 hours (Fig. 5). Compared with the medium containing 100 mg/dl glucose, the medium containing 450 mg/dl glucose increased TGF-β_1 mRNA levels by 80% and 76% at 72 and 96 hours, respectively ($N = 3$). These studies on non-transformed cells indicate that the effects of high glucose concentration on TGF-β_1 mRNA are not related to viral transformation of the cells.

Effect of neutralizing anti-TGF-β antibody on cell proliferation

To further test our hypothesis, we reasoned that a neutralizing anti-TGF-β antibody may abolish the late inhibitory effects of high glucose on MMCs proliferation. We have previously shown that the used lot of the rabbit antibody exhibited a neutralizing effect on TGF-β action [27]. The inhibitory effects of 450 mg/dl glucose on basal thymidine incorporation after 72 hours can be totally prevented by the addition of 30 μg/ml anti-TGF-β antibody (Fig. 6). Incubation of MMCs in the presence of anti-TGF-β antibody in 100 mg/dl glucose media had no significant effect on thymidine incorporation. The observed effects were specific for the anti-TGF-β antibody since the addition of 30 μg/ml normal rabbit IgG had no significant effect on basal thymidine incorporation (450 mg/dl glucose: 13.8 $\pm$ 0.3 $\times$ 10^3 cpms/well, 450 mg/dl glucose + 30 μg/ml rabbit IgG for 72 hr: 13.6 $\pm$ 0.4 $\times$ 10^3 cpms/well, not significant, $N = 6$).

Effect of exogenous TGF-β on cellular proliferation

Although the majority of studies show that TGF-β generally inhibits proliferation of mesangial cells [28–30], some authors have suggested a biphasic response with inhibition of proliferation at low plating density and stimulation when the cells are plated at high density [31]. We therefore examined whether exogenous TGF-β (1 ng/ml) exhibits an inhibitory effect on MMCs. Cells incubated for 24 hours with TGF-β were clearly growth-suppressed (controls in high glucose: 12.5 $\pm$ 0.7 $\times$ 10^3 cpms/well, TGF-β in high glucose: 7.0 $\pm$ 0.3 $\times$ 10^3 cpms/well, $P < 0.02$, $N = 6$). A similar growth inhibitory effect of TGF-β was present in MMCs grown in normal glucose medium (data not shown). The anti-proliferative effect of TGF-β was documented by direct cell counts (data not shown). The plating density of MMCs and the incubation time with TGF-β in normal or high glucose media was varied in some additional experiments (data not shown); however, we found no stimulatory effect of TGF-β on the proliferation of MMCs.

Cell cycle analysis

Cell cycle analysis was performed to confirm and extend the thymidine incorporation studies. Percentages of cells in G_1 and S-G_2-phase of the cell cycle was calculated by computer analysis using LYSYS II software. As shown in Figure 7 A and B, MMCs incubated in 450 mg/dl glucose for 24 hours have a greater percentage of cells in the S-G_2-phase of the cell cycle compared with MMCs grown in normal glucose, indicating that the cells were undergoing DNA-replication (100 mg/dl glucose

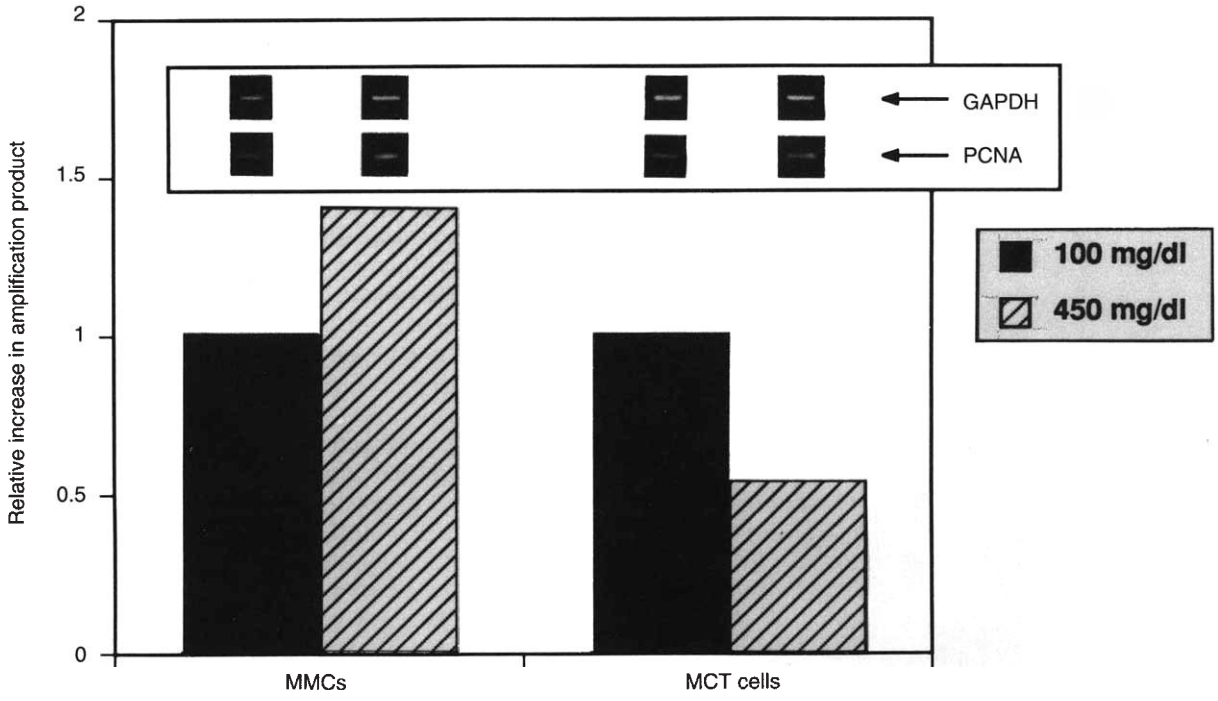

Fig. 3. *Quantitative cDNA amplification for the proliferating cell nuclear antigen (PCNA) which is expressed during the S-phase of the cell cycle.* PCNA was induced in mesangial cells (MMCs) grown for 24 hr in high glucose-containing medium (450 mg/dl), whereas expression was markedly suppressed in a proximal tubular cell line (MCT cells) incubated for the same time in high glucose. Expression of GAPDH was not significantly influenced by the medium glucose concentration. The insert shows the amplification product of one representative experiment, and the graph represents the mean of three independent experiments with separate treatment and reverse transcription reaction.

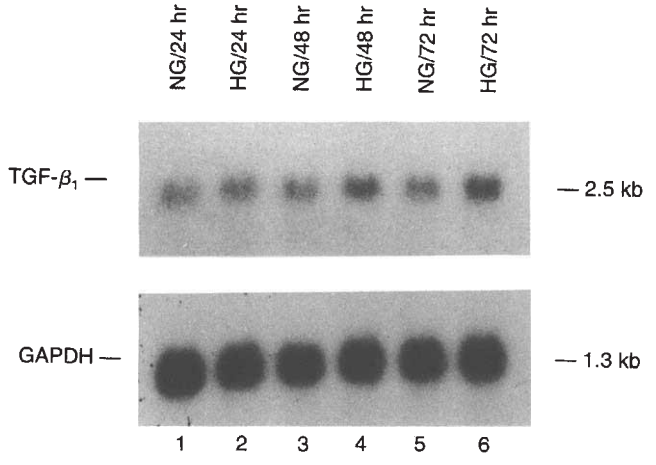

Fig. 4. *Northern blot of RNA harvested from transformed mesangial cells (MMCs) grown for different times in medium with either 100 mg/dl or 450 mg/dl D-glucose (NG or HG, respectively).* Expression of TGF-β_1 mRNA was significantly stimulated by more than 50% after 48 to 72 hr incubation of the cells in high glucose vs. normal glucose medium. This effect was not observed after 24 hr of exposure to high glucose. The blot was reprobed with GAPDH cDNA and is representative for four independent experiments.

for 24 hr: 47.4% cells in S-G_2, 450 mg/dl glucose for 24 hr: 62.7% cells in S-G_2). However, after a 48 hour incubation in high glucose the percentage of MMCs in S-G_2 phases of the cell cycle was markedly diminished (Fig. 7 C and D, 100 mg/dl glucose for 48 hr: 46.1% cells in S-G_2, 450 mg/dl glucose for 48 hr: 43.2% cells in S-G_2). Incubation of MMCs for 48 hours with 30 μg/ml neutralizing anti-TGF-β antibody in high glucose medium resulted in a sharp increase in the number of cells in S-G_2 (Fig. 7 E and F, 100 mg/dl glucose for 48 hr + anti-TGF-β antibody: 49.0% cells in S-G_2, 450 mg/dl glucose for 48 hr + anti-TGF-β antibody: 52.5% cells in S-G_2). Normal rabbit IgG did not significantly influence the cycling of MMCs (Fig. 7 C and D, and data not shown).

Discussion

Our present study provides evidence that the ambient glucose concentration of the culture media, in the absence of serum or any additional factors, has a biphasic effect on proliferation of a murine mesangial cell line. Raising the D-glucose concentration in the medium for 24 hours had a significant proliferative effect with an optimum response at a glucose concentration of 450 mg/dl (Fig. 1A). The proliferative effect was independent of the medium osmolarity and was not observed with L-glucose. Furthermore, the induced proliferation was associated with the predicted expression of immediate early genes (Fig. 2) as well as the proliferating cell nuclear antigen (PCNA) which is activated during the S-phase of the cell cycle (Fig. 3). However, after 72 hours of exposure to high glucose-containing media a growth suppressive effect on MMCs was observed (Fig. 1B). Our experiments using the anti-TGF-β antibody convincingly demonstrate that the later growth-inhibitory effect of high glucose is due to endogenous activation of TGF-β (Fig. 6). Furthermore, expression of steady-state RNA for TGF-β_1 was increased in high-glucose at 48 to 72 hours but not at 24 hours (Fig. 4). Production of TGF-β by mesangial cells has been

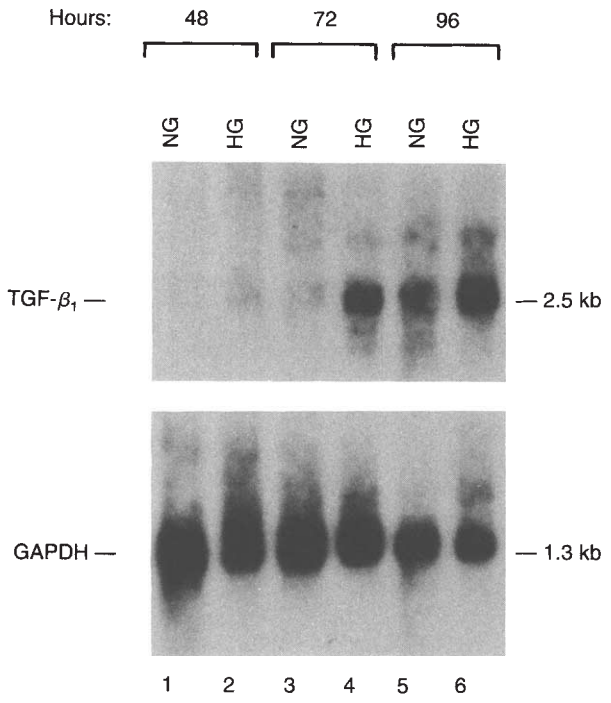

Fig. 5. *Northern blot of RNA harvested from non-transformed mesangial cells derived from MRL*-lpr/lpr *mice prior to the clinical development of lupus nephritis.* The cells were grown in serum-free medium for different time periods in medium with either 100 mg/dl or 450 mg/dl D-glucose (NG or HG, respectively). Expression of TGF-β_1 mRNA was stimulated by more than 80% after 72 to 96 hr incubation of the cells in high glucose vs. normal glucose medium. This effect was not observed at earlier time periods. The blot was reprobed with GAPDH cDNA and is representative of three different experiments.

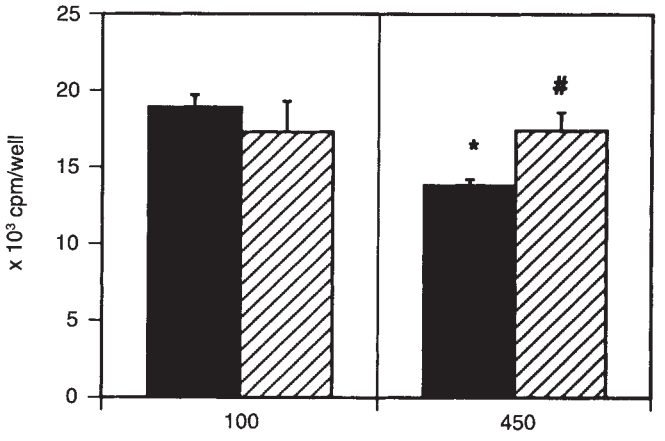

Fig. 6. *Thymidine incorporation into mesangial cells (MMCs) incubated for 72 hours in normal (100 mg/dl) or high (450 mg/dl) glucose-containing medium.* The suppressed thymidine incorporation in the high glucose medium was totally abolished by treatment with 30 μg/ml neutralizing anti-TGF-β antibody. Addition of 30 μg/ml normal rabbit IgG had no significant effect on thymidine incorporation ($N = 6$, $^*P <$ 0.01 vs. cells grown in 100 mg/dl glucose, $\#P < 0.01$ vs. controls in 450 mg/dl glucose medium without anti-TGF-β antibody.)

previously shown by various investigators [reviewed in 30]. The majority of studies report a general growth-inhibitory effect of exogenous TGF-β on mesangial cell proliferation, although some authors have reported a biphasic response [28–31]. We have not observed a stimulatory effect of TGF-β on MMCs proliferation in our cell culture system. The discrepancy of our observations in serum-free medium with the study of MacKay and co-workers [31] describing the biphasic effect of TGF-β in murine mesangial cells might be explained by the presence of fetal calf serum or other factors.

The mechanisms of the growth-inhibitory effect of TGF-β are complex and have been more recently elucidated [30]. For example, TGF-β has been shown to decrease transcription of c-*myc* by interacting with a *cis*-regulatory element in the c-*myc* promoter [32]. Furthermore, TGF-β prevents phosphorylation of the protein product of the retinoblastoma gene (pRB) which is a negative growth regulator [33, 34]. Under-phosphorylated pRB arrests cells in the G_1 phase of the cell cycle. Moreover, TGF-β has no growth suppressive effects in derivative cell lines which are insensitive to the TGF-β mediated under-phosphorylation of pRB [33, 35].

The influence of glucose on mesangial cell proliferation, in the absence of serum or other factors, has not been previously studied. One study reports no significant change in cell number of rat mesangial cells incubated for prolonged times in medium containing 10 mM (180 mg/dl) glucose [8]. However, this study was done in the presence of 20% fetal calf serum which may mask any stimulatory effects of elevated glucose levels and might easily abolish the growth suppressive effects of activated endogenous TGF-β. A recent study has demonstrated that in vitro nonenzymatic glycosylation of the support matrix for mesangial cells in culture significantly inhibited thymidine incorporation into the cells [11]. This interesting study suggests that nonenzymatic glycosylation of matrix which is induced by prolonged exposure to elevated glucose levels has a growth inhibitory effect on rat mesangial cells in culture. It has been reported that extracellular matrix can bind, store and release TGF-β [36]. One can speculate that nonenzymatic glycosylation leads to an increased affinity of extracellular matrix components for TGF-β. Such a system would then allow the storage of glucose-induced TGF-β with the possibility of long-term release and subsequent growth inhibition of mesangial cells. However, this intriguing hypothesis remains to be tested. Furthermore, as with all in vitro studies utilizing cell culture models, the immediate relevance of our short-term observations to the problem of human diabetic glomerulopathy requires confirmatory investigations in vivo.

How the elevated glucose concentration can stimulate early proliferation with subsequent induction of TGF-β is unclear, but some suggestions can be made. High glucose levels increase the activity of the polyol pathway in the kidney including cells of the glomerular tuft [37, 38]. There is clear evidence for the existence of the polyol pathway in cultured mesangial cells [38]. Cells incubated in medium containing high glucose accumulate large amounts of intracellular sorbitol [15, 39]. This may lead to disturbances in *myo*-inositol levels with subsequent alterations in signal pathways and associated growth regulation [15]. Moreover, the proteoglycan decorin has TGF-β binding activity and can neutralize this cytokine [40]. Possible interference of glucose with decorin production may modulate the levels of active TGF-β. The observed increase in steady-state mRNA for TGF-β can be due to an increase in transcriptional activity

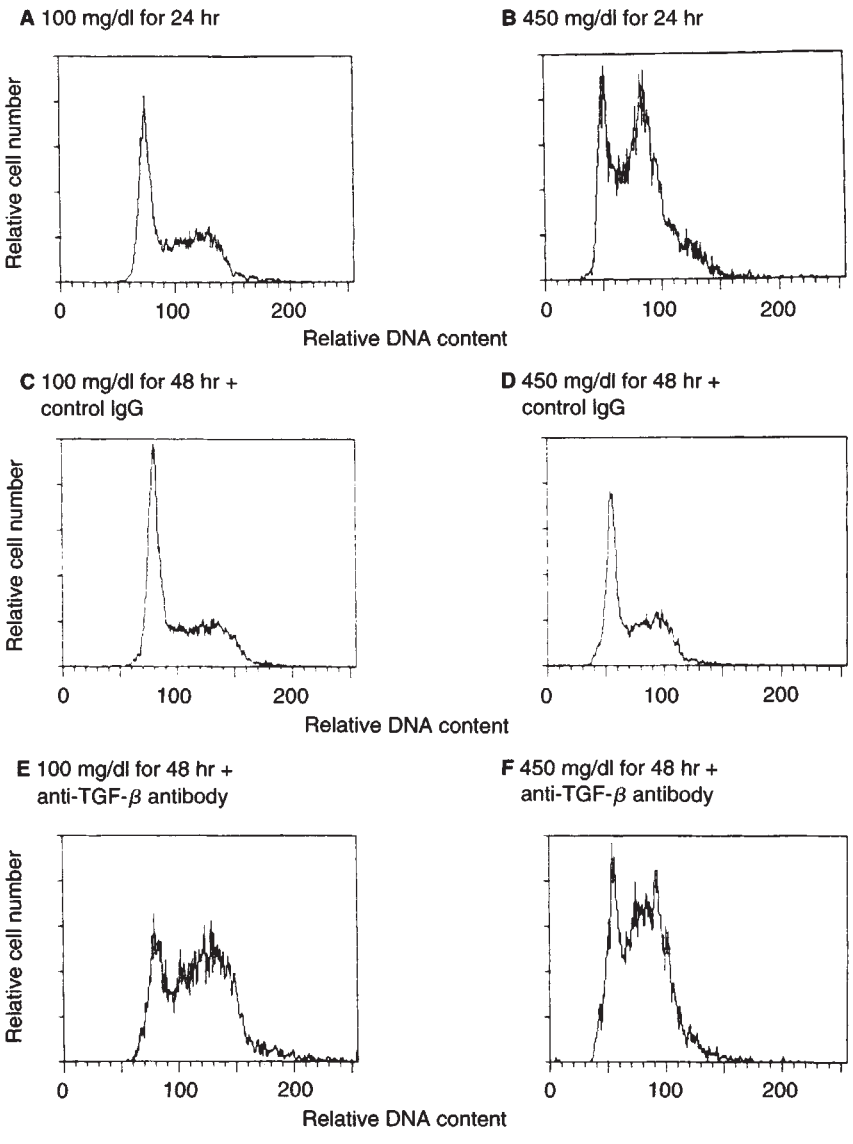

Fig. 7. *Cell cycle analysis of mesangial cells (MMCs) grown for different times in normal or high glucose medium.* As described in the **Methods** section, cellular DNA was stained with propidium iodide. Gated data are represented as histograms after computer analysis. The Y-axis represents the relative cell number whereas the X-axis refers to the relative DNA content of the cells. The first peak represents cells in the G_0/G_1-phase of the cell cycle, whereas cells in the second peak have progressed into G_2-phase after DNA-replication. **A and B.** Cells incubated for 24 hr in high glucose have a greater percentage of members in the S-G_2-phase compared to normal glucose-containing medium, indicating successful completion of DNA-replication and progression towards mitosis. However, the ratio was reversed after 48 hr in high glucose with arrest of cells in G_1 **(C and D)**. Neutralizing anti-TGF-β antibody (30 μg/ml) totally abolished the G_1-arrest of cells grown for 48 hr in high glucose **(F)**, but had only little stimulatory activity on cells grown in normal glucose **(E)**.

and/or post-transcriptional stabilization of the mRNA. Furthermore, it can be speculated that high glucose may activate latent, inactive forms of TGF-β and may thus increase the total available pool of bioactive TGF-β. Further studies are certainly necessary to elucidate the mechanisms of glucose-induced increase in TGF-β.

Autocrine regulation of mesangial cell growth may also underlie the early phase of growth stimulation of the cells following exposure to high glucose. The nature of the growth stimulant has not been investigated in this study, although several candidate factors may be considered. For instance, insulin-like growth factor I (IGF-I) is synthesized by mesangial cells and stimulates mitogenesis in an autocrine fashion [41, 42]. It has been reported that intrarenal levels of IGF-I are elevated in diabetic animals [43]. The IGF-I receptor density and the receptor mRNA are increased in kidneys of streptozotocin-induced diabetic rats and in mesangial cells from diabetic (*db/db*) mice [44]. In addition, mouse mesangial cells produce and release a non-glycosylated IGF-binding protein [42]. Thus, high glucose may stimulate early production of IGF-I with autocrine-induced proliferation of mesangial cells. This response can be modified by IGF-I receptor density, the synthesis of IGF-binding proteins and the subsequent induction of TGF-β which mediates the delayed growth-inhibitory effects of elevated glucose concentration. Moreover, elevated glucose levels might have several other effects in mesangial cells including stimulation of Na^+/H^+ exchange and an increase in the pentose-pathway activity [5, 39]. For example, an increase in glomerular RNA with a stimulated incorporation of orotate was observed 24 hours after the onset of diabetes [45]. Interestingly, it has been also reported that diabetic mesangial cells proliferate much faster than normal cells in the presence of high glucose [44].

It is well established that diabetic glomerulosclerosis is characterized by mesangial accumulation of extracellular matrix proteins including fibronectin, laminin, and type IV collagens [5]. Ayo and colleagues [7, 8] as well as others [46] have convincingly shown that long-term incubation (up to 4 weeks)

of cultured rat mesangial cells in high glucose leads to an increase in extracellular matrix proteins. Increased steady-state levels of fibronectin, laminin and type IV collagen were demonstrated after three days but not one day of exposure to high glucose [8]. The increased synthesis of matrix proteins in various cells exposed to high glucose media seems to be mediated, at least partly, by increased transcription [8, 14, 47]. An increasing body of evidence has established the pivotal role of TGF-β in the regulation of extracellular matrix proteins in various cell types including renal cells [30, 48, 49]. This regulation involves transcriptional and post-transcriptional steps of matrix metabolism [30]. Therefore, it is possible that the autocrine activation of TGF-β by high glucose levels in our mesangial cells may lead to stimulation of extracellular matrix accumulation as observed by others. Preliminary experiments (unpublished observations) indicate that extracellular matrix synthesis is stimulated after 72 hours, but not 24 hours after incubation in high glucose in our system. Moreover, this response can be abolished by neutralizing anti-TGF-β antibody (unpublished observations).

The induction of TGF-β by high glucose is not a feature specific for mesangial cells. We have previously demonstrated that high glucose concentration stimulates TGF-β mRNA expression in cultured syngeneic MCT proximal tubular cells [27]. However, in contrast to MMCs, MCT cell proliferation in high glucose media is not stimulated initially, but in fact is significantly suppressed in a sustained fashion [14, 15]. Moreover, the observed high glucose-induced cellular hypertrophy in MCT cells [14] suggests fundamental differences in the growth response to ambient glucose in the two different syngeneic renal cell lines. The growth modulating response to elevated glucose levels in the two cell culture lines is demonstrable in the absence of serum supplementation or the addition of other factors (such as insulin) which are known to alter growth of the cells [13, 16, 50].

Intriguingly, angiotensin II (Ang II) has distinct growth effects in the different cell lines, quite similar to high glucose. Ang II induces hypertrophy in the epithelial MCT cells but mitogenesis in MMCs [13, 16, 51]. Furthermore, the hypertrophogenic action of glucose [14] is potentiated in MCT cells by Ang II [52]. It is of considerable interest to further study the relationship between the growth promoting effects of Ang II and high glucose in MMCs, especially in light of the possibility that the activity of the renin-angiotensin system may be altered in diabetes mellitus [53].

Acknowledgments

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