High glucose-induced transforming growth factor beta1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells.

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Research Article

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High Glucose-induced Transforming Growth Factor β1 Production Is Mediated by the Hexosamine Pathway in Porcine Glomerular Mesangial Cells

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Abstract

Previous studies revealed that exposure of mesangial cells to high glucose concentration induces the production of matrix proteins mediated by TGF-β1. We tested if structural analogues of D-glucose may mimic the high glucose effect and found that D-glucosamine was strikingly more potent than D-glucose itself in enhancing the production of TGF-β protein and subsequent production of the matrix components heparan sulfate proteoglycan and fibronectin in a time- and dose-dependent manner. D-Glucosamine also promoted conversion of latent TGF-β to the active form. Therefore, we suggested that the hexosamine biosynthetic pathway (the key enzyme of which is glutamine:fructose-6-phosphate amidotransferase [GFAT]) contributes to the high glucose-induced TGF-β1 production. Inhibition of GFAT by the substrate analogue azaserine or by inhibition of GFAT protein synthesis with antisense oligonucleotide prevented the high glucose-induced increase in cellular glucosamine metabolites and TGF-β1 expression and bioactivity and subsequent effects on mesangial cell proliferation and matrix production. Overall, our study indicates that the flux of glucose metabolism through the GFAT catalyzed hexosamine biosynthetic pathway is involved in the glucose-induced mesangial production of TGF-β leading to increased matrix production. (*J. Clin. Invest.* 1998. 101:160–169.) Key words: diabetic nephropathy • glomerulosclerosis • fibronectin • antisense oligonucleotide • glutamine:fructose-6-phosphate amidotransferase

Introduction

Enhanced accumulation of mesangial matrix is a hallmark in diffuse and nodular diabetic glomerulosclerosis (1–3). The degree of mesangial expansion in diabetic patients correlates closely with the progressive decline in the glomerular capillary surface area available for filtration, and therefore with the glomerular filtration rate (1, 2, 4, 5). Previous immunohistochemical (3, 6, 7) and biochemical (8) studies on the composition of human glomerular matrix indicated an enhanced deposition of various components of the extracellular matrix in diabetic nephropathy. In experimental animal models evidence has been accumulating that increased renal synthesis of components of the extracellular matrix, particularly biglycan, collagen IV, laminin, and fibronectin, is an early event in diabetic nephropathy (9–13). It has been proposed that hyperglycemia per se is an important factor in the pathogenesis of diabetic nephropathy. Numerous studies provided evidence that elevated glucose levels can cause an increase in the accumulation and expression of collagen IV and VI, laminin, and fibronectin in cultured mesangial cells (10, 14–23). However, different pathobiochemical pathways have been suggested to be involved in the hyperglycemia-induced matrix production (9, 24–26); these include increased nonenzymatic glucosylation end products (24–26), activation of the polyol pathway (24–26), and high glucose-induced stimulation of protein kinase C (14, 24–26). The involvement of growth factors in the development of diabetic nephropathy has also been suggested (12, 17, 21, 26–28).

Recent in vitro studies in murine mesangial cells revealed that elevated D-glucose concentration (25 mM) resulted in sustained growth inhibition (29). This effect was mediated by autocrine TGF-β since TGF-β antibody attenuated the observed growth inhibition. In a succeeding study these authors showed that the stimulation by high glucose of endogenous TGF-β bioactivity in mesangial cells is largely responsible for the observed increase in collagen gene expression (21), thus connecting the known matrix-stimulating properties of TGF-β with its antiproliferative activity.

Our recent studies in porcine mesangial cells showed that high glucose concentrations caused a dose-dependent increase in the production of matrix proteins, particularly basement membrane–associated heparan sulfate proteoglycan (HSPG)[1] and fibronectin, while cellular proliferation was inhibited concomitantly (30). Furthermore, we could demonstrate that de novo TGF-β1 synthesis is necessary for the high glucose-induced effects since addition of antisense TGF-β1 oligonucleotide attenuated the observed effects. Overall, the data indicated that high glucose induces a coordinate increase in TGF-β1 mRNA, enhanced TGF-β1 protein production, and bioactivation of latent TGF-β protein in cultured mesangial cells. In turn, the autocrine bioactive TGF-β induces the synthesis and accumulation of matrix protein components and inhibits mesangial cell proliferation. However, the precise mech-

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1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcN-6-P, glucosamine-6-phosphate; HSPG, heparan sulfate proteoglycan (Perlecan); UDP-GlcNAc, UDP-*N*-acetylglucosamine.

anism by which high glucose concentrations induce TGF-β1 expression in mesangial cells is unknown.

It was our aim to study the molecular mechanism(s) underlying the effects of ambient glucose concentration on TGF-β1 induction. In this report we show that glucosamine mimics the high glucose effects by inducing autocrine TGF-β activity. Furthermore, our studies reveal that the hexosamine biosynthetic pathway is involved in the high glucose-induced increased expression and bioactivation of TGF-β1 in cultured mesangial cells.

Methods

Reagents

Recombinant human TGF-β1 and chicken anti–TGF-β were obtained from R&D Systems (Bad Nauheim, Germany). Mouse monoclonal anti–TGF-β1 IgG was from Genzyme (Munich, Germany). Biotin anti–chicken antibody and streptavidin were purchased from Vector Laboratories (Burlingame, CA). RPMI 1640 tissue culture medium, nonessential amino acids, and FCS were from Seromed (Berlin, Germany). Other medium supplements and all other biochemicals were obtained from Sigma (Deisenhofen, Germany). [^{3}H]Thymidine was obtained from DuPont (Bad Nauheim, Germany), D-[6-^{3}H]glucose (sp act, 1.37 GBq/μmol) and D-[1-^{3}H]glucosamine hydrochloride (sp act, 0.13 GBq/μmol) from Amersham (Buckinghamshire, United Kingdom), and [α-^{32}P]UTP (800 Ci/mmol) was from Hartmann Analytic (Braunschweig, Germany).

Cell culture of porcine mesangial cells

Mesangial cells isolated from porcine glomeruli were cultured and characterized as described previously (30). The mesangial cells were grown in 35-mm dishes in RPMI 1640 medium containing 10 mM glucose, nonessential amino acids, 1 mM pyruvate, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% FCS (standard culture medium). For experiments, standard media contained 10 mM or elevated glucose or glucosamine concentrations with or without the tested additives for the time periods indicated. Control media contained equivalent amounts of mannitol for osmotic standardization. D-Glucosamine stocks and media containing azaserine were prepared freshly before each use. Although 10 mM glucose is high compared with normal blood levels, this is the amount necessary for mesangial cells to grow well in culture (30). Lactate was measured as described recently (31). For incubations with radiolabeled glucose, media containing 10 and 30 mM glucose concentration with 14.9 and 44.7 MBq/ml D-[6-^{3}H]glucose were prepared.

Determination of mesangial cell proliferation and cell growth

Cell proliferation was measured by [^{3}H]thymidine incorporation as described previously (30). Determination of DNA was performed by fluorometry with bisbenzimidazol and mesangial growth was assessed by counting the cells (30).

Extraction of extracellular matrix components and assays

Extracellular matrix components were extracted from the cell layers by the procedure described recently (32). Briefly, culture medium was removed, the cells were washed three times and extraction buffer (4 M guanidinium chloride with proteinase inhibitors) was added. After 1 h, the samples were centrifuged and supernatants were stored at −70°C until use. After dilution of the samples, HSPG and fibronectin contents were determined by ELISA as described (32).

Determination of TGF-β bioactivity produced by mesangial cells

Determination of autocrine TGF-β bioactivity. Autocrine TGF-β bioactivity produced by mesangial cells was determined by its ability to reduce mesangial proliferation in the same culture well. In this closed system, the endogenously produced and autocrine active TGF-β is assessed by its antiproliferative activity determined by concomitant [^{3}H]thymidine incorporation into the TGF-β–producing cells as described before. This sensitive functional assay for TGF-β (possibly reflecting the pathophysiological situation more closely than other tests) has been used successfully in earlier experiments (30).

Determination of TGF-β activity by its ability to stimulate matrix production and to inhibit growth in fresh mesangial cells. For determination of TGF-β bioactivity produced by mesangial cells, glucose- or glucosamine-conditioned media were prepared by collecting the respective spent media. Media were centrifuged for 5 min at 4°C and the supernatants were dialyzed against normal medium and used for treatment of fresh mesangial cells (bioactive TGF-β). To determine whether the cells had been producing latent TGF-β, latent TGF-β was converted to the active form by treating culture supernatant with 1 N HCl (pH < 2.0) for 30 min at room temperature, followed by neutralization with 1 N NaOH (33). These media were dialyzed for 24 h against standard culture media containing normal glucose concentration and added to fresh mesangial cell cultures. After 24 or 48 h, cells were counted and matrix components were extracted and determined as described above.

TGF-β bioassay (mink lung cell proliferation assay). Mink lung epithelial cells (Mv1Lu) were maintained in RPMI (Seromed) with 10% FCS and used for TGF-β growth inhibition assay essentially as described (34). For each assay, a standard curve was obtained with 0.01–1 ng/ml of human recombinant TGF-β1. To neutralize TGF-β activity, a rabbit anti–TGF-β antibody (R&D Systems) was added at a concentration of 10 μg/ml.

Immunological determination of total TGF-β protein

Total TGF-β protein content in supernatants was determined after acid activation by a sandwich ELISA as described previously by us (30).

RNase protection assay and preparation of riboprobes

cDNA fragments for porcine TGF-β1 (528b) and porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 211b) were obtained from mesangial cell RNA by reverse transcriptase-PCR amplification. The cDNA fragments were then cloned into the *SmaI* site of the transcription vector pGEM 3Z (Promega, Madison, WI). After linearization, the cDNAs were transcribed by SP6 or T7 RNA polymerase in the presence of [α-^{32}P]UTP to produce the labeled antisense RNA probes of defined length by use of the protocol and reagents of the Maxiscript kit (Ambion, Heidelberg, Germany). For determination of TGF-β1 mRNA by RNase protection assay, mesangial cells were grown in 154-cm^2 dishes. Cells were washed, and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (30). The RNase protection assay was performed with the RPA II kit from Ambion according to the manufacturer's protocol. Briefly, for each protection assay, 10 μg total RNA was hybridized at 45°C overnight with the riboprobes for TGF-β1 and GAPDH and then digested for 40 min at 37°C with a 1:200 dilution of the RNase A/T$_1$ mixture. Protected RNA fragments were separated on denaturing 5% polyacrylamide gels and detected by autoradiography.

Oligonucleotide synthesis and treatment of cells

S-Oligonucleotides were synthesized, purified, and characterized in our laboratory (30). We used the same TGF-β1 oligonucleotides as described previously (30). The *S*-antisense glutamine:fructose-6-phosphate amidotransferase (GFAT) AS2 oligonucleotide had the following sequence (35): 5′-ACC-AAT-CTC-AGG-ACC-AGC-3′ (aa 466–471). Sense, reverse antisense, and/or scrambled oligonucleotides were used as additional controls. *S*-Oligonucleotides were typically added to cells for 48 h. The effect of the GFAT oligonucleotides on GFAT protein synthesis was assessed by determination of GFAT in mesangial extracts by ELISA. For this assay, polyclonal peptide antibodies against the COOH-terminal part of GFAT (aa 666–681) were produced in rabbits. The obtained antiserum was characterized by ELISA, Western blotting, and immunohistochemistry, and specificity

was assessed by competition with the peptide (36). Furthermore, when the full-length cDNA of GFAT was overexpressed in 293 fibroblasts, a > 20-fold enhanced immunoreactivity was found with our antibody indicating specificity.

Determination of the hexosamine metabolites

Glucosamine-6-phosphate (GlcN-6-P) was determined in mesangial cell lysates after derivatization with *o*-phthaldialdehyde as described by Daniels et al. (37) except that the gradient for HPLC elution contained 1% acetonitrile/isopropanol at the starting point. The fluorophor-labeled GlcN-6-P was detected and quantified by the fluorescence detector F1000 (Merck, Darmstadt, Germany). For determination of radiolabeled GlcN-6-P, cells were lysed with 0.5% Triton X-100 containing 60% acetonitrile. After lyophilization and redissolving in 0.1 ml 60% acetonitrile in H_2O, the solution was centrifuged and samples were applied on a Partisil 10 SAX column (Grom, Herrenberg, Germany) and GlcN-6-P was eluted with 30 mM potassium phosphate, pH 3.5. 1-ml fractions were collected and counted for radioactivity. For determination of UDP-*N*-acetylglucosamine (UDP-GlcNAc), cells were lysed as described above and UDP-hexosamines were separated by capillary zone electrophoresis on the CES I system (Dionex, Sunnyvalley, CA). Briefly, 30-nl samples were injected hydrodynamically into a 50 μm × 700 mm fused silica capillary. Electrophoresis was performed in 20 mM sodium borate buffer, pH 9.0, at 20 kV. UDP-hexosamines were detected and quantified at 262 nm. Commercially available UDP-hexosamines were used for calibration. The migration times for UDP-GlcNAc, UDP-GalNAc, UDP-Glc, and UDP-Gal were 12.3, 12.8, 13.1, and 13.6 min, respectively.

Statistical analysis

All values are means±SE. When multiple groups were compared, one-way ANOVA was performed initially to confirm the presence of significant differences. Individual comparisons were performed with Student's *t* test. Statistically significant differences between groups were defined as *P* values < 0.05 and are indicated in the legends to figures and tables. Each experiment was performed independently for a minimum of three times up to eight times.

Results

Effect of structural analogues of D-glucose on mesangial growth. We have shown previously that high D-glucose concentrations induce growth inhibition in mesangial cells (30). To examine the possible molecular mechanism of this effect, structural analogues of D-glucose were investigated for their ability to mimic the glucose effect. Of all hexose analogues tested, only D-glucosamine, but not its acetyl derivative, was able to mimic the glucose-induced growth inhibition on mesangial cells (Table I). Glucosamine (12 mM) inhibited mesangial growth in a more pronounced manner than high glucose levels (30 mM), indicating that glucosamine was more effective.

Table I. Effect of Structural Analogues of D-Glucose on Mesangial Growth

Component	Concentration	Percent of control value
	mM	
D-Glucose (control)	10	100%±1.7
D-Glucose	30	72%±1.4*
L-Glucose	30	96%±2.5
Sorbitol	30	95%±10
3-*O*-Methyl-D-glucose	30	102%±7.1
D-Glucosamine	12	64%±1.6*
N-Acetyl-glucosamine	12	100%±4.2

Mesangial cells were grown in the presence of structural analogues of D-glucose in concentrations as indicated. After 48 h, cells were counted as described (30). Each value represents the mean±SE; $n = 3$ dishes; $*P <$ 0.05 compared to control value.

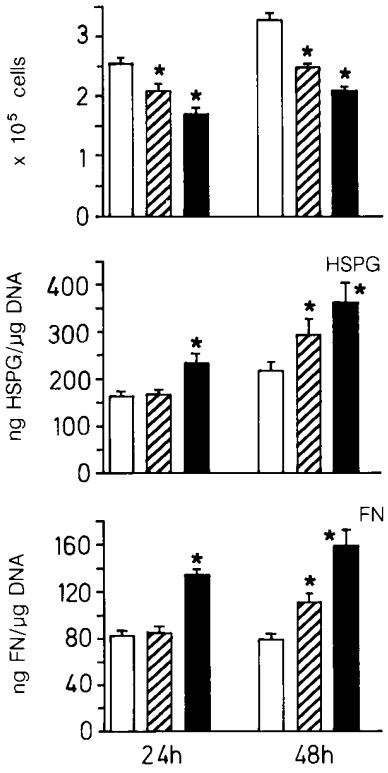

Figure 1. Effect of glucosamine on mesangial growth and mesangial HSPG and fibronectin accumulation. Cells were grown in standard culture medium containing 10 mM (*open bars*) and 30 mM D-glucose (*hatched bars*), and 12 mM glucosamine (*closed bars*), respectively. After 24 and 48 h, cell number, mesangial DNA content, and mesangial HSPG and fibronectin accumulation were determined as described in Methods. Values are means±SE; $*P < 0.05$, $n = 4$ dishes for HSPG or fibronectin determinations and $n = 3$ for DNA determinations and cell count.

Glucosamine inhibits mesangial cell proliferation and increases accumulation of matrix components. Incubation of mesangial cells with 12 mM glucosamine revealed a time-dependent increase in the production of extracellular matrix proteins while mesangial growth was inhibited (Fig. 1). In comparison, treatment with 30 mM glucose was less effective. Tracer stud-

Table II. Time-dependent [^{3}H]Thymidine Incorporation into the DNA of Cells Incubated in the Presence of D-Glucosamine or Elevated Glucose Concentrations

	[^{3}H]Thymidine incorporation (% of control)	
Time	Glucosamine (12 mM)	Glucose (30 mM)
h		
5	82±2.0*	88±3.0*
16	69±5.2*	83±4.1*
24	46±3.0*	81±2.9*
48	41±1.6*	75±6.1*

Porcine mesangial cells from the ninth passage were plated in standard culture medium. After 3 h, cells were incubated with medium containing 10 mM glucose with or without 12 mM D-glucosamine or with 30 mM glucose. [^{3}H]Thymidine was added and cells were harvested after the time periods indicated. Incorporation of [^{3}H]thymidine was determined as described. Values are expressed as percentages of control (10 mM D-glucose). Data represent means±SE; $*P < 0.05$ compared to control value, $n = 4$ dishes.

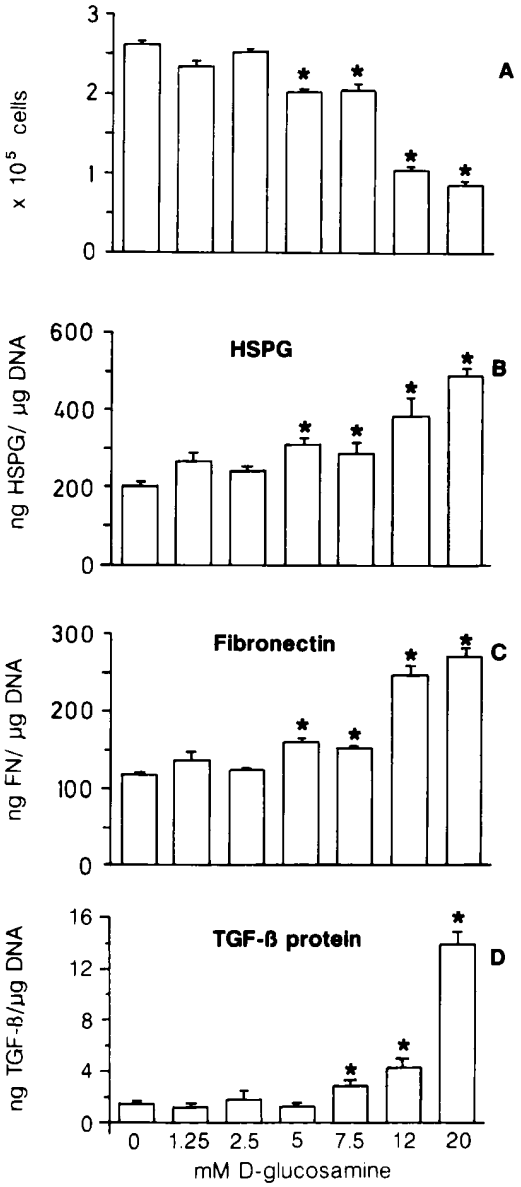

Figure 2. Dose-dependent effects of glucosamine on mesangial growth, mesangial HSPG, and fibronectin production and on TGF-β protein production. Growing mesangial cells were treated without or with increasing D-glucosamine levels. After 48 h, the cells were harvested and the cell count (*A*) and HSPG (*B*) and fibronectin (*C*) production of the cells were measured. Total TGF-β protein (*D*) was determined in the supernatant media (24–48 h) by sandwich ELISA after acidification. Values are means±SE; $*P < 0.05$, $n = 4$ dishes.

ies with [^{3}H]thymidine confirmed the more potent inhibitory effect of glucosamine on mesangial cell growth (Table II). The effects of increasing glucosamine concentrations on mesangial growth and production of matrix proteins are shown in Fig. 2. Exposure to glucosamine levels > 2.5 mM caused a dose-dependent increase in mesangial production of HSPG and fibronectin, whereas mesangial growth was concomitantly inhibited. The FCS content was not critical for the appearance of the glucosamine effects since similar effects were obtained with media containing 2% FCS. Treatment of the mesangial cells with 30 mM glucose or 12 mM glucosamine resulted in a significant increase in lactate production (24–48-h period) of 142±2.7% and 196±8.3% indicating stimulation of the glycolytic activity.

Effect of glucosamine-conditioned media on mesangial growth and matrix accumulation. To examine if the presence of glucosamine is necessary for the observed effects, conditioned media were prepared and tested for their ability to affect mesangial HSPG and fibronectin production as well as mesangial growth. Incubation of fresh mesangial cells with glucosamine-conditioned medium increased mesangial HSPG and fibronectin production 1.4- and 2.3-fold, respectively (i.e., from 633±79 to 878±53 ng HSPG/µg DNA and from 170±6 to 390±53 ng fibronectin/µg DNA; $n = 4$, $P < 0.05$). Cell number was concomitantly reduced by 39% (i.e., from 5.3±0.1 to 3.2±0.1 × 10^5; $n = 4$, $P < 0.05$). Acid activation of the glucosamine-conditioned medium increased the effect on mesangial cell growth and stimulation of matrix production only marginally. These results suggest that glucosamine induces the production of a nondialyzable factor in mesangial cells which stimulates the production of extracellular matrix and concomitantly inhibits mesangial growth. Since these properties are typical for bioactive TGF-β, we examined whether glucosamine, similar to high glucose, induces the expression, secretion, and bioactivation of this cytokine in mesangial cells.

Glucosamine induces the formation of TGF-β in mesangial cells. To evaluate whether the suggested production and secretion of TGF-β protein mediates the effects of glucosamine on mesangial proliferation, i.e., if glucosamine induces the formation of autocrine bioactive TGF-β, glucosamine-treated cells were coincubated with neutralizing TGF-β antibody. This treatment abrogated the glucosamine-induced inhibition of mesangial cell proliferation at 7 mM glucosamine and significantly attenuated the effect at 12 mM glucosamine while control IgG had no effect (Fig. 3).

To further verify that glucosamine induces TGF-β expression, levels of bioactive TGF-β, TGF-β protein, and TGF-β mRNA were monitored. Addition of glucosamine caused a time- and dose-dependent increase in mean active TGF-β concentration (Fig. 4). These glucosamine-induced levels are well within the range needed to increase mesangial matrix synthesis (38). To establish that the formation of TGF-β1 bioactivity

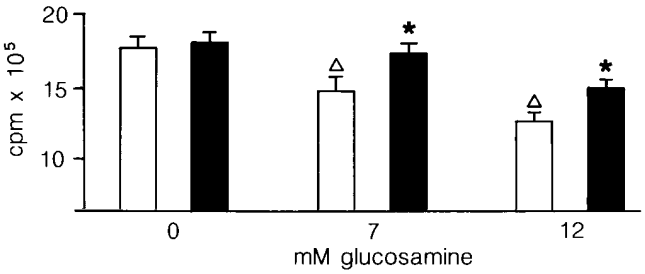

Figure 3. Effect of coincubation with TGF-β antibody on mesangial proliferation in glucosamine-treated mesangial cell cultures. Mesangial cells were incubated with 0, 7, or 12 mM glucosamine in the presence of control IgG (*open bars*) or in the presence of 0.1 mg/ml neutralizing TGF-β antibody (*closed bars*) for 48 h. Antiproliferative autocrine TGF-β activity on mesangial cell proliferation was determined by mesangial [^{3}H]thymidine incorporation. Values are means±SE; $*P < 0.05$ vs. without TGF-β antibody, $\Delta P < 0.05$ vs. without glucosamine; $n = 4$ dishes.

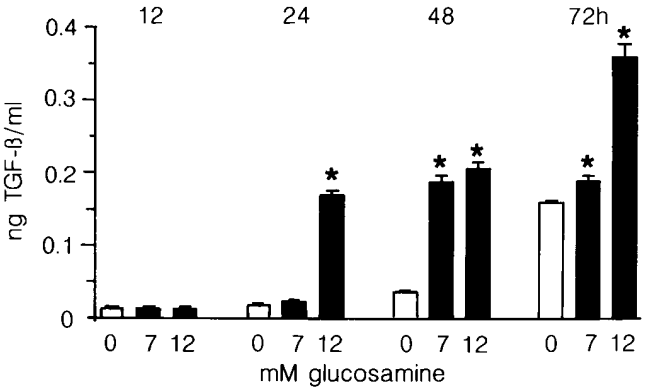

Figure 4. Effect of glucosamine on TGF-β bioactivity secreted by mesangial cells as measured by the standard mink lung cell proliferation assay. Mesangial cells were incubated with 0, 7, or 12 mM glucosamine and the supernatant media were tested for their bioactive TGF-β content by the bioassay as described in Methods. Since media were exchanged every 24 h, values represent formation of bioactive TGF-β in the respective time periods. Values are means±SE; $*P < 0.05$, $n = 5$ dishes.

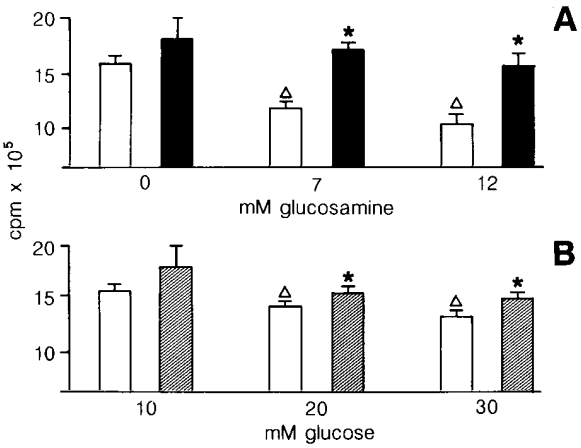

Figure 6. Effect of TGF-β antisense oligonucleotide on glucosamine-depressed [^{3}H]thymidine incorporation. Mesangial cells were incubated with 10 mM glucose containing 0, 7, or 12 mM glucosamine (*A*) or with 10, 20, or 30 mM glucose (*B*). [^{3}H]Thymidine incorporation was determined as described in Methods. Media also contained either 3.3 μM oligonucleotide reverse (*open bars*) or antisense (*closed* or *hatched bars*) to TGF-β1. Values are means±SE; $*P < 0.05$ vs. with reverse oligonucleotide; $\Delta P < 0.05$ compared with control values (10 mM glucose), $n = 4$ dishes.

was accompanied by the synthesis and secretion of TGF-β, protein media saved from the experiment shown in Fig. 2 were investigated for their total TGF-β protein content by sandwich ELISA. Fig. 2 *D* demonstrates that raising the glucosamine concentration from 7.5 to 20 mM glucosamine induced an exponential increase in TGF-β protein production. No significant alteration of TGF-β protein production was found at lower glucosamine concentrations. Similar results were obtained when the effect of glucosamine on TGF-β mRNA expression was examined by RNase protection assay (Fig. 5) or in situ hybridization (not shown).

To evaluate if induction of TGF-β1 transcription is necessary for the glucosamine-induced effects, antisense TGF-β1 oligonucleotides were added to mesangial cells. The results are shown in Fig. 6. As also observed earlier (30), using the same oligonucleotides, the high glucose-induced production of autocrine TGF-β was prevented by coincubation with antisense TGF-β1 oligonucleotide (Fig. 6 *B*). Although glucosamine induced a more pronounced antiproliferative activity, e.g., more autocrine active TGF-β in mesangial cells, presence of the antisense TGF-β1 oligonucleotide in the medium of glucosamine-treated cells resulted in complete protection against the glucosamine-induced effect at 7 and 12 mM glucosamine (Fig. 6 *A*). Addition of reverse oligonucleotide did not have such a protective effect.

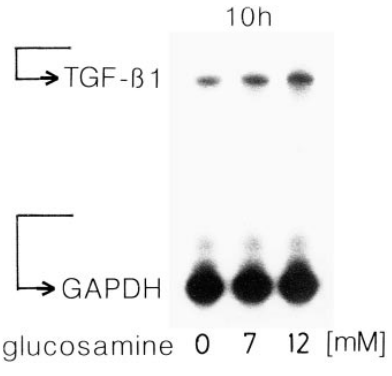

Figure 5. Effect of glucosamine on TGF-β1 mRNA levels determined by RNase protection assay. Mesangial cells were exposed to increasing glucosamine concentrations. After 10 h, cells were harvested and RNase protection assay was performed as described in Methods. Labeled cRNA probes for both TGF-β1 and GAPDH were added to each sample. Arrows on the left indicate the shift of full-length cRNA probes for TGF-β1 (592b) and GAPDH (241b) to protected cRNA of TGF-β1 (528b) and GAPDH (211b) after RNase A/T_1 digestion. No undigested probe is visible in the lanes of the samples. One of three typical experiments is shown.

Effect of glucosamine, high glucose, and GFAT inhibitors on mesangial content of hexosamine metabolites. Our results demonstrating that exposure of mesangial cells to glucosamine potently mimics the high glucose effects suggest that these were mediated by the hexosamine biosynthesis pathway. In this pathway, fructose-6-phosphate is first converted to GlcN-6-P by GFAT (E.C. 26.1.16), the first and rate-limiting enzyme of the hexosamine biosynthesis pathway (39, 40). These studies showed that almost all glucosamine taken up by adipocytes is phosphorylated and therefore enters the hexosamine pathway distal to GFAT, thus explaining the increased potency of glucosamine compared with glucose. Before concluding that the hexosamine pathway is involved in glucosamine- or high glucose-induced mesangial TGF-β1 production, it has to be shown that glucosamine or high glucose leads to increased levels of hexosamine metabolites in mesangial cells and that the high glucose-induced increase is prevented if GFAT is inhibited. First, we studied, by using radiolabeled glucose, if increasing concentrations of glucose result in increasing amounts of GlcN-6-P, the product of the GFAT-catalyzed conversion (Fig. 7, *top*). In addition, mesangial content of unlabeled GlcN-6-P was determined by HPLC. As shown in Fig. 7, elevated glucose levels lead to elevated GlcN-6-P levels after 24 h and this elevation is abolished by coincubation with the GFAT inhibitors *O*-diazoacetyl-L-serine (azaserine) or antisense GFAT oligonucleotide. The rationale for using azaserine is that this glutamine analogue inhibits GFAT on the substrate level, while

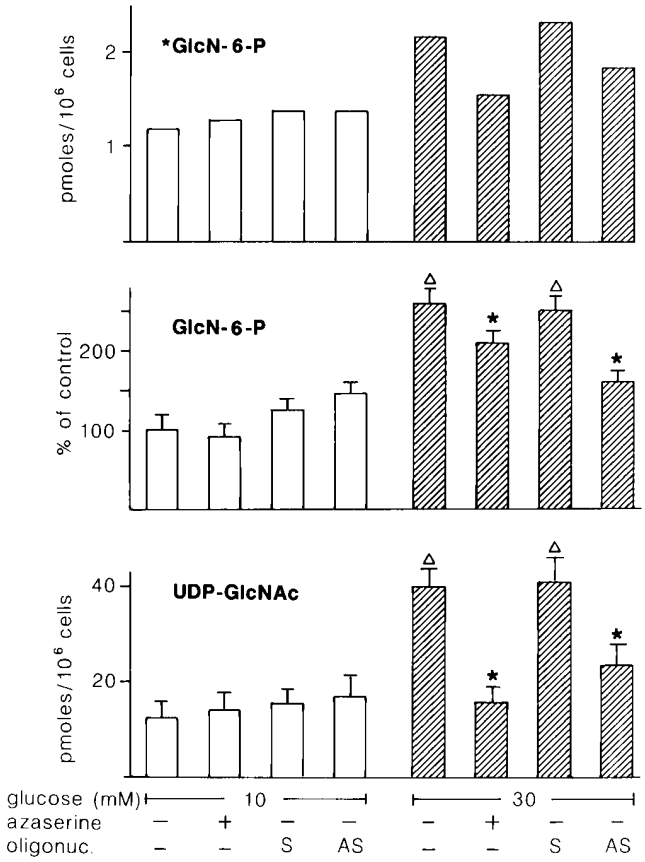

Figure 7. Effect of elevated glucose concentrations and GFAT inhibitors on mesangial hexosamine metabolite content. Mesangial cells were incubated with or without the GFAT inhibitors azaserine and antisense GFAT oligonucleotide (*AS*) with 10 (*open bars*) or 30 mM (*hatched bars*) glucose for 24 h and the cellular content of the hexosamine metabolites GlcN-6-P and UDP-GlcNAc was determined as described in Methods. The conversion of glucose to GlcN-6-P was studied by incubation with radiolabeled glucose. The amount of radiolabeled *GlcN-6-P formed was determined by counting the radioactivity incorporated into GlcN-6-P after HPLC separation and normalized to cell number. In addition, the content of GlcN-6-P was determined after derivatization and HPLC separation (*middle*) and UDP-GlcNAc was measured by capillary zone electrophoresis (*bottom*). Values are means±SE; $\Delta P < 0.05$; 30 vs. 10 mM glucose, $^*P < 0.05$ without vs. with GFAT inhibitors, $n = 4$ dishes; except values of the radioactive experiments, which are means of two determinations.

the oligonucleotide attenuates GFAT protein synthesis (see also Fig. 10). The increase in incorporated radioactivity in GlcN-6-P indicates that more GlcN-6-P is formed from glucose if high glucose levels are present extracellularly. Furthermore, we analyzed the mesangial content of UDP-GlcNAc, the most abundant hexosamine metabolite that accumulates in the cell. We found a significant increase in UDP-GlcNAc content after incubation with 30 mM glucose for 24 h, which was prevented by coincubation with the GFAT inhibitors (Fig. 7, *bottom*). Experiments were also performed for 4 h, but in no case was an increase in GlcN-6-P or UDP-GlcNAc found. Upon incubation with 7 mM glucosamine, we found 2- and 4.3-fold increases for 4 and 10 h, respectively. These data clearly show that glucosamine causes increases in hexosamine metabolites much more rapidly and to a higher extent when compared with high glucose levels and that a concentration of 7 mM glucosamine is sufficient to substantially increase intracellular glucosamine metabolite levels. Experiments performed with radiolabeled glucosamine according to the method described by Marshall et al. (39) showed that glucosamine enters the mesangial cell at a rate of 0.21 when compared with glucose in the absence of insulin, a value similar to that found for glucosamine uptake in adipocytes (39).

Ability of azaserine to attenuate the high glucose-induced TGF-β expression. We then investigated if the high glucose-induced stimulation of TGF-β protein and bioactivity is prevented by inhibition of GFAT. When mesangial cells were grown in the presence of high glucose concentrations, a significant decrease in the incorporation of [^{3}H]thymidine into newly synthesized DNA occurred indicating time-dependent production of autocrine TGF-β bioactivity (Fig. 8). This production of autocrine TGF-β was attenuated by coincubation with azaserine. It is noteworthy that the inhibitor azaserine increased cellular proliferation.

In a similar experiment, high glucose-treated cells were coincubated with azaserine and conditioned acidified media were prepared and tested for their ability to affect mesangial HSPG and fibronectin production as well as cellular growth. As reported previously (30), high glucose-conditioned media had a stimulatory effect on HSPG and fibronectin production and an inhibitory effect on mesangial growth. Both effects were prevented in the presence of azaserine indicating that azaserine prevented the production of bioactive TGF-β in mesangial cells (data not shown). Azaserine also prevented the increase in TGF-β protein induced by 30 mM glucose. For example from 0–24, 24–48, or 48–72 h, mesangial cells generated 1.06±0.02, 1.22±0.01, and 0.45±0.06 ng/ml TGF-β. In the presence of azaserine, TGF-β levels were reduced to 0.12±0.02, 0.27±0.03, and 0.18±0.02, respectively. Similarly, azaserine inhibited the high glucose-induced increase (1.9-

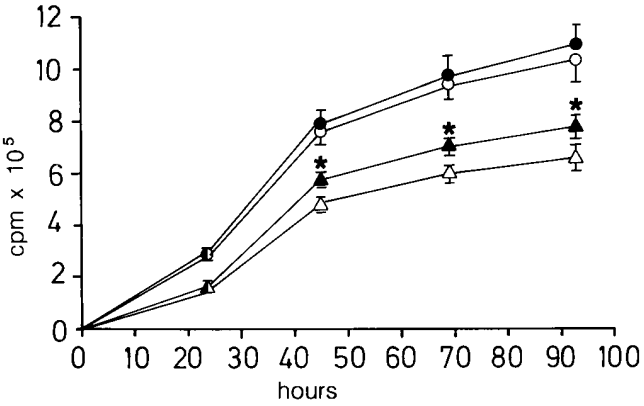

Figure 8. Effect of azaserine on high glucose-induced formation of autocrine TGF-β bioactivity. Mesangial cells were plated in standard culture medium. After 3 h, cells were incubated with medium containing 10 (*circles*) or 30 mM D-glucose (*triangles*) with (*closed*) or without (*open*) 5 μM azaserine. [^{3}H]Thymidine was added and incorporation of [^{3}H]thymidine was determined at the time points indicated. Data are expressed as cumulative [^{3}H]thymidine incorporation values. Data represent means±SE; $^*P < 0.05$ compared with control value, $n = 4$ dishes.

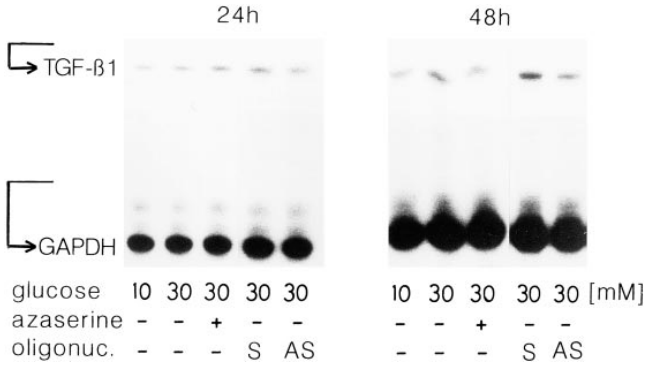

Figure 9. Effect of high glucose concentrations and GFAT inhibitors on TGF-β1 mRNA levels determined by RNase protection assay. Mesangial cells were exposed to increasing glucose concentrations with or without GFAT inhibitors. After the time points indicated, cells were harvested and mRNA levels were determined as described in the legend to Fig. 5. The effect of elevated glucose concentrations with or without azaserine (5 μM) and antisense GFAT (*AS*) oligonucleotide (3.3 μM) on TGF-β1 mRNA levels is shown in the left (24 h) and the right (48 h) panel of the figure. Sense GFAT oligonucleotide (*S*) was used as control. One of three typical experiments is shown.

fold) in TGF-β mRNA levels after 48 h (Fig. 9). We found no significant effect of elevated glucose concentrations with or without azaserine on TGF-β mRNA levels after 24 h.

The effect of azaserine appeared to be specific and nontoxic since presence of the glutamine analogue azaserine had no effect on TGF-β mRNA levels or mesangial proliferation of the control cells. Furthermore, azaserine did not influence the metabolic activity of the cells as assessed by determination of the glycolytic activity (lactate production) of the cells (data not shown). Taken together, the results suggest that azaserine inhibits the high glucose-induced production of TGF-β mRNA, TGF-β protein, and bioactivation of TGF-β.

Antisense GFAT oligonucleotide inhibits high glucose-induced TGF-β expression. Since the glutamine analogue azaserine may also affect other glutamine-dependent metabolic pathways, we applied antisense GFAT oligonucleotides to reduce the flux through the hexosamine pathway specifically. Preliminary experiments revealed that only the antisense oligonucleotide GFAT AS2 consistently inhibited the high glucose-induced effect on mesangial cells. A time course and dose-dependent study demonstrated that optimal inhibitory effects were obtained with 3.3 μM oligonucleotide without any lipid additive (data not shown). The efficiency of antisense GFAT oligonucleotide on GFAT synthesis was assessed by determination of GFAT by ELISA. Presence of antisense GFAT AS2 reduced GFAT synthesis by 2, 14, and 31% for 10, 20, and 30 mM glucose, respectively, while reverse GFAT AS2 had no effect (Fig. 10 *A*). Fig. 10 *B* shows that presence of the antisense GFAT oligonucleotide prevented the high glucose-induced autocrine TGF-β bioactivity, whereas reverse GFAT AS2 oligonucleotide had no effect. Similarly, sense GFAT or scrambled oligonucleotides had no effect (not shown). It is noteworthy, in some experiments, that the inhibitory effect of antisense GFAT oligonucleotide was significant at 10 mM glucose, indicating that the flux through the hexosamine pathway may be also enhanced at this glucose concentration. Similar results were obtained when the effect of antisense GFAT AS2 on high glucose-induced TGF-β1 mRNA expression was examined by RNase protection assay (Fig. 10) and by in situ hybridization (not shown).

For control, we also studied if azaserine and antisense GFAT oligonucleotide, the two GFAT inhibitors used in this study, affect the glucosamine-induced effects. Clearly, coincubation with azaserine or antisense GFAT AS2 oligonucleotide did not prevent the formation of autocrine TGF-β1 bioactivity induced by glucosamine (Fig. 11). These results support the idea that glucosamine acts at a site distal to the amination of fructose 6-phosphate by GFAT.

Discussion

In earlier investigations, it was demonstrated that prolonged exposure of growing mesangial cells to high glucose concentrations inhibits cellular proliferation and increases de novo synthesis of matrix proteins. Treatment of the cells with anti–TGF-β antibodies (21, 29) and antisense TGF-β1 oligonucleotides (30) partly or completely reversed the effects of high glucose suggesting that TGF-β1 may mediate, in an auto/paracrine fashion, some, if not all, of the biological effects of elevated ambient glucose on mesangial cells. Our current study pro-

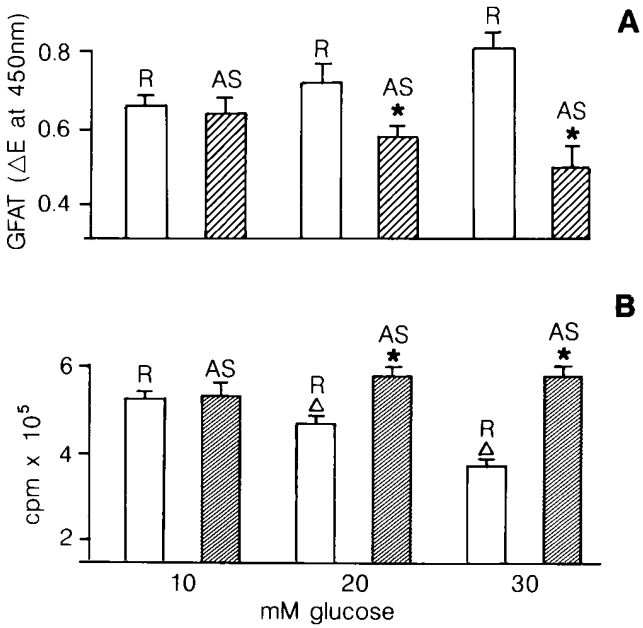

Figure 10. Antisense GFAT AS2 oligonucleotide reduces GFAT protein synthesis and concomitantly abrogates high glucose-induced autocrine TGF-β bioactivity in mesangial cells. Mesangial cells were incubated in standard culture medium for 3 h after seeding. Mesangial cells were then incubated with media containing normal or elevated glucose concentrations with 3.3 μM reverse (*R*, *open bars*) or with antisense GFAT AS2 oligonucleotide (*AS*, *hatched bars*) for 48 h. (*A*) GFAT protein content. For determination of GFAT protein content, cells were lysed and GFAT protein was determined by ELISA. Data are means±SE; $^*P < 0.05$ compared with reverse oligonucleotide, $n = 4$ dishes. (*B*) Autocrine TGF-β bioactivity. Autocrine TGF-β bioactivity was determined by [^{3}H]thymidine as described in Methods. Data are means±SE; $^*P < 0.05$ compared with reverse oligonucleotide; $\Delta P < 0.05$ compared with control value, $n = 4$ dishes.

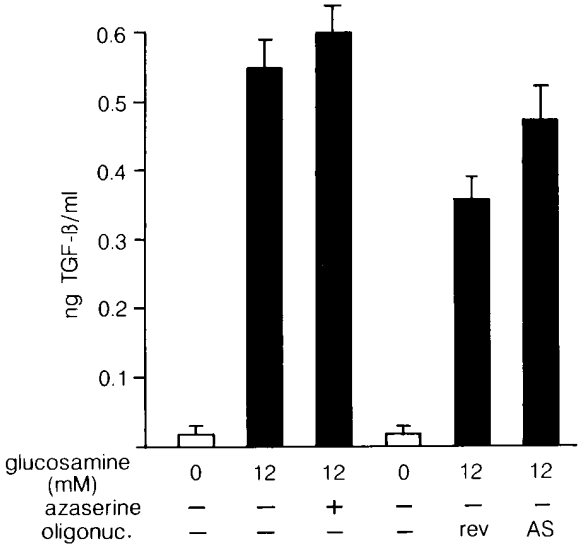

Figure 11. Effect of azaserine and antisense GFAT oligonucleotide on glucosamine-induced formation of bioactive TGF-β. Mesangial cells were incubated with (*closed bars*) or without (*open bars*) 12 mM glucosamine. As indicated, some incubation media contained 5 μM azaserine or 3.3 μM antisense GFAT AS2 oligonucleotide. For control, reverse GFAT AS2 oligonucleotide (*rev*) was used. Formation of bioactive TGF-β1 was determined by the mink lung epithelial cell bioassay as described in Methods. Data are means±SE; $n = 4$ dishes.

vides two lines of evidence indicating that the hexosamine biosynthetic pathway is involved in the glucose-induced expression and bioactivation of TGF-β1 in mesangial cells; glucosamine mimicked the effects of high glucose on cellular growth and matrix production time- and dose-dependently. Using two independent methods (coincubation with neutralizing TGF-β antibody and detection of TGF-β bioactivity) we demonstrated that glucosamine induced the production and secretion of bioactive TGF-β in mesangial cells. The suppression of glucosamine-induced autocrine TGF-β activity by antisense TGF-β oligonucleotide indicates that glucosamine exerts its effects on the transcriptional level. Accordingly, we found that glucosamine induced an increase in TGF-β1 mRNA levels and enhanced TGF-β1 protein production; inhibition of GFAT by azaserine or antisense GFAT oligonucleotide attenuated or abolished the high glucose-induced autocrine TGF-β bioactivity and subsequent effects on mesangial cells but not the glucosamine-induced effects. The results that azaserine or antisense GFAT oligonucleotide do not affect the control and that the two GFAT inhibitors used in this study increase mesangial proliferation cultured in high glucose medium (Figs. 8 and 10) exclude a possible direct inhibitory effect of the GFAT inhibitors (e.g., not via TGF-β inhibition).

As discussed below, glucosamine has been used in different cells and in whole animals to study the relevance of the hexosamine pathway for the development of insulin resistance. We used glucosamine to identify a metabolic pathway hitherto unknown to be involved in the hyperglycemia-induced stimulation of TGF-β1 in mesangial cells and therefore possibly involved in the pathogenesis of diabetic nephropathy. Since the level of glucosamine in blood is negligible (41), the use of glucosamine represents a scientific tool to mimic the high glucose-induced metabolic disturbances operating via the hexosamine pathway. Indeed, we found that glucosamine induces all effects observed with high glucose, but the effects are even earlier and more extensive. Glucosamine enters the cell via the glucose transporter in adipocytes (40, 41), in smooth muscle cells (42), and in mesangial cells (this study). To prove if the hexosamine pathway is involved in TGF-β1 overproduction, it is mandatory to demonstrate that hyperglycemic conditions lead to an increased flux through the hexosamine pathway via GFAT and that specific inhibition of this pathway reverses the increased hexosamine metabolite concentrations and subsequent biological effects. By using three different methods we could demonstrate that increased glucose levels cause an elevation in hexosamine metabolite concentration at 24 h thus preceding the subsequent increase in TGF-β1 expression observed after 48 h and that both increases could be prevented by inhibition of GFAT. Several observations indicate that also in vivo an increased glycolytic flux results in stimulation of the hexosamine pathway. A two- to threefold increase in UDP-*N*-acetyl hexosamines was found in muscles of glucose transporter 1–overexpressing animals (43) and a 40% increase in muscles of hyperglycemic diabetic animals was observed (44).

The involvement of the hexosamine pathway in the physiological regulation of the insulin-responsive glucose transport system has been discovered recently by Marshall et al. (39, 40). The results initiated a series of experimental studies presenting evidence that excess hexosamine flux causes insulin resistance in cultured cells, tissues, and intact animals (e.g., mice transgenic for GFAT in fat and skeletal muscle) as excellently reviewed by McClain and Crook (41). These authors have shown that the hexosamine pathway is involved in the glucose-induced TGF-α expression in vascular smooth muscle cells (42). Overexpression of GFAT in these cells revealed that the cells exhibited a glucose-dependent twofold increase in TGF-α activity (45). Of note, we found that glucosamine is a similar potent stimulator of TGF-β1 transcription in porcine aortic smooth muscle cells (our unpublished results). Together, the data indicate that the involvement of the hexosamine pathway in the mediation of the high glucose effects may be more general, possibly effective in adipocytes, skeletal muscle cells, mesangial cells, smooth muscle cells (42), and tubular cells (46). If so, these data and the present results suggest the possibility of a common molecular pathobiochemical pathway for the genesis of insulin resistance and vascular complications (41).

Our findings, together with earlier studies, strengthen the important role of hyperglycemia in the induction of the prosclerotic cytokine TGF-β in renal cells. These studies revealed that high glucose concentrations in the culture medium, independent of an osmotic effect, stimulated protein synthesis and mRNA levels of matrix proteins (15, 16, 21, 30). The causal role of increased glucose metabolism on matrix formation was shown unequivocally by using glucose transporter 1–transfected mesangial cells (47). These cells showed an increased flux in glucose metabolism and exhibited increased matrix production, again indicating that glucose must be metabolized to exert its effects.

TGF-β1, known to stimulate the synthesis of typical matrix proteins, also stimulates the synthesis of proteoglycans, e.g., the chondroitin sulfate containing decorin (38), biglycan (38), and HSPG (30). Although rat mesangial cells generate predominantly chondroitin/dermatan sulfate proteoglycans and

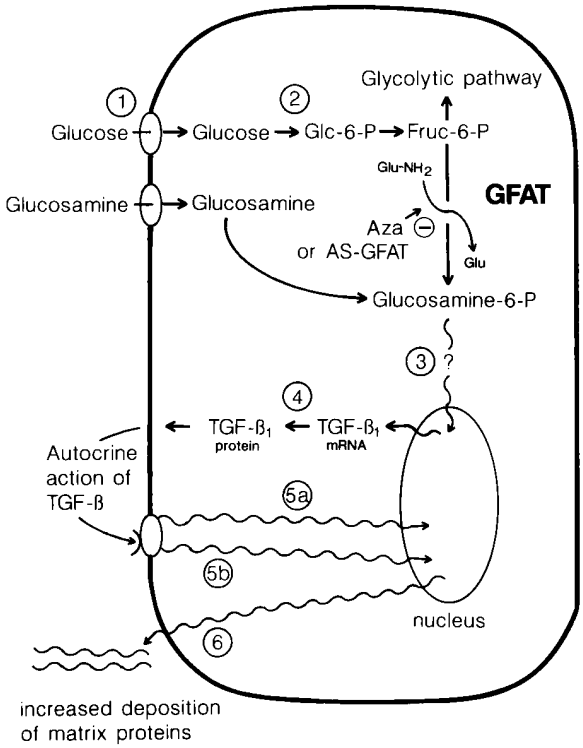

Figure 12. Pathobiochemical link between high glucose concentrations and increased production of extracellular matrix in mesangial cells. Elevated ambient glucose levels may induce TGF-β_1–mediated mesangial matrix production by the following sequence: (*1*) carrier-mediated uptake of D-glucose; (*2*) glucose (and glucosamine) are phosphorylated by hexokinase and elevated glycolytic glucose metabolites lead to increased synthesis of GlcN-6-P catalyzed by GFAT, which may be inhibited by azaserine (*Aza*) or antisense GFAT oligonucleotide (*AS-GFAT*); (*3*) unidentified glucosamine metabolite(s) induce(s) TGF-β1 expression and (*4*) secretion of bioactive TGF-β1; (*5a*) TGF-β1 inhibits cell proliferation; (*5b*) TGF-β1 induces production of HSPG and fibronectin and other matrix components; (*6*) expression, secretion, and extracellular deposition of matrix components. The conversion of glucosamine 6-phosphate to glycoproteins/lipids has been omitted for clarity.

less HSPG (48) we determined HSPG in addition to fibronectin since it had been shown that the HSPG content of the glomerular basement membrane, predominantly produced by endothelial and epithelial cells, and the HSPG content of the mesangial matrix are decreased in diabetic patients, whereas other matrix proteins are increased (3). However, we have found that TGF-β1 stimulates HSPG synthesis in cultured mesangial cells similar to other matrix proteins, e.g., fibronectin (30), indicating that this cytokine is not responsible for the proceeding reduction of HSPG in diabetic patients developing nephropathy. Therefore, the mechanism of the diabetes-induced glomerular HSPG reduction remains to be clarified.

Although the precise role of TGF-β in human renal disease remains to be established, studies with experimental animals, particularly studies in rats with transiently overexpressed TGF-β1 in the glomeruli, indicate that TGF-β is a critical determinant of matrix deposition (49). Recent studies showed that glomerular TGF-β mRNA and TGF-β protein are increased in diabetic rats and in diabetic patients with nephropathy (12, 50). The causal role of TGF-β may be estimated also from two recent reports showing that application of neutralizing anti–TGF-β antibody attenuates kidney hypertrophy and enhanced extracellular matrix gene expression in diabetic rats (51) and that treatment of diabetic rats with modified heparin, which prevents development of diabetic nephropathy, also prevents the induction of mesangial overexpression of α_1 (IV) collagen (52) and TGF-β1 (53).

In conclusion, we provide evidence that the high glucose-induced stimulation of TGF-β1 expression in cultured mesangial cells is mediated by the hexosamine biosynthetic pathway. The concept is schematically depicted in Fig. 12. We have further shown that specific inhibition of this pathway, e.g., by antisense GFAT oligonucleotide, prevents high glucose-induced TGF-β1 overexpression and subsequent matrix production. The data also suggest a new therapeutic way to prevent diabetes-associated sclerosis.

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References

1. Steffes, M.W., R.W. Bilous, D.E. Sutherland, and S.M. Mauer. 1992. Cell and matrix components of the glomerular mesangium in type I diabetes. *Diabetes.* 41:679–684.

2. Osterby, R., M.A. Gall, A. Schmitz, F.S. Nielsen, G. Nyberg, and H.H. Parving. 1993. Glomerular structure and function in proteinuric type 2 (non-insulin-dependent) diabetic patients. *Diabetologia.* 36:1064–1070.

3. Nerlich, A., and E. Schleicher. 1991. Immunohistochemical localization of extracellular matrix components in human diabetic glomerular lesions. *Am. J. Pathol.* 139:889–899.

4. Kreisberg, J.I., and S.H. Ayo. 1993. The glomerular mesangium in diabetes mellitus. *Kidney Int.* 43:109–113.

5. Osterby, R. 1992. Glomerular structural changes in type 1 (insulin-dependent) diabetes mellitus: causes, consequences, and prevention. *Diabetologia.* 35: 803–812.

6. Kim, Y., R. Butkowski, B. Burke, M.M. Kleppel, J. Crosson, A. Katz, and A.F. Michael. 1991. Differential expression of basement membrane collagen in membranous nephropathy. *Am. J. Pathol.* 139:1381–1388.

7. Nerlich, A.G., E.D. Schleicher, I. Wiest, U. Specks, and R. Timpl. 1994. Immunohistochemical localization of collagen VI in diabetic glomeruli. *Kidney Int.* 45:1648–1656.

8. Mohan, P.S., W.G. Carter, and R.G. Spiro. 1990. Occurrence of type VI collagen in extracellular matrix of renal glomeruli and its increase in diabetes. *Diabetes.* 39:31–37.

9. Abrass, C.K., C.V. Peterson, and G.J. Raugi. 1988. Phenotypic expression of collagen types in mesangial matrix of diabetic and nondiabetic rats. *Diabetes.* 39:1695–1702.

10. Ziyadeh, F.N. 1993. The extracellular matrix in diabetic nephropathy. *Am. J. Kidney Dis.* 22:736–744.

11. Fukui, M., T. Nakamura, I. Ebihara, I. Shirato, Y. Tomino, and H. Koide. 1992. ECM gene expression and its modulation by insulin in diabetic rats. *Diabetes.* 41:1520–1527.

12. Yamamoto, T., T. Nakamura, N.A. Noble, E. Ruoslahti, and W.A. Border. 1993. Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc. Natl. Acad. Sci. USA.* 90:1814–1818.

13. Ledbetter, S., E.J. Copeland, D. Noonan, G. Vogeli, and J.R. Hassell. 1990. Altered steady-state mRNA levels of basement membrane proteins in diabetic mouse kidneys and thromboxane synthase inhibition. *Diabetes.* 39:196–203.

14. Ayo, S.H., R. Radnik, J.A. Garoni, D.A. Troyer, and J.I. Kreisberg. 1991. High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am. J. Physiol.* 261:F571–F577.

15. Ayo, S.H., R.A. Radnik, W.F. Glass, J.A. Garoni, E.R. Rampt, D.R. Appling, and J.I. Kreisberg. 1991. Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. *Am. J. Physiol.* 260:

F185–F191.

16. Danne, T., M.J. Spiro, and R.G. Spiro. 1993. Effect of high glucose on type IV collagen production by cultured glomerular epithelial, endothelial, and mesangial cells. *Diabetes.* 42:170–177.

17. Doi, T., H. Vlassara, M. Kirstein, Y. Yamada, G.E. Striker, and L.J. Striker. 1992. Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 89:2873–2877.

18. Crowley, S.T., M. Brownlee, D. Edelstein, J.A. Satriano, T. Mori, P.C. Singhal, and D.O. Schlondorff. 1991. Effects of nonenzymatic glycosylation of mesangial matrix on proliferation of mesangial cells. *Diabetes.* 40:540–547.

19. Studer, R.K., P.A. Craven, and F.R. DeRubertis. 1993. Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes.* 42:118–126.

20. Nahman, N.S., Jr., K.L. Leonhart, F.G. Cosio, and C.L. Hebert. 1992. Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. *Kidney Int.* 41:396–402.

21. Ziyadeh, F.N., K. Sharma, M. Ericksen, and G. Wolf. 1994. Stimulation of collagen gene expression and protein synthesis in murine mesangial cells by high glucose is mediated by autocrine activation of transforming growth factor-beta. *J. Clin. Invest.* 93:536–542.

22. Wakisaka, M., M.J. Spiro, and R.G. Spiro. 1994. Synthesis of type VI collagen by cultured glomerular cells and comparison of its regulation by glucose and other factors with that of type IV collagen. *Diabetes.* 43:95–103.

23. Pugliese, G., F. Pricci, F. Pugliese, P. Mene, L. Lenti, D. Andreani, G. Galli, A. Casini, S. Bianchi, C.M. Rotella, and U. di Mario. 1994. Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. *Diabetes.* 43:478–490.

24. Larkins, R.G., and M.E. Dunlop. 1992. The link between hyperglycaemia and diabetic nephropathy [published erratum appears in *Diabetologia.* 35: 1100]. *Diabetologia.* 35:499–504.

25. Schleicher, E.D. 1994. Biochemical aspects of diabetic nephropathy. *In* The Kidney and Hypertension in Diabetes Mellitus. C.E. Mogensen, editor. Kluwer Academic Publishers, Boston/Dordrecht/London. 191–202.

26. Schleicher, E.D., and A. Nerlich. 1996. The role of hyperglycemia in the development of diabetic complications. *Horm. Metab. Res.* 28:367–373.

27. Nakamura, T., M. Fukui, I. Ebihara, S. Osada, I. Nagaoka, Y. Tomino, and H. Koide. 1993. mRNA expression of growth factors in glomeruli from diabetic rats. *Diabetes.* 42:450–456.

28. Sharma, K., and F.N. Ziadeh. 1995. Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-β as a key mediator. *Diabetes.* 44:1139–1146.

29. Wolf, G., K. Sharma, Y. Chen, M. Ericksen, and F.N. Ziyadeh. 1992. High glucose-induced proliferation in mesangial cells is reversed by autocrine TGF-beta. *Kidney Int.* 42:647–656.

30. Kolm, V., U. Sauer, B. Olgemoller, and E.D. Schleicher. 1996. High glucose-induced TGF-β1 regulates mesangial production of heparan sulfate proteoglycan. *Am. J. Physiol.* 270:F812–F821.

31. Olgemoller, B., E.D. Schleicher, S. Schwaabe, H.J. Guretzki, and K.D. Gerbitz. 1990. High concentrations of low density lipoprotein decrease basement membrane-associated heparan sulfate proteoglycan in cultured endothelial cells. *FEBS Lett.* 264:37–39.

32. Olgemoller, B., E. Schleicher, A. Nerlich, E.M. Wagner, and K.D. Gerbitz. 1989. Isolation, characterization and immunological determination of basement membrane-associated heparan sulfate proteoglycan. *Biol. Chem. Hoppe Seyler.* 370:1321–1329.

33. Coimbra, T., R. Wiggins, J.W. Noh, S. Merritt, and S.H. Phan. 1991. Transforming growth factor-beta production in anti-glomerular basement membrane disease in the rabbit. *Am. J. Pathol.* 138:223–234.

34. Kagami, S., W.A. Border, D. Miller, and N.A. Noble. 1994. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor β expression in rat glomerular cells. *J. Clin. Invest.* 93: 2431–2437.

35. McKnight, G.L., S.L. Mudri, S.L. Mathewes, R.R. Traxinger, S. Marshall, P.O. Sheppard, and P.J. O'Hara. 1992. Molecular cloning, cDNA sequence, and bacterial expression of human glutamine:fructose-6-phosphate amidotransferase. *J. Biol. Chem.* 267:25208–25212.

36. Nerlich, A.G., V. Kolm, U. Sauer, E. Wagner, and E.D. Schleicher. 1998. Expression of glutamine:fructose-6-phosphate amidotransferase in human tissues: evidence for high variability and distinct regulation in diabetes. *Diabetes.* In press.

37. Daniels, M.C., T.P. Ciaraldi, S. Nikoulina, R.R. Henry, and D.A. McClain. 1996. Glutamine:fructose-6-phosphate amidotransferase activity in cultured human skeletal muscle cells. *J. Clin. Invest.* 97:1235–1241.

38. Border, W.A., S. Okuda, L.R. Languino, and E. Ruoslahti. 1990. Transforming growth factor-β regulates production of proteoglycans by mesangial cells. *Kidney Int.* 37:689–695.

39. Marshall, S., V. Bacote, and R.R. Traxinger. 1991. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J. Biol. Chem.* 266:4706–4712.

40. Marshall, S., W.T. Garvey, and R.R. Traxinger. 1991. New insights into the metabolic regulation of insulin action and insulin resistance: role of glucose and amino acids. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:3031–3036.

41. McClain, D.A., and E.D. Crook. 1996. Perspectives in diabetes. Hexosamines in insulin resistance. *Diabetes.* 45:1003–1009.

42. McClain, D.A., A.J. Paterson, M.D. Roos, X. Wei, and J.E. Kudlow. 1992. Glucose and glucosamine regulate growth factor gene expression in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 89:8150–8154.

43. Buse, M.G., K.A. Robinson, B.A. Marshall, and M. Mueckler. 1996. Differential effects of *GLUT1* or *GLUT4* overexpression on hexosamine biosynthesis by muscles of transgenic mice. *J. Biol. Chem.* 271:23197–23202.

44. Rosetti, L., M. Hawkins, W. Chen, J. Gindi, and N. Barzilai. 1995. In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. *J. Clin. Invest.* 96:132–140.

45. Daniels, M.C., P. Kansal, T.M. Smith, A.J. Paterson, J.E. Kudlow, and D.A. McClain. 1993. Glucose regulation of transforming growth factor α expression is mediated by products of the hexosamine biosynthesis pathway. *Mol. Endocrinol.* 7:1041–1048.

46. Rocco, M.V., Y. Chen, S. Goldfarb, and F.N. Ziyadeh. 1992. Elevated glucose stimulates TGF-beta gene expression and bioactivity in proximal tubule. *Kidney Int.* 41:107–114.

47. Heilig, C.W., L.A. Concepcion, B.L. Riser, S.O. Freytag, and P. Cortes. 1995. Overexpression of glucose transporters in rat mesangial cells cultured in a normal glucose milieu mimics the diabetic phenotype. *J. Clin. Invest.* 96:1802–1814.

48. Silbiger, S., D. Schlondorff, S. Crowley, L. Rosenberg, H. Choi, V. Hatcher, and P. Gordon. 1993. The effect of glucose on proteoglycans produced by cultured mesangial cells. *Diabetes.* 42:1815–1822.

49. Isaka, Y., Y. Fujiwara, N. Ueda, Y. Kenada, and E. Imai. 1993. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-β or platelet-derived growth factor gene into the rat kidney. *J. Clin. Invest.* 92:2597–2601.

50. Iwano, M., A. Kubo, T. Nishino, H. Sato, H. Nishioka, Y. Akai, H. Kurioka, Y. Fujii, M. Kanavachi, S. Shiki, and K. Dohi. 1996. Quantification of glomerular TGF-β1 mRNA in patients with diabetes mellitus. *Kidney Int.* 49: 1120–1126.

51. Sharma, K., J. Guo, and F.N. Ziyadeh. 1996. Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and enhanced extracellular matrix gene expression in streptozotocin-diabetic mice. *Diabetes.* 45: 522–530.

52. Ceol, M., A. Nerlich, B. Baggio, F. Anglani, U. Sauer, E.D. Schleicher, and G. Gambaro. 1996. Increased glomerular α1 (IV) expression and deposition in long-term diabetic rats is prevented by chronic glycosaminoglycan treatment. *Lab. Invest.* 74:484–495.

53. Schleicher, E.D., M. Ceol, U. Sauer, A. Nerlich, B. Baggio, F. Anglani, and G. Gambaro. 1996. TGF-β1 renal overexpression in long-term diabetic rats and in high glucose mesangial cell cultures: inhibition by heparin. *Diabetologia.* 39 (Suppl. 1):A38. (Abstr.)