Glucose Stimulation of Transforming Growth Factor- β Bioactivity in Mesangial Cells Is Mediated by Thrombospondin-1

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Glucose is a key factor in the development of diabetic complications, including diabetic nephropathy. The development of diabetic glomerulosclerosis is dependent on the fibrogenic growth factor, transforming growth factor- β (TGF- β). Previously we showed that thrombospondin-1 (TSP-1) activates latent TGF- β both *in vitro* and *in vivo*. Activation occurs as the result of specific interactions of latent TGF- β with TSP-1, which potentially alter the conformation of latent TGF- β . As glucose also up-regulates TSP-1 expression, we hypothesized that the increased TGF- β bioactivity observed in rat and human mesangial cells cultured with high glucose concentrations is the result of latent TGF- β activation by autocrine TSP-1. Glucose-induced bioactivity of TGF- β in mesangial cell cultures was reduced to basal levels by peptides from two different sequences that antagonize activation of latent TGF- β by TSP, but not by the plasmin inhibitor, aprotinin. Furthermore, glucose-dependent stimulation of matrix protein synthesis was inhibited by these antagonist peptides. These studies demonstrate that glucose stimulation of TGF- β activity and the resultant matrix protein synthesis are dependent on the action of autocrine TSP-1 to convert latent TGF- β to its biologically active form. These data suggest that antagonists of TSP-dependent TGF- β activation may be the basis of novel therapeutic approaches for ameliorating diabetic renal fibrosis. (*Am J Pathol* 2000, 157:1353–1363)

Diabetic nephropathy is the main cause of end-stage renal failure in patients with type 1 and 2 diabetes in the United States requiring chronic, costly dialysis treatments. The degree of hyperglycemia is a predictor of diabetic renal complications.¹ Diabetic nephropathy is characterized by thickening of the glomerular basement

membranes, accumulation of extracellular matrix (ECM) proteins, and hypertrophy of glomerular and tubular elements that lead to proteinuria, hypertension, and kidney failure.^{2–4}

Hyperglycemic conditions stimulate elevated expression of the fibrogenic cytokine, transforming growth factor- β (TGF- β).^{4,5} Either acute or chronic high glucose exposure stimulates TGF- β transcription which leads to an increased pool of bioactive TGF- β as well.^{6–10} However, the posttranslational mechanisms involved in regulating the activation step are not fully understood.

TGF- β s are a family of multifunctional regulators of cellular growth, gene expression, and differentiation. TGF- β is secreted by most cells as an inactive precursor consisting of an active peptide noncovalently associated with a precursor portion, termed the latency-associated peptide (LAP).^{11,12} This inactive form must be converted to an active form that can bind to its receptors and elicit cell responses.

Multiple lines of evidence suggest that TGF- β is a major mediator of ECM deposition in the diabetic kidney.¹³ Sharma et al^{4,14} have demonstrated that the actions of high glucose on renal cells are similar to those of TGF- β . The fibrogenic effects of TGF- β are because of its up-regulation of ECM components, including collagen, fibronectin, osteopontin, and the down-regulation of matrix-degrading enzymes.^{15–18} It has been shown by Oh et al,¹⁶ that high glucose increases TGF- β mRNA followed by subsequent increases in fibronectin (FN) mRNA levels, consistent with TGF- β -dependent regulation of FN expression. Furthermore, TGF- β 1-specific neutralizing antibodies diminish overproduction of ECM proteins secreted by mesangial cells incubated with high glucose levels.^{13,15,16} Increased expression of both active and total TGF- β in glomeruli occurs in both patients and in experimental animal models of diabetes.^{13,19} Patients with type 2 diabetes have elevated urinary and renal vein levels of total TGF- β .²⁰ In rats and mice with streptozotocin-induced diabetes, there is an increased expression

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of TGF- β in the renal cortex and glomeruli as early as 2 to 3 days after drug administration that is accompanied by increased ECM production.^{4,21,22}

Activation of latent TGF- β is induced by factors that alter the interaction of the LAP with the mature domain of TGF- β . This can occur by proteolysis of the LAP; by denaturing factors such as heat, chaotropic agents, detergents, and extreme pH; or by altering the conformation of the latent complex as is thought to occur through binding of thrombospondin-1 (TSP-1), interactions with the $\alpha_v\beta_6$ integrin, or possibly modification by reactive oxygen species.^{23–31} We previously showed that platelet- and endothelial cell-derived TSP-1 activates latent TGF- β as a result of binding interactions between these two proteins.^{32–34} TSP-mediated activation of latent TGF- β is a complex process. Initially, the WXXW motif in the type 1 repeats of TSP binds to the active portion of TGF- β , acting as a docking site to orient the TSP molecule in such a way as to facilitate interactions of the KRFK sequence in the type 1 repeats of TSP-1 with the LAP.²⁹ Interaction of the KRFK sequence of TSP-1 is sufficient to induce activation, potentially by inducing conformational changes in the latent TGF- β complex.²⁹ A site near the amino terminus of the LAP is important for TSP-mediated activation, because a synthetic peptide of this site (L₅₄SKL₅₈) can competitively inhibit TSP-LAP interactions and TGF- β activation both *in vitro* and *in vivo*.^{33,35}

Data suggest that TSP-1 plays a role in diabetic nephropathy. Patients with type 1 diabetes have elevated plasma levels of TSP-1 as do platelets from patients with diabetes.^{36,37} Data from *in vitro* studies demonstrate that TSP-1 expression by mesangial cells is up-regulated at both the mRNA and protein levels under pathological glucose concentrations.^{8,38} Mesangial cells exposed to high glucose concentrations for either the short term (6 days) or long term (up to 4 weeks) show elevated expression of TSP-1 and other ECM proteins.^{8,38}

Although it is well documented that mesangial cells exposed to pathological levels of glucose, both *in vitro* and *in vivo*, express increased TGF- β protein and activity, these studies have primarily focused on alterations in TGF- β message, total protein, and downstream matrix synthesis. Consequently, the mechanism by which the latent TGF- β protein becomes activated under high glucose conditions is primarily unknown. Because glucose can regulate TSP-1 expression and TSP-1 is a major physiological activator of latent TGF- β , we sought to determine whether endogenous TSP-1 is mediating activation of TGF- β stimulated by pathological levels of glucose. Our studies show that rat mesangial cells (RMCs) cultured with high glucose express increased levels of both TSP-1 and TGF- β . Antagonists of TSP-dependent TGF- β activation block glucose stimulation of both TGF- β activity and ECM protein synthesis. Therefore, these results suggest that activation of latent TGF- β by endogenous TSP-1 is responsible for the increased levels of TGF- β activity stimulated by high glucose concentrations and for the corresponding up-regulation of ECM protein production by mesangial cells.

Materials and Methods

Peptides, Antibodies, and Other Reagents

The peptides (high pressure liquid chromatography purified) were synthesized by the University of Alabama at Birmingham Comprehensive Cancer Center/Peptides Synthesis and Analysis shared facility. RPMI 1640 medium with L-glutamine without glucose was purchased from Life Technologies, Inc. (Gaithersburg, MD), insulin-transferrin-sodium selenite liquid media supplement, minimal essential medium nonessential amino acid solution, and sodium pyruvate solution were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antiserum against rat osteopontin was a generous gift from Dr. Pi-Ling Chang (University of Alabama at Birmingham). Rabbit polyclonal antiserum against rat fibronectin was purchased from Life Technologies, Inc. Monoclonal anti-TGF- β 1-3 antibody was purchased from R&D Systems (Minneapolis, MN). Nonimmune mouse IgG was purchased from Sigma Chemical Co. Monoclonal antibody 133 raised against human platelet TSP-1 stripped of TGF- β activity was purified by our lab in a joint effort with the University of Alabama at Birmingham Hybridoma Core Facility.³² Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Purification of Fab Fragments

Two mg of IgG 133 (monoclonal mouse antibody raised against TSP-1) was incubated with 20 μ g of papain, 50 mmol/L of L-cysteine, 1 mmol/L of ethylenediaminetetraacetic acid in buffer for 2 hours and 30 minutes at 37°C. Sodium acetate (0.1 mol/L) was used to adjust the pH of the solution to 5.5. The reaction was stopped by adding 75 mmol/L of iodoacetamide. The sample was dialyzed overnight at 4°C against 1 mmol/L NaCl, 10 mmol/L Tris-base, pH 8.3. After dialysis, the sample was purified on a diethylaminoethyl (DEAE) column. Fab fragments were eluted from the DEAE column with a gradient of NaCl (1 mmol/L to 300 mmol/L NaCl in 10 mmol/L Tris-base, pH 8.3). Activity of the Fab fragments was confirmed by Western blot analysis.

Cells

RMCs were the generous gift from Dr. Anne Woods, University of Alabama at Birmingham. Cells were cultured according to the previously published protocol in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 5 mmol/L D-glucose, 2 mmol/L L-glutamine, 1% (v/v) nonessential amino acids, 2 mmol/L sodium pyruvate, 10 μ g/ml transferrin, 5 ng/ml sodium selenite, and 0.6 IU/ml insulin.^{6,39} Human mesangial cells were purchased from Clonetics Corp. (Walkersville, MD) and grown in the manufacturer's growth media according to instructions (media contained 8 to 10 mmol/L of glucose). Both rat and human mesangial cells were passaged at 80% confluency. Experiments in this study were performed on cells between the fifth and tenth passages.

NRK-49F cells (CRL-1570) were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum.

Conditioned Medium Assay

Mesangial cells were seeded in six-well plates (100,000 cells/well). Cells were grown in the growth medium containing 20% fetal bovine serum for 3 days until cells reached 80% confluence. Cells were then made quiescent by culturing in serum- and insulin-free RPMI media containing 5 mmol/L of glucose, 10 μ g/ml of transferrin, and 5 ng/ml of sodium selenite for 48 hours. Cells were treated for the next 48 hours with serum-free media containing either 5 mmol/L or 30 mmol/L of glucose. Media were changed every 24 hours to maintain glucose levels. Peptides or aprotinin were added during this period of time. Concentrations of reagents are specified in the figure legends. Conditioned media were harvested and analyzed for active and total TGF- β in the normal rat kidney fibroblasts soft agar assay. To measure total TGF- β (active and latent), conditioned media samples were heat activated for either 5 minutes at 80°C or 3 minutes at 100°C. Each experiment was performed in triplicate on at least three separate occasions. Exposure of RMCs to high glucose concentrations for 48 hours decreased cell number by $18 \pm 3\%$ as compared to cells cultured with 5 mmol/L of glucose.

NRK Colony Assay for TGF- β Activity

TGF- β activity was assayed by measuring colony formation by NRK cells in soft agar as previously described.³² Briefly, 5% Noble agar (Difco, Detroit, MI) was diluted 10-fold in 10% calf serum/DMEM and 0.5 ml of this 0.5% agar dilution was added per well to 24-well plates as a base layer, and allowed to harden. The sample (0.4 ml) containing 5 ng of epidermal growth factor (EGF) (Life Technologies, Inc.) was combined with 1.2 ml of 0.5% agar and 0.4 ml (2.5×10^3) of a NRK cell suspension in 10% calf serum/DMEM. Then 0.5 ml of this 0.3% agar sample solution was added to the cooled base layer and the plates were incubated for 7 days at 37°C, 5% CO₂. The number of colonies >62 μ m in diameter were counted. Experiments were performed in triplicate on at least three separate occasions.

PAI-1 Promoter Luciferase Assay

Mink lung epithelial cells (clone 32) transfected with the TGF- β response element of the PAI-1 promoter linked to a luciferase reporter construct, were a generous gift from Dr. D. B. Rifkin, New York Medical Center. Cells were cultured according to the published protocol.⁴⁰ Briefly, mink lung epithelial cells were plated in DMEM supplemented with 10% calf serum and L-glutamine and incubated at 37°C for 4 hours for optimal attachment. The serum-containing media were aspirated and conditioned media were added. Samples and TGF- β standards were incubated overnight at 37°C. After incubation, cells were lysed with lysis buffer (Promega Corp., Madison, WI) and luciferase activity was measured using luciferase assay substrate (Promega Corp.).

Figure 1. The effect of increasing concentrations of glucose on activation of mesangial cell-derived TGF- β . RMCs (which were quiescent for 2 days) were treated for 48 hours with increasing glucose concentrations (5 mmol/L to 40 mmol/L of glucose) as indicated in Materials and Methods. **A:** Conditioned media were harvested and analyzed for TGF- β activity in the NRK soft agar assay. Total TGF- β was determined in media that had been heat-treated at either 80°C for 5 minutes or 100°C for 3 minutes to activate latent TGF- β . Active TGF- β was determined in media without additional processing. Results are expressed as the mean number of colonies of triplicate determinations $\pm$ SD. The baseline colony formation in this assay in wells containing 2.5 ng/ml of EGF without conditioned media was 12 ± 3 colonies. *, Active TGF- β at 5 mmol/L versus 30 mmol/L of glucose, $P < 0.001$; total TGF- β at 5 mmol/L versus 30 mmol/L of glucose, $P < 0.005$. **B:** The percent of TGF- β in the active form as a function of increasing glucose concentrations (percent activity equals number of colonies from active TGF- β samples/number of colonies from total TGF- β samples times 100). *, Percent of TGF- β in the active form in RMC cultured with 30 mmol/L versus 5 mmol of glucose, $P < 0.005$. Results are representative of three separate experiments. The Student's *t*-test was used to analyze data.

Western Blot Analysis

Protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The difference in protein concentration among samples was not significant ($<10\%$); therefore, equal volumes of media were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Conditioned media were harvested and subjected to SDS-PAGE under reducing conditions and then proteins were transferred to nitrocellulose (100 V, 2 hours). To detect fibronectin and thrombospondin, nitrocellulose membranes were blocked with 0.5% bovine serum albumin in Tris-buffered saline/Tween-20 (TBS-T) overnight at 4°C to block nonspecific protein-binding sites present in the membranes. Membranes were then incubated with primary antibodies diluted in TBS-T (rabbit anti-rat fibronec-

tin antiserum used at 1:1,000 dilution or mouse monoclonal anti-TSP antibody 133 at 0.05 $\mu\text{g/ml}$) for 4 hours at room temperature. After extensive washing, membranes were incubated with peroxidase-conjugated secondary antibodies diluted in TBS-T (goat anti-rabbit IgG used at 0.1 $\mu\text{g/ml}$ or goat anti-mouse IgG used at 0.1 $\mu\text{g/ml}$) for 1 hour at room temperature. To detect osteopontin, after proteins were transferred to nitrocellulose the membrane was incubated with 10% nonfat milk for 2 hours at room temperature. The membrane was then incubated with rabbit anti-rat osteopontin antibody used at 1:4,000 dilution overnight at 4°C. After washing, the membrane was incubated with peroxidase-conjugated secondary antibody diluted in TBS-T (goat anti-rabbit IgG used at 0.1 $\mu\text{g/ml}$) for 1 hour at room temperature. Immunoreactive bands were visualized using the chemiluminescent detection system (Pierce, Rockford, IL) according to the manufacturer's instructions. Multiple exposures were obtained to assure linearity of the response.

Densitometry

Immunoblots were analyzed by scanning densitometry and quantified by one-dimensional gel analysis (One-Dscan version 1.31, Scanalytics, Fairfax, VA).

Statistical Analysis

Statistical comparisons were done using the one-tail Student's *t*-test or one-way analysis of variance test where appropriate.

Results

High Glucose Concentrations Up-Regulate TGF- β Bioactivity and TGF- β Protein Production

It is well documented that high glucose concentrations stimulate TGF- β secretion and bioactivity.^{6,10} Therefore, we first evaluated whether high glucose concentrations had a stimulatory effect on TGF- β bioactivity and protein expression in a RMC experimental system. Stimulation of RMCs with increasing concentrations of D-glucose (5 to 40 mmol/L) for 48 hours resulted in a concentration-dependent up-regulation of both TGF- β bioactivity and total (active and latent) TGF- β protein production in conditioned media as measured in the NRK colony-forming assay. The maximal response occurred with 30 to 40 mmol/L of glucose (Figure 1A). Therefore, 30 mmol/L of glucose was used in all subsequent experiments.

The proportion of TGF- β that is in the active form is increased at elevated glucose concentrations. The majority of TGF- β (67%) secreted into the conditioned media of cells cultured with 30 mmol/L of glucose is in the active form as compared to 42% in the active form in cells grown in physiological (5 mmol/L) glucose concentrations (Figure 1B). When the amount of TGF- β activity was calculated in terms of pg/0.4 ml sample instead of colonies/well, 25 or 48% of TGF- β is active in cultures treated

Figure 2. Time-dependent activation of TGF- β and stimulation of TSP-1 expression in media conditioned by mesangial cells in the presence of high glucose levels. RMCs were treated as indicated in Materials and Methods. Conditioned media were harvested on days 1 and 2 and analyzed for levels of active (A) or total (B) TGF- β in samples by the NRK soft agar assay. Results are expressed as the mean number of colonies from triplicate determinations $\pm$ SD. Results are representative of three separate experiments. The baseline colony formation in this assay in wells containing 2.5 ng/ml EGF without conditioned media was 12 ± 2 colonies. *, For active TGF- β in RMC cultured with 30 mmol/L versus 5 mmol/L, $P < 0.001$. *, For total TGF- β in RMC cultured with 30 mmol/L versus 5 mmol/L, $P < 0.005$. The Student's *t*-test was used to analyze data. C: Conditioned media were harvested on days 1 and 2, and a 60- μl aliquot of 1 ml of conditioned medium loaded in each well was subjected to 8% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and analyzed by Western blotting with mouse 133 monoclonal antibody to thrombospondin as indicated in Materials and Methods. Lane 1, conditioned media harvested on day 1 from RMC cultured with 5 mmol/L of glucose; lane 2, conditioned media harvested on day 1 from RMC cultured with 30 mmol/L of glucose; lane 3, conditioned media harvested on day 2 from RMC cultured with 5 mmol/L of glucose; and lane 4, conditioned media harvested on day 2 from RMC cultured with 30 mmol/L of glucose. Immunoblots were analyzed by scanning densitometry and quantified by one-dimensional gel analysis (One-Dscan version 1.31; Scanalytics). Results are representative of three separate experiments.

Figure 3. Anti-TSP antibody and antagonist peptides (GGWSHW and LSKL) block TSP-mediated activation of latent TGF- β secreted by RMCs under high glucose conditions. RMCs were made quiescent for 48 hours in serum-free media. Cells were then stimulated for 48 hours with either 5 mmol/L or 30 mmol/L of glucose in the presence of TSP antagonist peptides (LSKL at 1 μ mol/L, GGWSHW at 10 μ mol/L), control peptides (SLLK at 1 μ mol/L, GGWSHW at 10 μ mol/L), or aprotinin (200 μ g/ml). Aliquots of conditioned media (0.4 ml) were assayed for either (A) active or (B) total TGF- β levels using the NRK soft agar assay. The dashed line represents the baseline colony formation in this assay in wells containing only 2.5 ng/ml EGF without conditioned media (12 ± 2 colonies). Results are expressed as the mean number of colonies from triplicate determinations $\pm$ SD. One-way analysis of variance analysis was used to determine significance: *, $P < 0.05$ for 30 mmol/L of glucose control versus LSKL peptide or GGWSHW peptide in 30 mmol/L of glucose. Results for samples treated with either LSKL or GGWSHW peptides in 30 mmol/L of glucose were not statistically different from control samples in 5 mmol/L of glucose. Results are representative of three separate experiments. C: RMC stimulated with 5 mmol/L or 30 mmol/L of glucose were treated with Fab fragments (24 μ g/ml) purified from 133 anti-TSP1 antibody (α -TSP). Results are expressed as the mean number of colonies from triplicate determinations $\pm$ SD. *, $P < 0.01$ for 30 mmol/L of glucose control versus Fab fragments in 30 mmol/L of glucose. D: Media from RMC grown in 5 or 30 mmol/L of glucose were treated with either 15 μ g/ml anti-TGF- β 1-3 antibody (α -TGF) or 15 μ g/ml nonimmune IgG (NI) before addition to the NRK assay. Results are the means of triplicate determinations.

with either 5 mmol/L or 30 mmol/L of glucose, respectively. This increase in TGF- β activity and protein expression was specific to D-glucose and was not due to a rise in the osmolarity, because the addition of 25 mmol/L of L-glucose did not significantly stimulate TGF- β bioactivity (data not shown).

Kinetics of High Glucose-Induced TGF- β Activity and TSP-1 Protein Production in RMCs

RMCs cultured in media containing high glucose (30 mmol/L) concentrations demonstrated a time-dependent increase in the levels of active and total TGF- β (Figure 2). Production of bioactive and total TGF- β by mesangial

cells cultured in 30 mmol/L of glucose increased progressively throughout 2 days as shown in Figure 2, A and B. NRK colony formation in soft agar, an indicator of TGF- β activity, increased $\sim$ 100 to 200% by day 2 in cultures stimulated with 30 mmol/L of glucose, whereas colony formation in cultures stimulated with 5 mmol/L of glucose increased only by 40% by day 2 (Figure 2A). Total TGF- β expression, measured in media that been heat treated to activate latent TGF- β , was also up-regulated by glucose, although to a lesser extent than was TGF- β activity. This is consistent with the data reported in Figure 1 showing that the proportion of TGF- β that is in the active state increases with glucose concentration. Total TGF- β expression as measured by colony formation increased by

~60% in cultures stimulated with 30 mmol/L of glucose and only by ~10% in cultures treated with 5 mmol/L of glucose (Figure 2B). Thus, glucose not only stimulates expression of TGF- β , but it significantly increases the proportion of TGF- β that is in the bioactive form. Using another assay for TGF- β activity, the PAI-1 promoter luciferase reporter assay, similar results were obtained. Glucose stimulated a 300% increase in the level of active TGF- β and an ~200% increase in total TGF- β protein (data not shown).

To confirm the regulation of TSP-1 protein expression by glucose in conditioned media, we performed immunoblot analysis. TSP was detectable in serum-free control media containing 5 mmol/L of glucose. Stimulation of RMCs with 30 mmol/L of D-glucose resulted in a time-dependent up-regulation of TSP protein at day 1 with further increases at day 2 (Figure 2C). Treatment of mesangial cells with 30 mmol/L of glucose stimulated approximately a 4.6- $\pm$ 3.5-fold increase in immunostaining of TSP-1 protein on day 1 and 6.4- $\pm$ 1.5-fold increase on day 2 as compared to cells treated with 5 mmol/L of glucose. This is consistent with previous reports showing a nearly eightfold stimulation of TSP-1 mRNA with high glucose.⁴¹

High Glucose Induces TGF- β Activity in a TSP-Dependent Manner

We demonstrated that TSP-1 activates latent TGF- β , both *in vitro* and *in vivo*.^{32,35,42} Therefore, we tested the hypothesis that up-regulation of endogenous TGF- β bioactivity in the presence of 30 mmol/L of glucose occurs in a TSP-dependent manner. Peptides that are antagonists of TSP-mediated TGF- β activation were used to evaluate this hypothesis. Previously, we showed that the peptides, LSKL, derived from the precursor portion of latent TGF- β , and GGWSHW, from the type 1 repeats of TSP-1, inhibit the activation of TGF- β by TSP-1.^{29,33} As shown in Figure 3A, incubation of the inhibitory peptides with RMCs cultured in 30 mmol/L of D-glucose reduced the stimulatory effect of high glucose on TGF- β activity. TGF- β activity was not blocked entirely, but was reduced to the level of TGF- β activity observed in mesangial cells cultured with 5 mmol/L of glucose (basal levels). Inhibition of glucose-stimulated TGF- β activation was observed by treatment with 1 μ mol/L of LSKL peptide and 10 μ mol/L of GGWSHW peptide. Levels of total TGF- β were unaffected by these peptides (Figure 3B). Control peptides (SLLK and GGYSHW) had no effect on either basal or glucose-stimulated levels of TGF- β activity or total TGF- β (Figure 3, A and B). Additionally, Fab fragments raised against TSP-1 also inhibited TGF- β activity in cells treated with 30 mmol/L of glucose (Figure 3C). Fab fragments did not have any effects on the basal level of TGF- β activity or total TGF- β protein production. The NRK soft agar assay is specific for TGF- β because anti-TGF- β antibody inhibited colony formation; nonimmune IgG did not have any effect on the colony formation (Figure 3D). The role of TSP-1 as an effector of glucose-stimulated TGF- β activity is not unique to RMCs because similar results were ob-

tained with cultures of human mesangial cells treated with the LSKL peptide (Figure 4).

To establish optimal concentrations of inhibitory peptides, RMCs were incubated with increasing concentrations of LSKL or SLLK peptides (1 nmol/L to 10 μ mol/L). Activation of TGF- β in cultures treated with 30 mmol/L of glucose was inhibited by the LSKL peptide in a concentration-dependent manner. The maximum inhibition of TGF- β activation was achieved with 0.1 μ mol/L of LSKL peptide (Figure 5A). The IC₅₀ of the LSKL peptide in this system is ~0.05 μ mol/L. This inhibitory peptide did not alter basal TGF- β activity levels or total TGF- β in cultures treated with 5 mmol/L of glucose (Figure 5, A and B). An inactive analogue of LSKL, SLLK, had no effect on total or active TGF- β in cultures with either 5 or 30 mmol/L of glucose when tested throughout the same concentration range as the LSKL peptide (Figure 5, C and D).

Moreover, increases in TGF- β activity under high glucose conditions seem to be predominantly mediated by TSP, because aprotinin (an inhibitor of plasmin) had no inhibitory effect on TGF- β bioactivity (Figure 3, A and B). Therefore, these data indicate that TSP is a major activator of TGF- β in our model system.

Induction of Mesangial Cell ECM Protein Expression by High Glucose Is Dependent on TSP-Mediated Activation of TGF- β

We next examined whether blocking TSP-mediated TGF- β activation would similarly inhibit glucose-dependent stimulation of matrix protein synthesis. To explore the role of autocrine TSP-mediated activation of TGF- β in the regulation of matrix protein expression, RMCs were incubated with 30 mmol/L of D-glucose in the presence or

Figure 4. Activation of TGF- β produced by human mesangial cells cultured with high glucose is inhibited by the LSKL peptide. Normal human mesangial cells were grown as indicated in Materials and Methods. Normal human mesangial cells were made quiescent under serum-free and low glucose conditions for 48 hours. Cells were then stimulated for an additional 48 hours with either 10 mmol/L or 30 mmol/L of glucose in the presence or absence of 1 μ mol/L LSKL peptide. Media were replaced at 24 hours. Aliquots of conditioned media (0.4 ml) were harvested and assayed for active TGF- β levels using the NRK soft agar assay. Results are expressed as the mean number of colonies from triplicate determinations $\pm$ SD. Results are representative of two separate experiments.

Figure 5. The LSKL but not the SLLK peptide inhibits activation of TGF- β in a dose-dependent manner under high glucose conditions. RMCs were made quiescent for 48 hours in serum-free media. Cells were then stimulated for 48 hours with either 5 mmol/L or 30 mmol/L of glucose in the presence of increasing concentrations (1 nmol/L to 10 μ mol/L) of LSKL (**A** and **B**) or SLLK (**C** and **D**) peptides. Conditioned media were assayed for active (**A** and **C**) and total (**B** and **D**) TGF- β levels using the NRK soft agar assay. To measure total TGF- β , samples were heat-activated for 3 minutes at 100°C. Results are expressed as the mean number of colonies from triplicate determinations $\pm$ SD. Results are representative of two separate experiments. The **dashed line** represents the baseline colony formation in wells containing 2.5 ng/ml EGF without conditioned media (12 ± 2 colonies).

absence of peptides (LSKL and GGWSHW) and conditioned media were analyzed for relative levels of the secreted matrix proteins, fibronectin, and osteopontin. Glucose-stimulated fibronectin and osteopontin synthesis was reduced by the addition of LSKL or GGWSHW peptides to mesangial cell cultures (Figure 6, A and B). The level of type IV collagen secreted by RMCs cultured with 30 mmol/L of glucose was also reduced by inhibitory peptides (data not shown). In some experiments, aliquots of the same conditioned media were analyzed for both levels of ECM proteins and TGF- β activity in the NRK assay. Increased levels of ECM proteins in the conditioned media correlate with up-regulated TGF- β activity and correspondingly, decreased levels of ECM proteins correlate with decreased NRK colony formation in samples treated with antagonist peptides (data not shown). The inactive peptides (SLLK or GGYSHW) did not decrease fibronectin, osteopontin, or type IV collagen levels secreted by RMC cultured with serum-free media con-

taining either 5 or 30 mmol/L of glucose. Together, this data strongly suggests that glucose-stimulated matrix protein expression is dependent on TSP-1 stimulation of latent TGF- β activation.

Interestingly, the expression of TSP-1 by mesangial cells cultured with either 5 or 30 mmol/L of glucose was not blocked by the antagonist peptides (Figure 6C) or by anti-TGF- β antibody (data not shown), suggesting that glucose-dependent regulation of TSP-1 expression most likely occurs via a TGF- β -independent mechanism.

Discussion

Although the importance of TGF- β in the development of diabetic nephropathy is well established, the molecular mechanisms that regulate the activation of the latent TGF- β complex secreted in response to glucose stimulation are primarily unknown.^{5,43} Our current data dem-

Figure 6. Extracellular matrix protein expression is up-regulated by TGF- β in a TSP-dependent manner under high glucose conditions. RMCs were made quiescent for 48 hours in serum-free media. Cells were then stimulated for 48 hours with either 5 mmol/L or 30 mmol/L of glucose in the absence or presence of TSP antagonist peptides (LSKL and SLLK at 1 μ mol/L, GGWSHW and GGYSHW at 10 μ mol/L). Conditioned media were harvested and subjected to SDS-PAGE under reducing conditions for either fibronectin, thrombospondin (8% SDS-PAGE) or for osteopontin detection (10% SDS-PAGE), transferred to nitrocellulose, and analyzed by Western blotting as indicated in Materials and Methods section. **Left**, immunoreactive bands were developed by enhanced chemiluminescence according to the manufacturer's instructions. **Lanes 1–5**, conditioned media from RMCs cultured with 5 mmol/L of glucose in the presence or absence of antagonist peptides; **lane 1**, conditioned media from RMC cultured with 5 mmol/L of glucose only; **lane 2**, conditioned media from RMC cultured in the presence of LSKL peptide; **lane 3**, conditioned media from RMC cultured in the presence of SLLK peptide; **lane 4**, conditioned media from RMC cultured in the presence of GGWSHW peptide; **lane 5**, conditioned media from RMC cultured in the presence of GGYSHW peptide; **lanes 6–10**, conditioned media from RMC cultured with 30 mmol/L of glucose in the presence or absence of antagonist peptides; **lane 6**, conditioned media from RMC cultured with 30 mmol/L of glucose only; **lane 7**, conditioned media from RMC cultured in the presence of LSKL peptide; **lane 8**, conditioned media from RMC cultured in the presence of SLLK peptide; **lane 9**, conditioned media from RMC cultured in the presence of GGWSHW peptide; **lane 10**, conditioned media from RMC cultured in the presence of GGYSHW peptide. **Right**, relative protein levels as determined by scanning densitometry of immunoblots. The level of protein expressed under 5-mmol/L glucose conditions was arbitrarily set at one. Results are the means of three separate experiments $\pm$ SD. **A:** The level of fibronectin protein expression in media conditioned by RMC with either 5 mmol/L or 30 mmol/L of glucose in the presence or absence of inhibitory peptides. *, $P < 0.01$ for 30 mmol/L of glucose control versus LSKL peptide in 30 mmol/L of glucose, #, $P < 0.05$ for 30 mmol/L of glucose control versus GGWSHW peptide in 30 mmol/L of glucose. **B:** The level of osteopontin protein expression in media conditioned by RMC with either 5 mmol/L or 30 mmol/L of glucose in the presence or absence of inhibitory peptides. *, $P < 0.01$ for 30 mmol/L of glucose control versus LSKL peptide in 30 mmol/L of glucose. **C:** The level of TSP-1 protein expression in media conditioned by RMC with either 5 mmol/L or 30 mmol/L of glucose in the presence or absence of inhibitory peptides. The Student's *t*-test was used to analyze data.

onstrates the role of TSP-mediated activation of TGF- β under high glucose conditions and the concomitant up-regulation of ECM expression. Two peptide antagonists of TSP-mediated TGF- β activation were able to block glucose stimulation of TGF- β activity and also glucose-dependent expression of the ECM proteins, fibronectin, osteopontin, and type IV collagen secreted by cultured RMCs. It has been shown that glucose up-regulates TGF- β bioactivity and TGF- β protein production leading to the excessive deposition of ECM proteins in the diabetic kidney and fibrotic end-stage renal disease.^{6-9,13,16,19} High glucose also increases TSP-1 both at mRNA and protein levels.^{8,38} The data presented in this report support our hypothesis that under high glucose conditions, increased TSP-1 protein expression induces TGF- β bioactivity, which leads to the onset of glomerulosclerosis (Figure 7).

There are other potentially physiological means of activating latent TGF- β , including proteolysis by plasmin, alteration of the latent complex by binding to integrin, or by oxidative modification.^{23,24,30,31} Aprotinin had no effect on glucose-stimulated levels of TGF- β activity, indicating that it is unlikely that plasmin is involved in this particular activation process. Other data are consistent with this finding, as it has been shown that high glucose inhibits plasmin activity.⁴⁴ Furthermore, TSP inhibits plasmin activity both directly by binding to plasminogen and indirectly by stimulation of PAI-1 synthesis.^{45,46} In addition, PAI-1 expression is increased in diabetic patients in response to high glucose levels.^{7,47} TGF- β can also be activated by binding of the latent complex to the $\alpha_v\beta_6$ integrin.³⁰ However, expression of this integrin is primarily restricted to epithelial cells.^{48,49} Because, there is no evidence for $\alpha_v\beta_6$ expression on mesangial cells, this mechanism is unlikely to be involved.⁵⁰ It remains to be determined whether oxidative modification of the latent complex is also involved in glucose-stimulation of TGF- β activation. However, activation by TSP-1 does seem to be sufficient to account for the increase in TGF- β activity stimulated by glucose, suggesting that TSP-1 is the primary mediator of glucose-stimulated mesangial cell-derived TGF- β activation.

TSP-1 is secreted by a variety of cells including RMCs and its expression by human mesangial cells is up-regulated by elevated glucose concentrations.^{8,38,51} Additionally, TSP has been shown to be increased in the kidneys of diabetic patients.⁵² Therefore, TSP protein expression is increased in renal tissue under diabetic conditions. Tada and Isogai³⁸ demonstrated that addition of exogenous TSP to mesangial cell cultures increased endogenous TGF- β bioactivity and increased production of fibronectin by mesangial cells. This observation is consistent with the earlier report that the expression of TSP-1 in injured kidneys often precedes and predicts foci of subsequent fibrosis.⁵³ In our current studies we similarly showed that TSP-1 expression by RMCs is up-regulated in response to glucose.

TSP-1 expression can be up-regulated by many factors, including TGF- β itself. TGF- β increases TSP-1 expression at both the mRNA and protein levels.⁵⁴ Conditioned media from RMCs stimulated with high glucose concentrations show both increased TSP protein and

TGF- β activity, raising the possibility that TSP protein is up-regulated primarily by TGF- β in a positive autocrine manner. However, neither the LSKL and GGWSHW peptides (inhibitors of TGF- β activation) nor anti-TGF- β antibody altered TSP protein levels in mesangial cells cultured with 30 mmol/L of D-glucose, suggesting that, unlike fibronectin, osteopontin, and collagen IV, glucose regulates TSP protein expression via a TGF- β -independent mechanism. It is known that glucose also alters the oxidative balance of cells.^{55,56} We are currently investigating the possibility that glucose-mediated regulation of TSP-1 expression occurs through mechanisms that involve modification of the intracellular redox state. Our current studies, however, do not eliminate the possibility that basal TGF- β activity occurring in cultures exposed to 5 mmol/L of glucose might contribute to basal TSP-1 expression.

It is interesting to note that only stimulated levels of TGF- β activity up-regulated by pathological glucose levels were diminished by the peptides, whereas the basal levels of TGF- β activity in RMCs cultured with physiological levels of glucose were unaffected by the TSP antagonist peptides. Additionally, total TGF- β production was not affected by inhibitory peptides. This would suggest that during short-term exposure to high glucose, the positive feedback loop is not the major mechanism of increased TGF- β expression. Therefore, it is possible that these antagonist peptides might act as selective tools to inhibit only the undesirable levels of TGF- β activity without altering the physiological levels of TGF- β required for normal tissue homeostasis.

Ongoing studies will determine whether TSP-dependent stimulation of TGF- β activation is involved in animal models of diabetic nephropathy and whether these antagonist peptides are therapeutically useful in blocking

Figure 7. A model of TSP-dependent regulation of TGF- β activation by high glucose. Glucose induces the increased expression of both latent TGF- β and TSP-1 by mesangial cells. Conversion of latent TGF- β to its bioactive form by TSP-1 results in stimulation of matrix protein synthesis by mesangial cells and the consequent development of fibrotic end-stage renal disease. Peptide antagonists (LSKL, GGWSHW) of TSP-1 mediated TGF- β activation have the effect of reducing matrix protein expression and possibly, renal fibrosis.

the toxic effects of not only glucose, but also of advanced glycosylation end products, in affected organ systems.

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