Thrombospondin-1 Is the Key Activator of TGF-β1 in Human Mesangial Cells Exposed to High Glucose

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Abstract. Elevated levels of transforming growth factor-β1 (TGF-β1) are synthesized by human mesangial cells that are cultured in medium that contains high concentrations of glucose and mediate increased synthesis of fibronectin (FN), plasminogen activator inhibitor-1 (PAI-1), and changes in the expression of other genes. TGF-β1 is synthesized as a latent complex. Previous work indicated that high-glucose conditions also upregulate expression of thrombospondin-1 (TSP-1), a potential activator of latent TGF-β1. With the use of the synthetic peptide GGWSHW, an inhibitor of the TSP-1 activation mechanism, endogenous TSP-1 is shown to be responsible for converting high levels of latent TGF-β1 to bioactive growth factor over 3 wk of exposure of mesangial cells to 30 mM D-glucose. Peptide inhibition of TGF-β1 activation by TSP-1 in high-glucose conditions completely suppresses increases in FN and PAI-1 expression. Treating mesangial cells maintained in high glucose with a TSP-1 antisense oligonucleotide reduced TSP-1 expression to levels found in 4 mM D-glucose cultures, prevented TGF-β1 activation, and normalized expression of FN.

Expansion of the glomerular mesangial matrix is a major feature of diabetic nephropathy, a fibrotic disorder of the kidney (1). Elevated blood glucose levels in diabetes stimulate increased expression of transforming growth factor-β1 (TGF-β1) in mesangial cells, which, in turn, promotes the synthesis of matrix proteins and the inhibition of matrix turnover. TGF-β1 is thought to be the key mediator of matrix accumulation in this disorder (2). However, TGF-β1 is secreted as a latent complex (3) that must be activated before it can bind to its receptors. Thus, changes in expression of latent TGF-β1 will have no biologic effect unless mechanisms for converting it to the active form are operational.

There are two types of latent TGF-β1 complex. A small latent complex consists of TGF-β1 associated noncovalently with a disulfide-linked dimer of the N-terminal part of pro-TGF-β1, referred to as the latency-associated peptide (LAP) (4,5). A large complex also contains latent TGF-β1-binding protein (LTBP) disulfide-linked to LAP. LTBP does not itself play any role in latency but is involved in the secretion of TGF-β1, storage in the extracellular matrix, and eventual activation of the factor (6).

In vitro studies show that the latent TGF-β1 complex can be activated by conformational changes induced by pH or high temperature (7), limited proteolysis or deglycosylation of LAP (8–10), the effect of reactive oxygen species on redox-sensi-

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based on the results of cell-free experiments with synthetic TSP-1–derived KRFK and GGWSHW peptides (22). It was shown that the minimal sequence able to activate TGF-β1 is KRFK. GGWSHW peptide, while unable to activate TGF-β1, is able to bind to the latent complex. Moreover, it blocks activation by TSP-1 and increases activation by KRFK peptide (22).

Treatment of cultured mesangial cells with TSP-1 leads to an increase in active TGF-β1, elevating the production of fibronectin (14). TGF-β1 expression is increased in stretched mesangial cells, and extracellular matrix synthesis is enhanced. The stretch-induced matrix expression is inhibited by TSP-1–blocking peptide (24). TSP-1 is also a major activator of TGF-β1 in vivo as demonstrated with TSP-1 null mice (12). In addition, TSP-1–blocking peptides are active in vivo, inhibiting mesangial cell proliferation in the anti-Thy1 model of glomerulonephritis in rats, a process driven by TGF-β1 (25). Moreover, TSP-1 expression is related closely to the development of fibrosis in proliferative glomerulonephritis (26) and in tubulointerstitial fibrosis (27). Collectively, this evidence suggests a major role for TSP-1 in pathologic fibrosis by its activation of TGF-β1. However, direct evidence for TSP-1–activating TGF-β1 in mesangial cells in high-glucose conditions is lacking. Thus, we investigated TGF-β1 activation by endogenous TSP-1 in human mesangial cell cultures maintained in media containing normal (4 mM) and elevated (30 mM) glucose levels. We used the GGWSHW peptide to inhibit activation by TSP-1 and TSP-1 antisense oligonucleotide to inhibit TSP-1 synthesis in the mesangial cells. Secreted and cell layer–associated fibronectin and plasminogen activator inhibitor-1 (PAI-1) were measured as markers of TGF-β1–dependent gene activation in the mesangial cell cultures. The results indicate that the elevated TGF-β1 activity in mesangial cells in high-glucose conditions is dependent on TSP-1 activation of the growth factor.

Materials and Methods

Materials

Primary normal human mesangial cells (HMC; CC-2259, lot 8F1510) were purchased from BioWhittaker (Wokingham, UK) and originated from a 62-yr-old Caucasian female. Special RPMI 1640 medium without d-glucose, fetal calf serum (FCS), antibiotics, ITS (insulin, transferrin, sodium selenite), and glutamine were from Life Technologies (Paisley, Scotland, UK), and Dulbecco’s modified Eagle’s medium (DMEM: with glutamax) was from Sigma Chemical Co. (Poole, Dorset, UK). Human TGF-β1 and the TGF-β1 immunoassay kit were supplied by R&D Systems (Minneapolis, MN), human platelet TSP-1 and human recombinant PAI-1 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA), and human fibronectin (FN) was from ICN Pharmaceuticals (Costa Mesa, CA). Mouse anti–thrombospondin-1 monoclonal antibody, rabbit anti-human FN antibody, and rabbit anti-actin were obtained from Sigma, and goat anti–PAI-1 antibody was supplied by American Diagnostics, Inc. (Dundee, UK). Secondary peroxidase-conjugated antibodies were purchased from DAKO (High Wycombe, UK). Western blotting detection reagents (ECL + Plus) were from Amersham Pharmacia Biotech Ltd. (Uppsala, Sweden), and the reverse transcription-PCR (RT-PCR) kits and 1-kb DNA ladder were supplied by Life Technologies.

Antisense (GTG CGG CGA CGT GT) phosphorothioate oligonucleotides directed to TSP-1 were designed and manufactured by Biognostik GmbH (Göttingen, Germany), who own the intellectual property rights of these sequences. Both blocking and control peptides were synthesized at our Advanced Biotechnology Center core facility.

Experimental Design

Early confluent cultures of HMC were maintained in either normal glucose (4 mM) or high-glucose (30 mM) containing media supplemented with 10% FCS for up to 3 wk (28). The TSP-1–blocking peptide GGWSHW or a negative control peptide, GGYSHW, was added to media throughout the experiment. Media were changed every 48 h. At the end of each week, the medium was changed to serum-free and ITS-free medium containing the appropriate concentration of glucose and peptide. After 24 h, secreted TSP-1, TGF-β1 (total and active), FN, and PAI-1 were measured in the medium by enzyme-linked immunosorbent assay (ELISA). The cell layers were used to estimate mRNA (RT-PCR) and protein (Western blot) levels of cell layer–associated TSP-1, FN, and PAI-1. The biologic activity of TGF-β1 was determined using an Mv1Lu mink lung epithelial cell growth inhibition assay (29).

A TSP-1 mRNA antisense oligonucleotide (2 μM, as recommended by the manufacturer) or a CG-matched randomized sequence oligonucleotide (negative control) was added directly to normal- and high-glucose cultures for 1 wk. The medium containing oligonucleotide was renewed every 48 h. All subsequent analytical procedures were as for the peptide addition experiments.

Mesangial Cell Culture

Primary HMC were maintained at 37°C with 5% CO2/95% air in RPMI 1640 medium, containing 4 mM glucose, 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 1 μg/ml Amphotericin B, and ITS (5 μg/ml, 5 μg/ml, 5 ng/ml, respectively). Cells were routinely passaged with a 1:4 split and were used for experiments at the seventh to ninth passages.

At the end of each experimental period, HMC-conditioned medium was collected under sterile conditions, centrifuged (3000 × g, 10 min), and aliquoted. Protease inhibitors were added to give final concentrations of 10 mM aminohexanoic acid, 1 mM ethylenediaminetetraacetate, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide. Inhibitors were not added to samples assigned for measurement of TGF-β1 bioactivity. Samples were stored at −70°C before ELISA assay or TGF-β1 bioactivity assay.

Cell layers (5 to 6 × 10⁵ cells) were washed extensively with phosphate-buffered saline at 4°C and were solubilized either in a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing protease inhibitors for subsequent analysis of protein expression by Western blotting or in RNaizolB (AMS Biotechnology, Oxfordshire, UK) for investigating gene expression by RT-PCR. In the first case, samples were frozen and stored at −20°C before analysis. In the second case, total RNA was extracted, dissolved in diethyl pyrocarbonate–treated water, and stored at −70°C before assay.

Assay for TGF-β1 Bioactivity

TGF-β1 bioactivity in the conditioned media was assayed by its ability to inhibit the growth of mink lung epithelial (Mv1Lu) cells (29). Mv1Lu cells were maintained in DMEM supplemented with...
10% FCS, 2 mM glutamine, and antibiotics (as above). Cells were passaged with a 1:10 split.

For bioassays, Mv1Lu cells (100 μl, 7 to 10 × 10⁴ cells/ml) were seeded in 96-well plates in DMEM with 10% FCS. After 2 to 3 h, the plates were washed and DMEM with 10% FCS (100 μl) was added to the wells. After 3 d, the number of cells in each well was determined by sulforhodamine B assay (30). A standard curve was constructed for 1 × 10⁻⁴ to 30 × 10⁻⁴ cells. A further standard curve was set up for each assay with 0.01 to 1 ng/ml of human platelet TGF-β1.

**Enzyme-Linked Immunosorbent Assay**

The concentration of TSP-1, FN, and PAI-1 in the conditioned media was measured by ELISA (28,31,32) with some modifications to the original method. Briefly, microtiter plates were coated overnight at 4°C with serial dilutions of standard proteins in coating buffer (0.15 M NaCl, 1.5 mM KH₂PO₄, 10.8 mM Na₂HPO₄, 2.7 mM KCl [pH 7.3] for TSP-1 and 15 mM Na₂CO₃, 35 mM NaHCO₃ [pH 9.6] for FN and PAI-1) and with HMC-conditioned media, diluted with the appropriate coating buffer. Nonspecific proteins were blocked with 2% bovine serum albumin at 37°C for 1 h. The optimal dilutions of the primary antibodies were found to be: 1:1000, 1:500, and 1:2000 for mouse monoclonal anti–TSP-1, goat anti–PAI-1, and rabbit anti-human FN, respectively. The dilutions of secondary antibodies were 1:2000 (rabbit anti-goat and swine anti-rabbit). The bound antibodies were detected with 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid at a wavelength of 405 nm.

TGF-β1 was determined by ELISA using TGF-β1–soluble receptor type II (33). To measure active TGF-β1 in the media, the samples were assayed directly, whereas to determine total TGF-β1, samples were acidified before the measurement.

**Western Blotting**

Samples were boiled for 5 min and diluted with reducing loading buffer to standardize for cell number. Samples were resolved by SDS-PAGE (15% wt/vol gels for PAI-1 and 7.5% for FN) (28). Prestained molecular-mass standards were used to monitor protein migration. Proteins were transferred onto poly(vinylidene difluoride) membrane (Immobilon-P; Millipore, Bedford, UK) with a Bio-Rad transfer apparatus (Hercules, CA). Immunodetection was performed as described (34), and bound antibodies were revealed using the enhanced chemiluminescence reagent ECL Plus (Amersham Pharmacia Biotech, Little Chalfont, UK). The results were normalized against the intensity of the β-actin band for each sample.

**Reverse Transcription-PCR**

Equal amounts (2 μg) of DNA-free total RNA from each sample was converted to cDNA by SuperScriptTM II RNase H⁻ reverse transcriptase (Life Technologies) with random primers in a 20-μl reaction volume. The reverse transcription reaction (0.5 μl) was subjected to PCR amplification using 2.5 U of Taq DNA polymerase in a 100-μl reaction volume with 0.5 μM of each dNTP, 0.5 μM of each specific primer, and 1.5 mM MgCl₂. The amount of amplified reverse-transcribed cDNA (0.5 μl) was determined to be nonsaturating. The “house-keeping” gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously with TSP-1, PAI-1, and FN genes.

The sequences of primers were designed from the human genes: TSP-1 (496 bp), sense: 5’-AAA GGC TCT TCA CCA GAG ACC T-3’, antisense: 5’-GCA GAT GGT TAA TGA GGT CTG ACA A-3’; PAI-1 (396 bp), sense: 5’-GTA TCT CAG GAA GTC CAG CC-3’, antisense: 5’-TCT AAG GTA GTT GAA TCC GAG C-3’; FN (639 bp), sense: 5’-CGA AAT CAC AGC CAG TAG-3’, antisense: 5’-ATC ACA GCC ACA CGG TAG-3’, GAPDH (195 bp), sense: 5’-CCA TGG AGA AGG CTG GGG-3’, antisense: 5’-CAA AGT GTG CAT GGA TGA CC-3’. The amplification program was 94°C for 3 min, 35 cycles consisting of 94°C for 60 s, 55°C for 60 s, 72°C for 90 s, and 72°C for 10 min.

Equal volumes of the amplification products were analyzed by agarose gel (1.2%) electrophoresis with ethidium bromide (0.5 μg/ml) staining. A 1-kb DNA ladder was used as a size marker. Gels were photographed and analyzed with Image software (NIH Image). The results were normalized to the intensity of GAPDH bands.

**Statistical Analysis**

Results were compared using unpaired t test. A P value of 0.05 or less was regarded as denoting a significant difference.

**Results**

Previously, we reported that prolonged exposure of HMC to high-glucose conditions over 3 wk stimulated continuous production of elevated levels of active TGF-β1, as detected by ELISA (28). Similarly, the expression of FN and PAI-1, two genes known to be controlled by TGF-β1 (35,36), was markedly increased in high-glucose conditions over the same period (28). We also found that mRNA levels for TSP-1, an activator of TGF-β1, were increased in HMC exposed to high-glucose conditions over 3 wk. To test whether TSP-1 is responsible for activating TGF-β1 in high-glucose mesangial cell cultures and, through this, for the downstream upregulation of FN and PAI-1 expression, we investigated the effect of the blocking peptide GGWSHW (22). We also investigated whether antisense oligonucleotide inhibition of TSP-1 expression modulated the activation of TGF-β1 and the expression of FN and PAI-1.

**TSP-1 Expression in Mesangial Cells Exposed to Low- and High-Glucose Conditions**

TSP-1 mRNA levels, measured by semiquantitative RT-PCR, were increased in HMC exposed to 30 mM d-glucose for 1, 2, and 3 wk over levels detected in cultures maintained in 4 mM d-glucose conditions (Figure 1, lanes 1 and 2). Densitometry of the cDNA bands from three independent experiments showed a mean increase of 1.8-fold after 1 wk, 2-fold at 2 wk, and 2.5-fold at 3 wk (P < 0.01 to 0.05; data not shown). Treatment of the 30 mM d-glucose cultures with either the TGF-β1 activation-blocking peptide (peptide W, 1 μM) or the control peptide (peptide Y, 1 μM) had no effect on the mRNA expression level of TSP-1 at any time point (Figure 1, lanes 5 and 6). However, treatment of the high-glucose cultures with TSP-1 antisense oligonucleotide for 1 wk reduced TSP-1 expression to approximately or below the level found in 4 mM d-glucose cultures (Figure 1, lane 3). A control oligonucleotide had no effect on TSP-1 mRNA expression level in high glucose (Figure 1, lane 4).

The increased levels of TSP-1 mRNA in high-glucose cultures were reflected in increased levels of TSP-1 protein in the culture media (Figure 2). The greatest increase occurred after 1 wk (2.3-fold, P < 0.001) and was 2.2-fold at 2 wk (P < 0.001) and 1.6-fold at 3 wk (P < 0.02), despite the gradually increas-
ing levels of TSP-1 mRNA in high-glucose cultures at these time points (see above). This may be because of increased retention of TSP-1 in the cell layer matrix of cultures with increasing time. Mesangial cell cultures treated with 4 mM D-glucose + 26 mM mannitol had the same levels of TSP-1 in the medium after 1, 2, and 3 wk as cultures maintained in 4 mM D-glucose alone (data not shown). Therefore, the increased levels of TSP-1 in cultures maintained in 30 mM D-glucose are not due to the hyperosmotic effect of high-glucose concentration.

Effect of Blocking Peptide W on TSP-1 and TGF-β1

The effect of peptide W on TSP-1 synthesis was investigated. Concentrations of the peptide up to 10 μM had no effect on TSP-1 levels in the media of either normal- or high-glucose cultures after 1 wk of treatment (data not shown). Moreover, neither 1 μM peptide W nor the control peptide Y had any significant effect on the elevated TSP-1 mRNA levels in high-glucose cultures (Figure 1, lanes 5 and 6) or on TSP-1 protein levels in normal- or high-glucose media (Figure 2) when cultures were treated with either peptide over 3 wk.

ELISA assay of culture media for total TGF-β1 showed that high-glucose–treated HMC synthesized 2.5-, 2.3-, and 1.8-fold, more growth factor after 1, 2, and 3 wk, respectively, than 4 mM D-glucose–treated cells (P < 0.01), and this was not affected by 1 μM peptide W (Figure 3) or concentrations of peptide up to 10 μM (data not shown). The amount of naturally “active” TGF-β1, detected by ELISA, accounted for less than 5% of total TGF-β1 in 4 or 30 mM D-glucose–treated cultures. The levels were not affected by the presence of the control peptide Y in either low- or high-glucose conditions. In contrast, concentrations of peptide W between 0.1 and 10 μM markedly reduced the level of “active” TGF-β1 in HMC cultures maintained in either 4 or 30 mM D-glucose. Maximum reduction was achieved with 1 μM peptide W (data not shown).

The levels of biologically active TGF-β1 produced in mesangial cell cultures maintained in high- and low-glucose conditions over 3 wk was investigated using the mink lung cell growth inhibition bioassay. Bioactive TGF-β1 levels increased by approximately 10-fold during the first 2 wk of exposure to 30 mM D-glucose and thereafter remained at the same high level (Figure 4). In contrast, bioactive TGF-β1 concentrations increased only marginally over 3 wk culture in 4.0 mM D-glucose conditions. Treatment of high-glucose cultures with peptide W reduced the level of bioactive TGF-β1 in these cultures at each time point over 3 wk to the same low levels found in 4 mM D-glucose cultures. In contrast, the control peptide Y had no effect on TGF-β1 bioactivity in high-glucose conditions.

Figure 1. Reverse transcription-PCR (RT-PCR) amplification of thrombospondin-1 (TSP-1), fibronectin (FN), plasminogen activator inhibitor-1 (PAI-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts in mesangial cells. Cells were incubated under the following conditions: 4 mM D-glucose (lane 1), 30 mM D-glucose (lane 2), 30 mM + TSP-1 mRNA antisense oligonucleotide (lane 3), 30 mM + TSP-1 control oligonucleotide (lane 4), 30 mM + TSP-1 blocking peptide W (lane 5), and 30 mM + control peptide Y (lane 6). RNA extraction and RT-PCR were performed as described after treatment periods of 1, 2, and 3 wk. Ten μl of each PCR reaction was electrophoresed through a 2% agarose gel with ethidium bromide.
cultures (Figure 4). The results indicate that all of the additional TGF-β1 bioactivity in high-glucose–treated cultures over baseline TGF-β1 bioactivity in 4 mM D-glucose cultures is dependent on TSP-1 activation of the growth factor.

Dependence of FN and PAI-1 Expression on Activation of TGF-β1 by TSP-1

We investigated next whether the increased expression of FN and PAI-1 in HMC cultures with prolonged exposure to high glucose is dependent on TSP-1 activation of TGF-β1. mRNA levels for FN and PAI-1 were increased in 30 mM compared with 4 mM D-glucose cultures at weeks 1, 2, and 3 (Figure 1, lanes 1 and 2 and Figures 5 and 6), confirming previous results (17,28). Treatment with peptide W reduced mRNA levels in 30 mM D-glucose, whereas control peptide Y had no significant effect (Figure 1, lanes 5 and 6). The protein levels of FN and PAI-1 were measured in the media of cultures by ELISA (Figures 5 and 6). In each case, the protein levels reflected the mRNA levels for FN and PAI-1, being elevated in high-glucose conditions and reduced to the basal levels of 4 mM D-glucose cultures when high-glucose cultures were treated with peptide W. They were unaffected by the control peptide Y. Thus, we conclude that the elevated expression of both FN and PAI-1 in mesangial cells exposed to high glucose is dependent on TGF-β1 activation by TSP-1.

Figure 2. The expression level of TSP-1 in mesangial cells exposed to high-glucose conditions in the presence or absence of W and Y peptides. Cells were incubated under the following conditions: 4 mM D-glucose (○), 30 mM D-glucose (■), 30 mM + TSP-1 blocking peptide W (□), and 30 mM + control peptide Y (●) for up to 3 wk. At the end of each week, the medium was changed to serum-free medium containing the same concentration of glucose and peptide. After an additional 24 h, media were collected and used to measure the secreted level of TSP-1 by enzyme-linked immunosorbent assay (ELISA) as described in the Materials and Methods section. The data represent mean ± SEM for three separate experiments with quadruplicate cultures for each condition in each experiment. TSP-1 levels are increased in 30 mM conditions compared with 4 mM conditions.

* P < 0.02; ** P < 0.001.

Figure 3. The level of the total transforming growth factor-β1 (TGF-β1) in the media of cultured mesangial cells exposed to high-glucose conditions under the 30 mM D-glucose conditions in the absence (○) or presence (■) of 1 µM TSP-1 blocking peptide for up to 3 wk. At the end of each week, the medium was changed to serum-free medium containing the same concentration of glucose and peptide. After an additional 24 h, media were collected, acidified, and used to measure the total level of TGF-β1 by ELISA as described in the Materials and Methods section. The data represent mean ± SEM for three separate experiments with quadruplicate cultures for each condition in each experiment. There were no significant differences between cultures maintained with or without the blocking peptide.

Figure 4. Effect of W and Y peptides on the level of biologically active TGF-β1 in the media of cultured mesangial cells exposed to high-glucose conditions. Cells were incubated in 4 mM (●) or 30 mM (■) D-glucose conditions or under 30 mM D-glucose conditions in the presence of 1.0 µM W (○) and Y (●) peptides for up to 3 wk. At the end of each week, the medium was changed to serum-free medium containing the same concentration of glucose and peptide. After an additional 24 h, media were collected and used to measure TGF-β1 bioactivity with the mink lung epithelial cell assay as described in the Materials and Methods section. The data represent mean ± SEM for three separate experiments with quadruplicate cultures for each condition in each experiment. Bioactive TGF-β1 is increased in 30 mM conditions compared with 4 mM conditions.

*, P < 0.01.
Effect of TSP-1 Deletion with an Antisense Oligonucleotide

Treatment of high-glucose cultures for 1 wk with a TSP-1 antisense oligonucleotide counteracted the overexpression of TSP-1 mRNA in these cells, whereas a randomized oligonucleotide used as a control had no effect (Figure 1, lanes 3 and 4). The level of TSP-1 protein in the medium of TSP-1 antisense oligonucleotide–treated cells decreased by 85% in high-glucose cultures and was 70% lower than in untreated 4.0 mM D-glucose cultures (Figure 7A). The total TGF-β1 level remained unchanged (Figure 7B), but levels of bioactive TGF-β1 fell dramatically in the 30 mM D-glucose–treated cells (Figure 7C). Moreover, treating HMC with the TSP-1 antisense oligonucleotide greatly reduced FN in mRNA levels in high-glucose cultures, whereas the control oligonucleotide had no effect (Figure 1, lanes 3 and 4). Protein FN levels were correspondingly reduced in the medium (2.5-fold) and cell layer of high-glucose cultures treated with antisense oligonucleotide but not with control oligonucleotide (Figure 7, D and E). Collectively, these results confirm a key role for TSP-1 activation of TGF-β1 and, downstream of this, for the increased expression and synthesis of FN in high-glucose conditions.

Discussion

TSP-1 is a multidomain protein that is synthesized by many cell types and exported to the extracellular matrix. It has roles in cell adhesion, cell movement, proliferation, and angiogenesis (20). Its potential ligands include integrins (37), type VII collagen, laminin α5β3 chain, fibrillin 2 (38), decorin (39), and TGF-β1 latency-associated peptide (23). Its interaction with the latent TGF-β1 complex induces activation of the growth factor in vitro (21–23), and it is a major activator of TGF-β1 in vivo (12).

Many studies show that TGF-1β is a profibrotic cytokine and an important mediator of kidney fibrosis (40,41). There is much evidence to implicate TGF-β1 as a factor that drives fibrosis in diabetic nephropathy (2,42). Increased levels of glomerular TGF-β1 mRNA in diabetic nephropathy biopsies correlate with the hyperglycemic status of the patient (43), and glomerular TGF-β1 protein is elevated in the disease (44).
Moreover, treatment of diabetic mice with anti–TGF-β1 antibodies attenuates renal expression of FN and type IV collagen and prevents glomerular hypertrophy (45). However, TGF-β1 is secreted in an inactive form (3), raising questions about the mechanism of its activation in the diabetic glomerulus. Previously, we showed that when human glomerular mesangial cells are exposed chronically, in vitro, to high concentrations of glucose, they continuously express increased levels of TGF-β1 mRNA and protein, of which at least a fraction is active, as detected by a specific ELISA (28). It seems unlikely that plasmin is involved in the activation process (8) because we also found that expression of PAI-1 is induced in these cells by high glucose, eliminating the activity of plasminogen activator in such conditions (17). However, we also found that TSP-1 expression is upregulated in mesangial cells that are exposed to high glucose (13), a finding confirmed subsequently by others (14,15). It is noteworthy that plasma levels of TSP-1 are significantly higher in patients who have diabetes mellitus with secondary complications, e.g., nephropathy, than in patients without such complications. The TSP-1 levels in the latter group are indistinguishable from the level in nondiabetic controls (46). Thus, TSP-1 seems to be a good candidate for activating latent TGF-β1 in glomerular mesangial cells that are exposed to high glucose in vitro or in the diabetic glomerulus in vivo.

Tada and Isogai (14) showed that when HMC cultures were treated with 1 to 5 µg/ml TSP-1, their active TGF-β1 levels increased by 1.3- to 2.1-fold without any concomitant increase in total TGF-β1 level. Moreover, the addition of TSP-1 to the cultures was accompanied by an increase in FN production, and this was blocked in the presence of an anti–TGF-β1 neutralizing antibody. This provided good evidence for activation of latent TGF-β1 produced by HMC by exogenous TSP-1. However, although these investigators showed that exposure to high-glucose conditions induced increased mesangial cell synthesis of TSP-1 (14), their experiments did not establish whether the endogenous protein was responsible for activating TGF-β1 produced in these conditions. The importance of our results is that they show that endogenous TSP-1 expression is upregulated in mesangial cells that are cultured in high glucose.
over 3 wk, simulating chronic hyperglycemia. Moreover, this TSP-1 activates all of the additional TGF-β1 produced by the cells in high glucose, generating chronically elevated levels of bioactive growth factor.

Although the greatest increase in mRNA and protein for TSP-1 occurred after 1 wk of exposure to high glucose (Figures 1 and 2), the production of bioactive TGF-β1 increased to reach plateau levels after 2 and 3 wk of exposure. However, as demonstrated by the blocking effect of peptide W, the increased bioactivity was totally dependent on TSP-1 activation of the growth factor at all times. Assuming that a similar situation occurs in vivo, prolonged periods of poor glycemic control in diabetics are likely to induce in mesangial cells levels of TSP-1 synthesis that are sufficient to activate the elevated levels of TGF-β1 produced during chronic hyperglycemia.

Inhibition of TSP-1 activation of TGF-β1 in high-glucose cultures by peptide W reduced the synthesis of both FN and PAI-1 to similar levels as were found in 4 mM D-glucose cultures, at all times up to 3 wk (Figures 6 and 7). Semiquantitative RT-PCR showed that this is due to normalization of gene transcription for FN and PAI-1 in high-glucose cultures treated with peptide W. Likewise, inhibiting the increase of TSP-1 transcripts in high-glucose cultures with an antisense oligonucleotide reduced FN synthesis to the same level as found in 4 mM D-glucose cultures. This is consistent with the notion that elevated FN transcription and synthesis in high glucose depends on TSP-1 activation of TGF-β1, which, after receptor binding, triggers intracellular signaling. The signaling pathway seems to involve activation of C-Jun N-terminal kinase in response to TGF-β1 and subsequent binding of C-Jun-ATF2 heterodimer to the FN promoter (47). However, this pathway, which is independent of smad 4, has not as yet been confirmed in mesangial cells.

Overall, our results indicate that endogenous TSP-1 plays a key role in activating TGF-β1 when HMC are exposed to high glucose in vitro. It is likely that TSP-1 has a similar role in hyperglycemic diabetic patients, promoting the mesangial fibrosis that is characteristic of diabetic nephropathy. Moreover, it is likely that TSP-1 is an intrinsic component of fibrosis in other regions of the kidney (27) and where fibrosis occurs in other organs. For example, liver homogenate from patients with congenital hepatic fibrosis have higher levels of TSP-1 and TGF-β1 than in normal livers (48).
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