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Glycosaminoglycan Therapy Prevents TGF-β1 Overexpression and Pathologic Changes in Renal Tissue of Long-Term Diabetic Rats

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Abstract. Chronic induction of the prosclerotic cytokine transforming growth factor β (TGF-β) has been implicated in the pathogenesis of diabetic nephropathy. In a rat model of diabetes mellitus-induced glomerulosclerosis, daily administration of a modified heparin (mH) glycosaminoglycan (GAG) preparation with low anticoagulant activity prevented glomerular and tubular matrix accumulation, as well as overexpression of TGF-β1 mRNA and albuminuria, without obvious side effects. To elucidate the molecular mechanisms of GAG/mH inhibitory actions on TGF-β1, studies using cultured mesangial cells were also performed. In these cells, high glucose-induced, dose-dependent increases in TGF-β1 mRNA and bioactive TGF-β protein expression were inhibited by GAG/mH treatment, whereas basal TGF-β1 expression was not affected. Both the heparin-derived GAG and dermatan sulfate were effective, indicating that the heparin chemical structure is not necessary for inhibitory activity. Coincubation of GAG with active TGF-β1 demonstrated no inhibitory effect on TGF-β1 bioactivity, excluding a neutralizing effect of GAG on TGF-β1 at the protein level. Furthermore, it was demonstrated that GAG inhibited phorbol myristate acetate-induced translocation of protein kinase C-α (PKC-α) and -β1 and activation of PKC-α, as well as high glucose-induced activation of PKC-α. These results suggest that GAG inhibit TGF-β1 overexpression at the transcriptional level, possibly via inhibition of high glucose-activated PKC. The findings indicate the potential of GAG therapy for the prevention of diabetic glomerulosclerosis by the inhibition of chronic disease-induced TGF-β1 mRNA overexpression.

Diabetic nephropathy is one of the leading causes of renal failure in western countries, where diabetic patients account for one-third of all patients undergoing hemodialysis. Progressive expansion of the mesangial matrix and thickening of the glomerular and tubular basement membranes, without signs of major cell proliferation, are hallmarks of human and experimental diabetic nephropathy (1–3). These lesions eventually lead to glomerular fibrosis, which is a central pathologic feature in many human acute and chronic kidney diseases; glomerular fibrosis progressively destroys the renal filtration unit and may finally cause renal failure.

Recent reports have indicated the involvement of cytokines and growth factors in the development of diabetic nephropathy and other nephropathies (4–7). In particular, transforming growth factor β (TGF-β) has been implicated in their pathogenesis (5–8). Latent TGF-β is the proform of the multifunctional cytokine TGF-β. Cells secrete latent TGF-β in a small or large form. The latter, containing a latent TGF-β-binding protein (LTBP), is secreted by glomerular cells (9). Among many diverse effects, TGF-β promotes the accumulation of extracellular matrix by increasing the synthesis of extracellular matrix components and reducing matrix degradation. Therefore, chronically enhanced induction of TGF-β, in response to injury or disease, is suggested to be the major mediator of progressive fibrosis in experimental animal and human diseases associated with sclerosis, such as diabetic nephropathy (10). The beneficial effects of inhibiting TGF-β action by injecting neutralizing antibodies have been demonstrated in various renal and nonrenal disease models and injuries, including diabetic nephropathy (11,12). Recent data convincingly demonstrated that inhibition of TGF-β activity by decorin, a proteoglycan that binds and neutralizes TGF-β via its core protein, resulted in the suppression of matrix accumulation in nephritic glomeruli (4,13,14). Similarly, administration of recombinant latency-associated peptide, an endogenous TGF-β-binding protein, reversed hepatic TGF-β1-induced effects (15). Furthermore, the use of antisense oligonucleotides against TGF-β1 mRNA

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blocked fibrosis in a mesangioproliferative nephritis model (16,17) and in cultured mesangial cells (18). Together, these data indicate that inhibition of TGF-β1 activity at the protein level or blockage at the translational level attenuates the fibrosis-promoting effects of TGF-β1.

We previously reported that chronic therapy with glycosaminoglycans (GAG) may prevent or cure experimental diabetic nephropathy (19,20). In this experimental model, daily treatment with low-molecular weight heparin or dermatan sulfate (DS) prevented structural changes in the glomerular basement membrane and changes in the albumin excretion rate, without affecting other parameters, *e.g.*, glycemia or GFR (19,20). Although a number of mechanisms have been proposed to explain the renoprotective effects of GAG, we wondered, in light of the hypothesized key role of TGF-β in diabetic nephropathy, whether these agents might be renoprotective because of their inhibition of the TGF-β cascade.

In this report, we demonstrate that chronic treatment with a modified heparin (mH) GAG preparation with low anticoagulant activity prevents the clinical and histologic signs of diabetic nephropathy in a well characterized rat model of diabetic glomerulosclerosis, mimicking most characteristics of human diabetic glomerulosclerosis, and we provide evidence that the disease-induced overexpression of TGF-β1 is suppressed by GAG/mH therapy. By studying the molecular mechanisms in an established cell culture system, we found that GAG/mH treatment of mesangial cells prevented the high glucose-mediated induction of TGF-β1 mRNA expression and the subsequent increase in TGF-β protein levels and bioactivity. Our data suggest the potential value of this treatment for diabetic patients with early signs of nephropathy and reveal that the renoprotective activity of heparin and GAG may be related to an anti-TGF-β effect.

Materials and Methods

Materials

Monoclonal anti-rat Thy-1.1 antibody was obtained from Camon (Wiesbaden, Germany), goat polyclonal antiserum against bovine and human type III collagen was purchased from Southern Biotechnology Associates (Birmingham, AL), and rabbit polyclonal antisera against human platelet-derived active TGF-β (LC 1–30) and LTBP (Ab39) were provided by Dr. K. Flanders and Dr. K. Miyazono (9), respectively. The digoxigenin RNA labeling kit and the gold-antidigoxigenin-antibody complex were from Boehringer (Mannheim, Germany), and the pGEM3Z plasmid was obtained from Promega (Madison, WI). The enzymes and all other biochemicals were from Sigma (Deisenhofen, Germany). Primers for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the *Taq*-specific antibody were purchased from Clontech (Palo Alto, CA). *Taq* polymerase and the RNA PCR kit were obtained from Perkin-Elmer (Foster City, CA). The GAG preparation used in the *in vivo* and *in vitro* studies was GAG/mH (10.4 kD) that had been chemically modified to yield a preparation with a sulfate/carboxylate ratio of 1.35, with very low anticoagulant activity (110 μg/ml was necessary to induce a doubling of the activated partial thromboplastin time) (19). In the *in vitro* study, a DS GAG preparation that had been characterized as described (20) was also used. GAG/DS is a nonanticoagulant GAG with a carbohydrate backbone different from that of heparin that was demonstrated, in a previous study, to be renoprotective in experimental diabetes mellitus (20). Both GAG/mH and GAG/DS were from Alfa Wassermann SpA (Bologna, Italy).

Study Design and Effect of GAG/mH Therapy on Albuminuria in Diabetic Rats

This section of the study involved the use of new data, particularly on TGF-β, from previous reports (19,21), to which readers are referred for more details on the protocol, metabolic control, renal function, and mortality rate. The study design is schematically depicted in Figure 1A. Diabetes mellitus was induced in 6-wk-old male Sprague-Dawley rats with streptozotocin. One-half of the diabetic rats and one-half of the control rats were treated subcutaneously with 15 mg/kg body wt per d GAG/mH, and the remaining one-half of each group were treated subcutaneously with 2 ml/kg per d saline solution, for 12 mo. None of the animals received insulin during the experiments.

Immunohistochemical Staining and Morphometric Analysis

Formaldehyde-fixed and paraffin-embedded tissue sections were deparaffinized and treated with phosphate-buffered saline containing 0.5% H_2O_2. Immunolocalization of collagen III was performed as described (22). Immunostaining for LTBP was performed similarly to the procedure used for α1 or α2 (IV) collagen staining (21), except that tissue sections were incubated with the rabbit LTBP-specific antiserum (1:80 dilution) at 4°C overnight; the same procedure was used for immunolocalization of TGF-β in tissue sections that had been pretreated with 1 mg/ml bovine hyaluronidase in 0.1 M sodium acetate (pH 5.5) containing 0.85% NaCl and incubated at 37°C for 30 min. Mesangial cells were identified by Thy-1.1 staining, as described (21). The avidin-biotin complexes were observed using 3,3'-diaminobenzidine, and the sections were counterstained with hematoxylin and evaluated by light microscopy. The sections from all experimental groups were incubated and developed simultaneously. The IgG fraction of rabbit serum was used as a negative control. Periodic acid-Schiff (PAS) and immunohistochemical staining was evaluated by

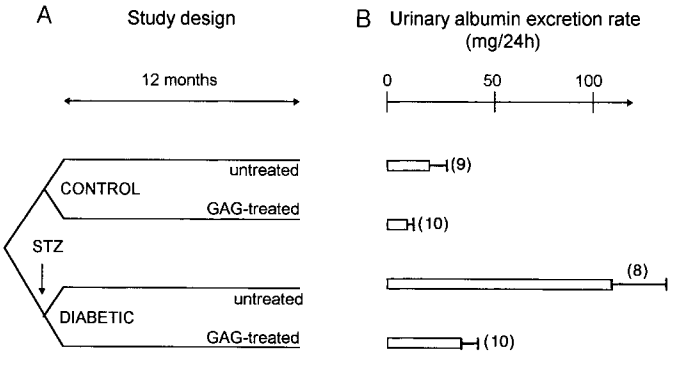

Figure 1. Experimental animal model, treatment protocol, and albumin excretion rate. (A) Diabetes mellitus was induced by the injection of streptozotocin (STZ), and control and diabetic animals were chronically treated subcutaneously with saline solution (untreated) or with glycosaminoglycan (GAG)/modified heparin (mH), respectively. Details are provided in the text. (B) After 12 mo, the 24-h urinary albumin excretion rate was determined for all animals. Data represent means ± SEM; the number of animals studied is shown in parentheses.

morphometric analysis, as described (21). The images of >20 systematically sampled glomeruli in each animal were digitized from the light microscope, using a video camera and a computer-based image analysis system (VIDAS System; Kontron, Eching, Germany). Glomerular cross-sections were displayed on the computer screen at a final magnification of ×400, and the area of the outlined polygon was measured with general-purpose image analysis software (VIDAS System; Kontron), using an interactive procedure. The positively stained mean glomerular areas were calculated for each animal.

Preparation of Riboprobes

cDNA fragments for TGF-β1 and GAPDH were obtained from mouse spleen and porcine mesangial cell RNA by reverse transcription (RT)-PCR amplification, according to the previously described protocol (18). The cDNA fragments were then cloned into the transcription vector pGEM3Z (Promega). The TGF-β1 probe (528 bp) is located near the 5'-terminus of the coding sequence (corresponding to amino acids 32 to 207). After linearization of the plasmid, single-stranded RNA probes complementary (antisense) or anticomplementary (sense, negative control) to cellular mRNA were obtained by run-off transcription using T7 or SP6 polymerase. For *in situ* hybridization, the probes were labeled with digoxigenin, using the digoxigenin RNA labeling kit (Boehringer); for the RNase protection assay, [α-^{32}P]UTP (800 Ci/mmol; Hartmann, Braunschweig, Germany) and the Maxiscript kit (Ambion, Heidelberg, Germany) were used.

In Situ Hybridization of TGF-β1 in Kidney Sections and Quantitative Analysis

Nonradioactive *in situ* hybridization and detection of the bound riboprobes by gold-silver enhancement was performed with digoxigenin-labeled sense and antisense TGF-β1 riboprobes, as described (21). Control experiments were performed with sense TGF-β1 and with hybridization buffer without probe. All *in situ* hybridization experiments, including the silver enhancement, were performed in parallel, to ensure identical reaction conditions. All animals were investigated at two per group in every assay session. The interassay variability was <10%. For quantitative analysis of the *in situ* hybridization data, sections from each animal were randomly selected, and grains were counted in glomerular and proximal tubular cells. Only cells for which the nucleus and cytoplasm were clearly attributable to that defined cell were evaluated. As many as 40 cells/glomerulus and 22 glomeruli/group were counted separately by two investigators. Both investigators were unaware of the treatments received by the different animals.

Mesangial Cell Culture

Porcine mesangial cells were isolated and characterized as described previously (23). For experiments, cells were grown in RPMI 1640 medium (10 to 30 mM glucose) or in medium with phorbol myristate acetate (PMA) (0.1 μM), without or with varying amounts of GAG, for the periods indicated. For experiments performed with elevated glucose concentrations, mannitol was added to the control medium. For TGF-β1 bioassays, the conditioned media were centrifuged for 5 min at 4°C. The pellet was discarded, and the supernatant was collected, divided into aliquots, and stored frozen at −20°C until the bioassay.

TGF-β1 Bioassay (Mink Lung Cell Proliferation Assay)

Mink lung epithelial cells (CCL; American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Seromed, Berlin, Germany) with 10% fetal calf serum (FCS) and were used for the TGF-β growth inhibition assay essentially as described by Itoh *et al.* (24). The cells were trypsinized, washed with DMEM, suspended in standard RPMI 1640 medium with 10% FCS, and seeded at 7×10^3 cells/0.2 ml in each well of 96-well dishes. After 3 h, mesangial cell supernatants were added. These supernatants had been extensively dialyzed against serum-free RPMI 1640 medium, for equilibration with the different glucose concentrations, and were supplemented with 1% FCS and 4 mM glutamine before being added to the mink lung cells. For determination of total (latent plus active) TGF-β levels, supernatants were heat-activated at 85°C for 5 min. After 22 h of incubation, the cells were pulsed for 2 h with 1.0 μCi [^{3}H]thymidine/well, washed twice with phosphate-buffered saline, trypsinized, collected using a microculture harvesting device, and counted. For each assay, a standard curve was established with 0.01 to 1 ng/ml human recombinant TGF-β1 (Life Technologies, Karlsruhe, Germany), with or without GAG/mH. Preliminary experiments were performed to exclude any direct inhibitory effects of GAG/mH on mink lung cell proliferation. Under the conditions used, GAG/mH only marginally inhibited proliferation (Table 1). To neutralize TGF-β activity, a rabbit anti-TGF-β antibody (R&D Systems, Minneapolis, MN) was added at a concentration of 10 μg/ml.

Determination of Mesangial Cell TGF-β1 mRNA Levels by RT-PCR

Mesangial cells were grown in 28-cm^2 dishes and washed, and total RNA was isolated as described (23). Two hundred nanograms of total RNA were used to synthesize cDNA with random priming, according to the instructions provided by the manufacturer for the RNA-PCR kit (Perkin-Elmer). Four microliters of the RT reaction mixture were used to amplify, in different tubes, TGF-β1 (161 bp) and the housekeeping gene GAPDH (983 bp). The PCR amplification was performed in a final volume of 50 μl, containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 U of *Taq* DNA polymerase from a freshly prepared 28:1 mixture of

Table 1. Effects of GAG preparations on the bioactivity of active TGF-β1[a]

Additions	Thymidine Incorporation (cpm × 10^3)	
	20×10^3 Cells/Well	40×10^3 Cells/Well
None	29.1 ± 5.2	91.5 ± 8.7
GAG/mH	31.2 ± 2.4	86.5 ± 8.6
GAG/DS	25.6 ± 4.2	81.7 ± 9.8
TGF-β1	4.1 ± 3.7[b]	31.5 ± 6.6[b]
TGF-β1 + GAG/mH	2.1 ± 1.9[b,c]	28.7 ± 3.9[b,c]
TGF-β1 + GAG/DS	1.8 ± 1.5[b,c]	27.9 ± 6.4[b,c]

[a] TGF-β1, transforming growth factor-β1; GAG, glycosaminoglycan; mH, modified heparin; DS, dermatan sulfate. TGF-β1 bioactivity was assessed using the mink lung cell assay, as described in Materials and Methods. Mink lung cells (20×10^3 or 40×10^3 cells/well) were incubated with growth medium, with or without 1 ng/ml recombinant active TGF-β1, in the presence or absence of 10 μg/ml GAG/mH or GAG/DS. After 24 h, the incorporated [^{3}H]thymidine (used as a marker of DNA synthesis) was measured. Values are the mean ± SEM of triplicate determinations.

[b] $P < 0.005$ *versus* no TGF-β1.

[c] $P > 0.05$, NS *versus* TGF-β1 without GAG.

Taq antibody and *Taq* polymerase, 0.4 μM primers, 50 mM KCl, and 10 mM Tris-HCl (pH 8). Kinetic analysis was performed using 24 to 32 amplification cycles (94°C for 45 s, 60°C for 45 s, and 72°C for 2 min), to obtain quantitative data. PCR fragments were separated by polyacrylamide gel electrophoresis and observed by silver staining. Quantification was performed by direct densitometric analysis of silver-stained bands (IBAS 2000; Kontron). Ratios of the OD values for the TGF-β1 (30 cycles) and GAPDH (24 cycles) PCR products were determined and compared.

Determination of TGF-β1 mRNA Levels by Northern Blotting and by RNase Protection Assays

For Northern analysis, RNA was prepared from cultured mesangial cells with the RNeasy kit (Qiagen, Hilden, Germany). Total RNA (20 μg) was separated on a formaldehyde-containing agarose gel and transferred to a nylon membrane. Digoxigenin-labeled RNA probes have been described. Hybridization was performed overnight at 68°C in 5× SSC, 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), 2% blocking reagent (Boehringer), and the filters were then washed with 2× SSC/0.1% SDS at room temperature and with 0.5× SSC/0.1% SDS at 68°C. For detection, a digoxigenin luminescence detection kit (Boehringer) was used. TGF-β1 mRNA levels were also assessed by RNase protection assays, as described previously (23).

Determination of Protein Kinase C (PKC) Isoform Translocation and PKC-α Activity in Mesangial Cells

Mesangial cells were stimulated with PMA for 0.5 h, in the presence or absence of 10 μg/ml GAG as indicated. Cellular fractions were prepared and Western blotting was performed as recently described (25). Isoform-specific antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The specificity was evaluated as described (25). For determination of PKC-α activity, mesangial cells were grown to subconfluency in plastic dishes in RPMI 1640 medium with 15% FCS, transferred to DMEM (without sodium pyruvate) with 0.5% FCS for synchronization, and cultured for 48 h, without or with 10 μg/ml GAG/mH, in normal- or high-glucose medium or in medium with PMA. Cells were then removed from culture dishes by scraping in test buffer [20 mM Tris-HCl, pH 7.4, 250 mM saccharose, 2 mM ethylenediaminetetraacetate, 8 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate, 20 mM mercaptoethanol, with protease inhibitor cocktail (Boehringer)], centrifuged at 2000 rpm for 3 min, and washed once with the same buffer. Packed cells were resuspended in 200 μl of test buffer, sonicated, and microfuged at 14,000 rpm for 20 min at 4°C. The supernatant was incubated overnight at 4°C with anti-PKC-α antibody (C-20 antiserum; Santa Cruz Biotechnology) bound to protein A-Sepharose. The immunocomplexes were washed three times with 50 mM Tris-HCl buffer (pH 7.5) containing the protease inhibitor cocktail. The anti-PKC-α-immunoprecipitated activity assay was performed for 10 min at 30°C in a 30-μl incubation mixture containing 50 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (specific activity, 2000 cpm/pmol; Amersham Pharmacia Biotech, Milan, Italy), 1 mM $CaCl_2$, 50 μg/ml phosphatidylserine, and 0.1 mg/ml myelin basic protein. Reactions were stopped by the addition of 1% SDS and 1% β-mercaptoethanol (final concentrations), followed by 5-min treatment at 100°C. Solubilized proteins were subjected to 0.1% SDS-15% polyacrylamide gel electrophoresis, dried, and counted for radioactivity in a Packard Instant Imager. The experiment was repeated three times.

Statistical Analyses

One-way ANOVA and ANOVA for repeated measures were used for morphometric analysis of *in situ* hybridization and immunohistochemical data and for the *in vitro* studies. Bonferroni's test for multiple comparisons was applied.

Results

Effects of Chronic GAG/mH Therapy on Metabolic (Laboratory) Parameters for Diabetic Rats

The effects of diabetes mellitus and GAG/mH treatment on weight, glycemia, plasma creatinine levels, GFR (creatinine clearance), and albuminuria at the end of the study were reported previously (19). Diabetes mellitus resulted in weight loss and hyperglycemia, whereas GFR remained unaffected. No significant effect of GAG/mH therapy on weight, glycemia, creatinine levels, or GFR was observed. After 12 mo of diabetes mellitus, albuminuria had increased sixfold (Figure 1B). Treatment with GAG/mH prevented the diabetes mellitus-induced increase in albuminuria, with little effect on control animals.

Effects of GAG/mH Therapy on Structural Changes and TGF-β Expression in the Kidneys of Diabetic Rats

Diabetic rats exhibited an accumulation of PAS-stained mesangial matrix (Figure 2A, b), which was not observed in the control animals (Figure 2A, a) or in the GAG/mH-treated animals (Figure 2A, c). Similarly, the tubular basement membranes stained more intensely in diabetic animals, whereas these structures appeared unchanged in GAG/mH-treated diabetic animals, compared with control animals. Morphometric quantification of the amount of PAS-positive glomerular matrix confirmed the aforementioned observations (Figure 2A, d). Because previous reports indicated that collagen III is virtually absent in normal glomeruli but is present at increased levels in diabetic glomeruli (22), we studied the effect of GAG/mH treatment on collagen III protein content. The immunohistochemical analysis of collagen III revealed positive staining in the glomerular mesangial matrix of untreated diabetic rats (Figure 2B, b); staining was significantly less extensive in normal glomeruli (Figure 2B, a) and in GAG/mH-treated diabetic rats (Figure 2B, c). In glomeruli from diabetic rats, collagen III staining was increased more than twofold, compared with both normal control animals ($P < 0.005$) and GAG/mH-treated diabetic rats ($P < 0.001$) (Figure 2B, d). A corresponding increase in staining in diabetes mellitus and its amelioration by GAG/mH treatment were also obvious in the peritubular matrix (Figure 2B). In earlier studies with the same animals, we demonstrated that glomerular and tubular α1 (IV) collagen expression and deposition are prevented by chronic GAG/mH treatment (19,21).

Although we observed clearly increased TGF-β1 protein staining in proximal tubular cells of diabetic animals (see below), insignificant TGF-β1 staining could be observed in the glomerular matrix or in glomerular cells in all animal groups studied. Therefore, glomerular LTBP expression was analyzed by immunohistochemical analysis. We observed a fourfold increase in LTBP-labeled glomerular cells in diabetic animals

(Figure 2C, b and d), whereas there was no significant difference between control and GAG/mH-treated rats (Figure 2C, a, c, and d). In addition to cellular LTBP staining, we occasionally observed extracellular deposition, which was enhanced in untreated diabetic animals. PAS, collagen III, and LTBP staining in GAG/mH-treated normal rats was unchanged, compared with control animals (data not shown).

In contrast to glomerular TGF-β1 protein deposition, long-term diabetes mellitus had an enormous stimulatory effect on TGF-β1 protein deposition in tubular epithelial cells (Figure 3). Because it appeared that whole cortical tubules, rather than single cells, were affected, we quantified the effect by counting TGF-β1-positive tubules. As shown in Figure 3, the large increase in the number of TGF-β1-positive tubules in animals with untreated diabetes mellitus (Figure 3B) was significantly reduced by GAG/mH treatment (Figure 3C). Furthermore, our data clearly demonstrated that no significant glomerular TGF-β1 staining could be observed for untreated animals,

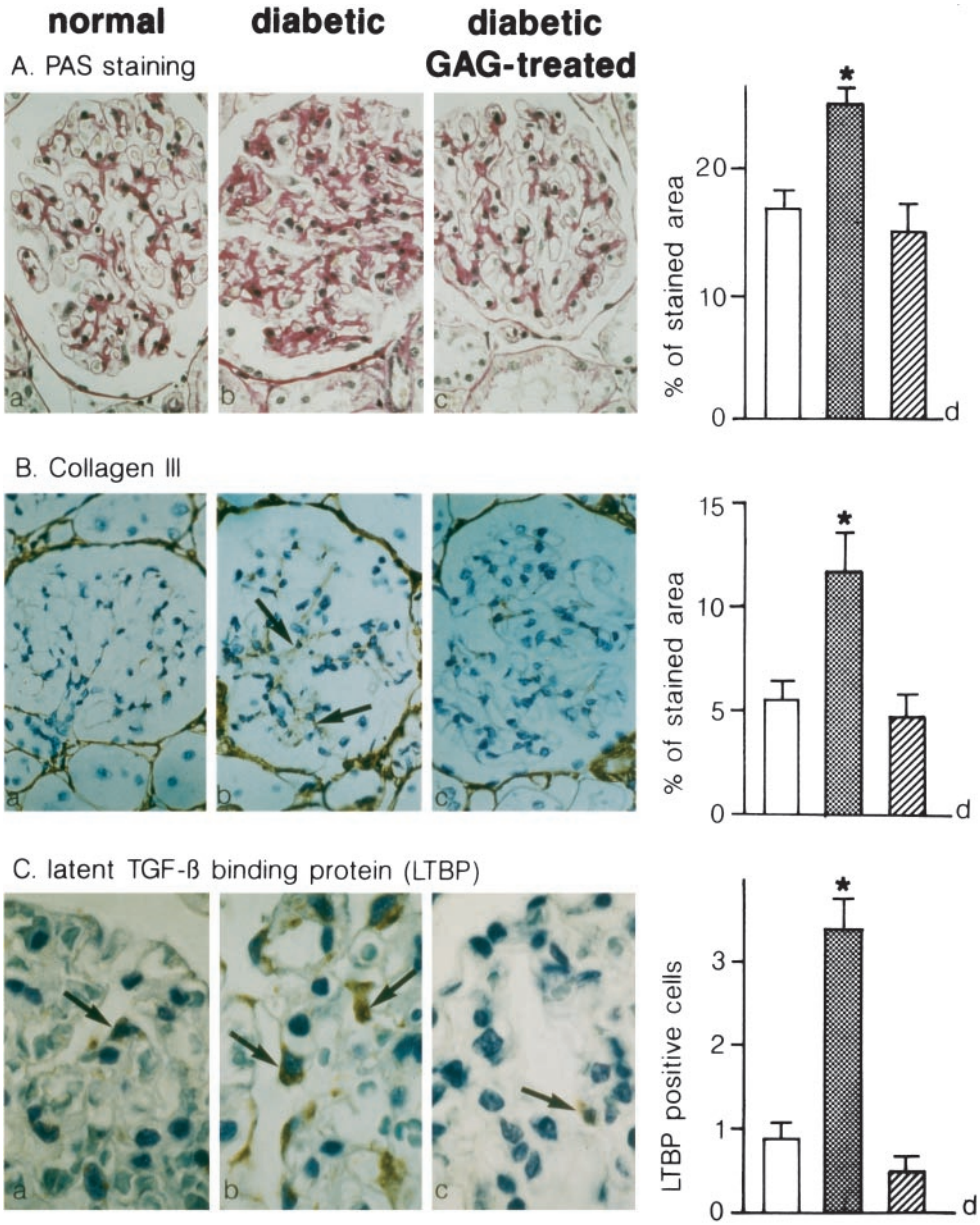

Figure 2. Histologic analysis of pathologic renal changes in normal and untreated or GAG/mH-treated long-term diabetic animals. (A) Representative photomicrographs of periodic acid Schiff (PAS)-stained renal sections from normal control animals (a), diabetic rats (b), and GAG/mH-treated diabetic animals (c). (B) Photomicrographs of renal sections from the same animals (a to c), immunohistochemically stained for collagen III. Arrows indicate marked accumulation of collagen III in the mesangium of diabetic animals. (C) Photomicrographs of renal sections from the same animals (a to c), stained for latent transforming growth factor β (TGF-β)-binding protein (LTBP). Prominent LTBP-positive cells are indicated by arrows. (d) Quantification of staining (percentage of stained area or number of positive cells per glomerular section, mean $\pm$ SEM) in glomeruli from normal (□), diabetic (▩), and GAG/mH-treated diabetic (▨) animals; 100 glomerular sections/animal were evaluated. *$P < 0.005$.

whereas intense TGF-β1 staining was observed in tubules in the same sections, confirming the activity of the TGF-β1-specific antibody used for staining. Despite several attempts to reveal TGF-β1 staining by treating the sections with urea, acid, or hyaluronidase or using antibodies from different sources, we could not detect glomerular TGF-β1 staining.

GAG/mH Prevention of Renal Overexpression of TGF-β1 mRNA in Long-Term Diabetic Rats

In situ hybridization of renal sections for determination of glomerular and tubular TGF-β1 expression is shown in Figure 4. Quantitative evaluation of glomerular TGF-β1 mRNA levels is presented in Figure 5. Control sections revealed a weak intracellular signal for TGF-β1 mRNA (Figure 4A), which was clearly greater than the sense control (Figures 4D and 5). In diabetic animals, a marked increase in TGF-β1 mRNA levels was observed in all glomerular cells (Figures 4B and 5), particularly mesangial cells (identified by Thy-1.1 staining) ($F = 5.89$, $P = 0.002$). In GAG/mH-treated diabetic rats, TGF-β1 mRNA expression appeared unchanged, compared with control animals (Figures 4C and 5) and GAG/mH-treated normal rats (Figure 5). Similarly, an increase in TGF-β1 mRNA expression in tubular epithelial cells of diabetic animals was observed (Figure 4F); this increase was prevented by GAG/mH therapy (Figure 4G). Quantification of tubular TGF-β1 mRNA expression confirmed TGF-β1 mRNA overexpression and its inhibition by GAG treatment (data not shown).

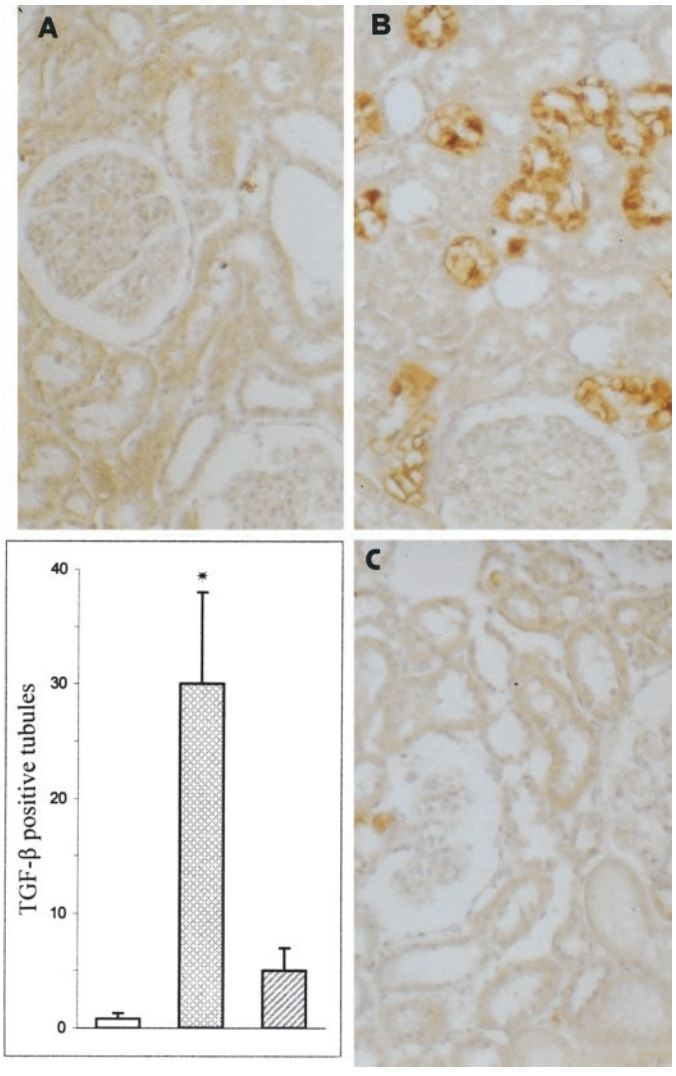

Figure 3. Immunohistologic analysis of renal TGF-β1 changes in normal and untreated or GAG/mH-treated long-term diabetic animals. Representative photographs of renal sections, stained with hematoxylin and anti-TGF-β1 antiserum, from normal control rats (A), diabetic animals (B), and GAG/mH-treated diabetic rats (C) are shown; 100 optical fields were evaluated. Magnification, ×25. In the histogram, quantification of staining (number of stained tubules, mean ± SEM) is expressed per optical field (□, control animals; ▩, diabetic rats; ▨, GAG/mH-treated diabetic rats). *$P < 0.0005$.

GAG/mH Inhibition of High Glucose-Induced Overexpression of TGF-β mRNA and Protein and TGF-β Bioactivity in Cultured Mesangial Cells

To elucidate the molecular mechanisms of the effects of GAG/mH treatment on diabetes mellitus-associated TGF-β1 mRNA induction, we studied the effects of GAG/mH on mesangial cells, the main target cells of diabetes mellitus in glomeruli. Preliminary studies using *in situ* hybridization indicated a dose-dependent, glucose-induced increase in TGF-β1 mRNA levels in cultured mesangial cells (data not shown). A dose-response analysis demonstrated that significant effects were obtained with 10 μg/ml GAG/mH. Therefore, this concentration was used in the *in vitro* experiments. To determine whether there was structural specificity of this GAG effect, GAG/DS was also studied. Quantitative analysis of the grains demonstrated a more than threefold increase (10 *versus* 30 mM glucose; $P < 0.005$), which was prevented by the addition of 10 μg/ml GAG/mH. In these preliminary experiments, we observed that GAG/DS, although less potent, was also active. Because data obtained by *in situ* hybridization are semiquantitative, we analyzed the effect by Northern blotting. As shown in Figure 6, both GAG compounds prevented the high glucose-induced TGF-β1 mRNA increase, with only little effect on basal levels.

To evaluate whether the effect of GAG/mH on TGF-β1 mRNA is translated to TGF-β1 protein, we determined cellular TGF-β1 mRNA and protein levels and TGF-β1 bioactivity in the supernatants of GAG/mH-treated mesangial cells stimulated with increased glucose concentrations. Determination of the TGF-β1 mRNA levels after RT-PCR amplification revealed a more than twofold, dose-dependent increase, which was prevented by 10 μg/ml GAG/mH ($F = 55.4$, $P = 0.000$) (Figure 7A). Furthermore, GAG/mH treatment attenuated high glucose-induced mesangial cell overproduction of TGF-β protein (Figure 7B) and the formation of bioactive TGF-β, without affecting basal levels (Figure 7C). To exclude a direct effect of GAG on TGF-β1 protein levels and bioactivity, 10 μg/ml GAG (*i.e.*, >1000-fold molar excess) was added to active recombinant TGF-β1 (1 ng/ml) and incubated with mink lung cells. TGF-β1 bioactivity was determined using the conventional mink lung cell proliferation assay. The results are presented in Table 1. Although the addition of 1 ng/ml TGF-β1

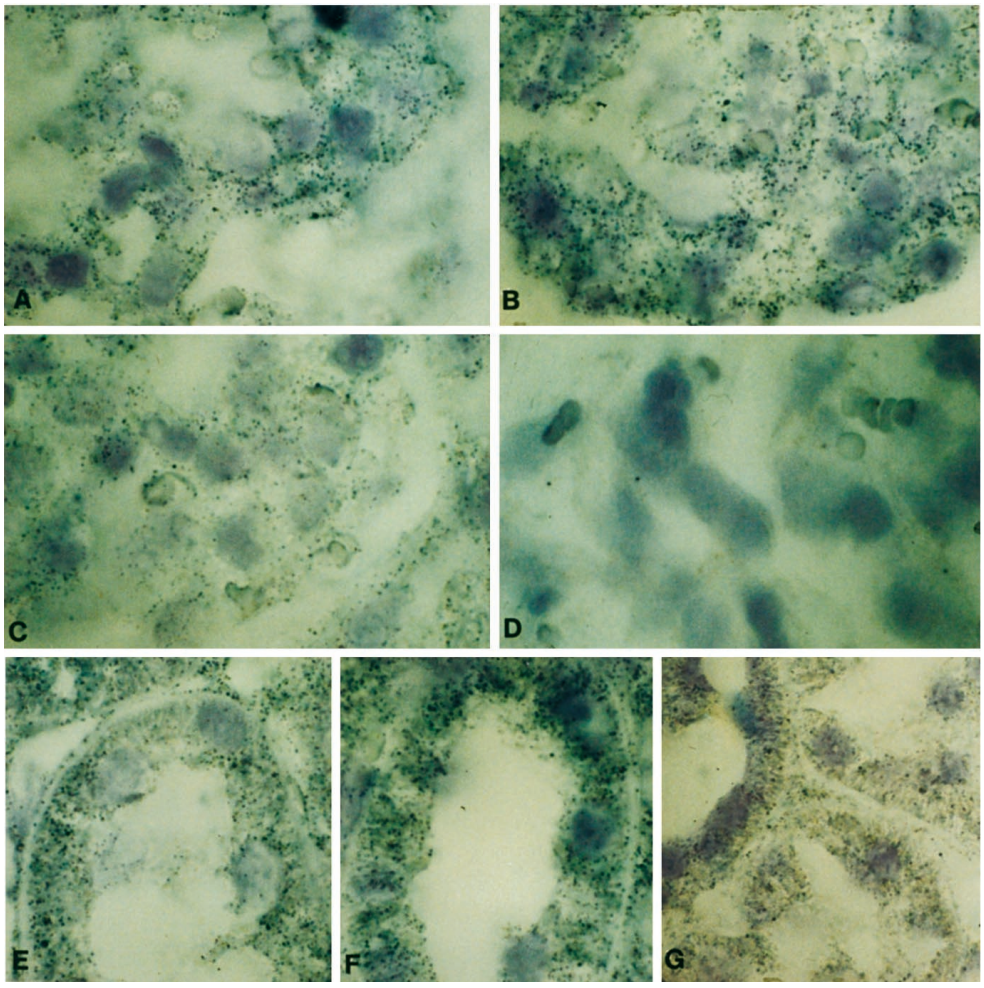

Figure 4. Renal overexpression of TGF-β1 mRNA in long-term diabetic rats and inhibition by GAG/mH therapy. *In situ* localization of TGF-β1 mRNA expression in glomeruli and tubules indicated that only a few positively labeled glomerular cells could be localized in normal animals (A) or GAG/mH-treated diabetic animals (C). The number of positive cells and the grain density/cell were increased in untreated diabetic animals (B). As a control for the hybridization procedure, renal tissue sections were hybridized with a sense riboprobe, which yielded essentially negative results (D). Significant and specific labeling was present in tubular epithelial cells from normal animals (E). The increase in intracellular grains in sections from diabetic animals (F) was prevented by GAG/mH treatment (G).

reduced DNA synthesis by 86 and 65% compared with controls under the experimental conditions used, the presence of either GAG preparation used in the animal studies had only marginal effects on TGF-β1 bioactivity. It is noteworthy that the addition of GAG had marginal effects on the proliferation of both control and TGF-β1-treated cells, and no TGF-β1-blocking effect, *i.e.*, abolishment of the antiproliferative effect of TGF-β1, was observed.

GAG/mH and GAG/DS Prevention of PMA-Induced Overexpression of TGF-β1 mRNA

To confirm the potency of GAG in preventing TGF-β1 mRNA overexpression, we used PMA, a known strong inducer of TGF-β1 expression. The addition of PMA increased mesangial cell TGF-β1 mRNA levels two- to three-fold after 9 to 12 h (Figure 8A). Coincubation with GAG/mH prevented PMA-induced TGF-β1 overexpression, without affecting basal expression. To demonstrate that the heparin structure and possible associated activities (*e.g.*, anticoagulation and growth factor binding) are not necessary for the inhibitory effect, a structurally unrelated GAG, *i.e.*, GAG/DS (see Materials and Methods), was used. The presence of increasing amounts of GAG/DS dose-dependently prevented the PMA-induced TGF-β1 mRNA increase, although GAG/DS was less efficient than GAG/mH (Figure 8B). Again, basal TGF-β1 mRNA levels were unchanged after the addition of GAG/DS.

Effects of GAG on Mesangial Cell PKC Translocation and PKC Activity

The inhibitory effect of GAG on PMA-induced TGF-β1 mRNA expression indicated the involvement of PKC. Therefore, the effects of GAG/mH on PMA-induced translocation of PKC-α, -β1, -δ, and -ϵ were studied by Western blotting. We observed that GAG/mH inhibited PKC-α and -β1 but not PKC-δ and -ϵ translocation to the mesangial cell plasma membrane. Data for PKC-α are presented in Figure 9A. To evaluate

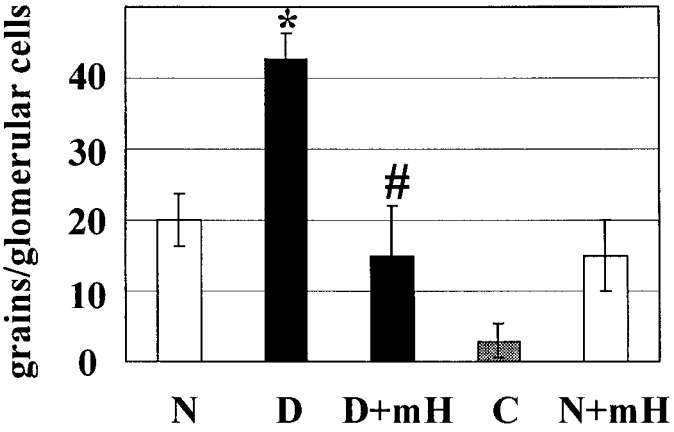

Figure 5. Quantitative analysis of the TGF-β1 mRNA signal in glomerular cells. For evaluation of the *in situ* hybridization experiments, the signals were quantified by counting the number of silver grains (see Materials and Methods). Significant levels of TGF-β1 mRNA were observed for normal control rats (N), with a significant increase for diabetic rats (D) ($t = 2.89$, $^*P < 0.05$). A significant reduction in TGF-β1 mRNA signal was observed for the GAG/mH-treated diabetic group (D+mH) ($t = 3.58$, $\#P < 0.05$). In normal rat cells hybridized with sense riboprobe (C), most cells exhibited fewer than five grains/cell; no effect was observed for GAG/mH-treated normal rats (N+mH) (not shown in Figure 4).

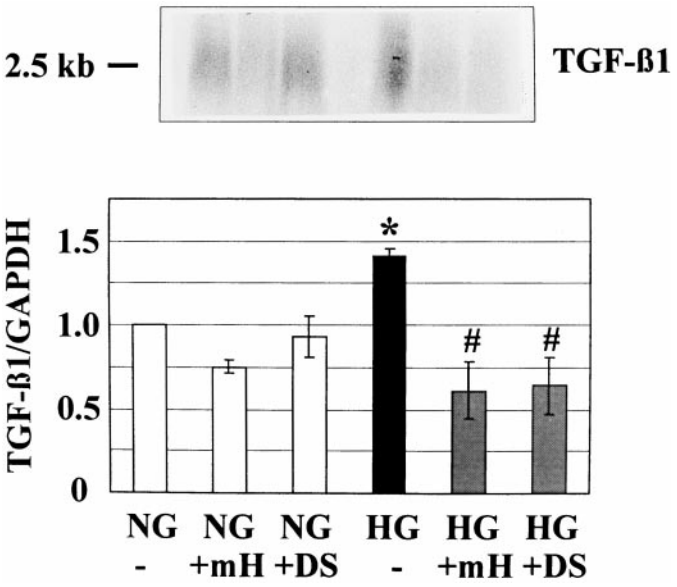

Figure 6. Effects of GAG/mH and GAG/dermatan sulfate (DS) treatment on hyperglycemia-induced TGF-β1 mRNA expression, as determined by Northern blotting. Mesangial cells were cultured for 24 h in normal-glucose (NG) or high-glucose (HG) medium, with or without 10 μg/ml GAG/mH or GAG/DS. Total RNA (20 μg) was separated on agarose-formaldehyde gels and transferred to nylon membranes for hybridization with digoxigenin-labeled TGF-β1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probes. Densities of the TGF-β1 mRNA signals were normalized to GAPDH mRNA signals. Results are expressed as the mean ± SEM of three separate experiments, compared with normal cells without GAG. $^*P < 0.01$ *versus* normal glucose, $\#P < 0.05$ *versus* high glucose without GAG.

whether the effect of GAG on PKC translocation reflects an effect on PKC activity, we studied the effect of GAG/mH on PKC-α activity. This particular isoform was studied more extensively because it has been reported that PKC-α expression is necessary for the inhibitory effect of heparin on cell proliferation (26). As shown in Figure 9B, GAG/mH inhibited PMA-induced PKC activity. Stimulation by a high glucose concentration was also studied, because this condition more closely resembles the pathophysiologic situation *in vivo*. The addition of GAG/mH completely prevented high glucose-induced PKC-α activation, whereas GAG/mH had no effect on basal PKC activity (Figure 9B).

Discussion

We and others recently observed that heparin, and more generally GAG, can prevent or cure experimental diabetic nephropathy (19–21,27,28). This study demonstrates that chronic GAG/mH treatment of long-term (12 mo) diabetic animals prevents albuminuria, glomerular increases in PAS staining, collagen III accumulation, and, as shown in earlier studies, enhanced collagen α1 (IV) accumulation and synthesis and basement membrane thickening (20,21). Together, these data demonstrate that the treatment ameliorates the functional and structural renal changes associated with diabetes mellitus.

To investigate the molecular mechanisms of the inhibitory effects of GAG, renal TGF-β1 expression was studied. In accordance with a previous study of short-term diabetic rats (29), increased glomerular and tubular expression of TGF-β1 mRNA was observed for our long-term diabetic animals. TGF-β1 protein levels were also increased in many cortical tubules of diabetic animals. However, we observed no significant glomerular TGF-β1 protein deposition in our normal and long-term diabetic rats, although some studies reported significant increases in glomerular TGF-β1 protein expression (5,29). It may well be that glomerular TGF-β1 protein expression is low after 12 mo of diabetes mellitus and is therefore indistinguishable from that of control animals. To circumvent this problem, we studied the accumulation of LTBP, a peptide whose strict functional and molecular association with TGF-β is known (9), and we observed significant increases in the glomeruli of diabetic animals, which were normalized by GAG/mH treatment (Figure 2C). The increased levels of LTBP-1 in diabetic rats could explain the increased TGF-β activation (30), which, notwithstanding low, immunohistochemically undetectable, glomerular levels of TGF-β protein, might produce increased local TGF-β bioactivity. It is noteworthy that the plasma half-life of active TGF-β1 is short; therefore, active TGF-β1 may be rapidly cleared from glomeruli, providing an explanation for our results with long-term diabetic rats. By using long exposure times, however, we could observe glomerular TGF-β1 mRNA expression, its increase in diabetes mellitus, and the inhibition by GAG/mH. Our finding that GAG/mH treatment prevented the diabetes mellitus-associated induction of glomerular and tubular TGF-β1 expression suggested that inhibition of TGF-β1 overexpression might be a

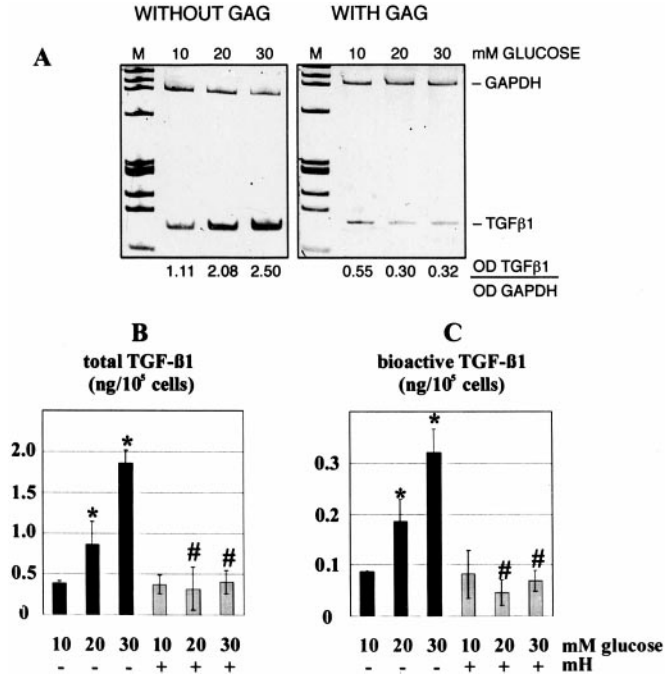

Figure 7. Dose-dependent increases in high glucose-induced TGF-β1 mRNA levels, total TGF-β protein production, and bioactive TGF-β formation and inhibition by GAG/mH therapy. (A) After reverse transcription of mRNA, samples were amplified by quantitative PCR with primers for TGF-β1 and GAPDH. The photographs show typical PCR products after 30 and 24 cycles, respectively. The numbers below each lane indicate the TGF-β1/GAPDH OD ratio obtained after densitometric scanning of the PCR products. Experiments were performed in triplicate. Increasing glucose concentrations induced statistically significant increases in the TGF-β1/GAPDH OD ratio (1.11 $\pm$ 0.06, 2.08 $\pm$ 0.02, and 2.50 $\pm$ 0.18, mean $\pm$ SEM, at 10, 20, and 30 mM glucose, respectively), although in the presence of GAG/mH there was no significant difference in TGF-β1 mRNA levels with increasing glucose concentrations (10, 20, and 30 mM glucose; F = 55.4, P = 0.000). (B and C) Mesangial cells were incubated with increasing glucose concentrations (10 to 30 mM) for 40 h, without or with 10 μg/ml GAG/mH. GAG/mH alone had no effect on mink lung cell proliferation (Table 1). Production of TGF-β protein (B) and bioactive TGF-β (C) was determined by bioassay (see Materials and Methods). *$P < 0.05$ *versus* 10 mM glucose; #$P < 0.05$ *versus* no GAG/mH addition (n = five dishes).

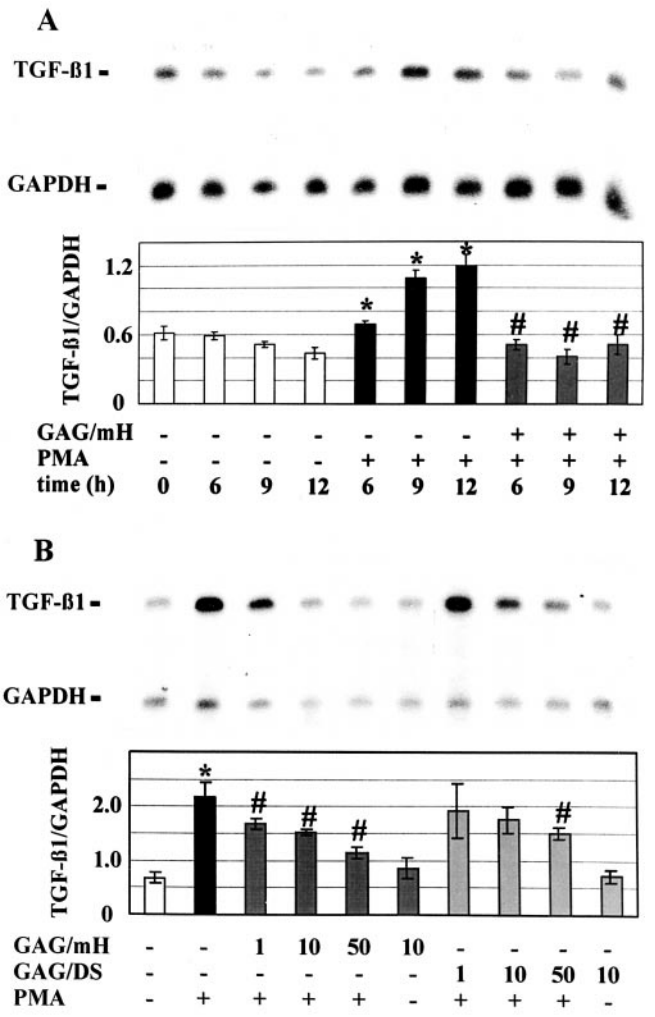

Figure 8. Dose-dependent inhibition of phorbol myristate acetate (PMA)-induced TGF-β1 mRNA overexpression by GAG/mH and GAG/DS in cultured mesangial cells. (A) Mesangial cells were grown in standard medium, stimulated with 0.1 μM PMA in the presence or absence of 10 μg/ml GAG/mH, and collected at the time points indicated, and RNase protection assays were performed as previously described (23). Labeled cRNA probes for both TGF-β1 and GAPDH were added to each sample. Densities of the TGF-β1 signals were normalized to corresponding GAPDH signals. Results are expressed as the mean $\pm$ SEM of three separate experiments. *$P < 0.05$ *versus* no PMA; #$P < 0.05$ *versus* no GAG/mH. A representative RNase protection assay is shown. Protected cRNA probes for TGF-β1 (307 bases) and GAPDH (211 bases) after RNase A/T1 digestion are indicated. (B) PMA-stimulated mesangial cells were treated for 12 h with increasing concentrations of GAG/mH or GAG/DS. Mesangial cell TGF-β1 mRNA levels were determined by RNase protection assays. For quantification, the autoradiograms from three different experiments were scanned. Graphs show the mean $\pm$ SEM of the TGF-β1/GAPDH ratios. *$P < 0.05$ *versus* the corresponding unstimulated values; #$P < 0.05$ for the difference between values for GAG-treated cells and the corresponding values for PMA-stimulated cells.

primary target of GAG/mH action. To investigate the molecular mechanisms of GAG/mH action in more detail, a well established *in vitro* model for the investigation of mesangial cell expansion, *i.e.*, mesangial cells cultured under high glucose conditions, was chosen. In these cells, we observed that GAG/mH, like TGF-β1 antisense oligonucleotides (18,23), suppressed high glucose-induced TGF-β1 mRNA and protein levels and bioactivity. Because the unequivocal demonstration of GAG inhibition of TGF-β1 mRNA overexpression is a crucial point for elucidation of the mode of action, we used four different methods to verify the inhibitory GAG effect on TGF-β1 mRNA levels. First, using *in situ* hybridization as a screening method, we could clearly demonstrate the inhibitory effect of GAG treatment on TGF-β1 mRNA levels in single cells. Second, the use of RT-PCR to assess the dose dependence of the glucose effect demonstrated the inhibition of increased TGF-β1 mRNA levels to basal levels. Although

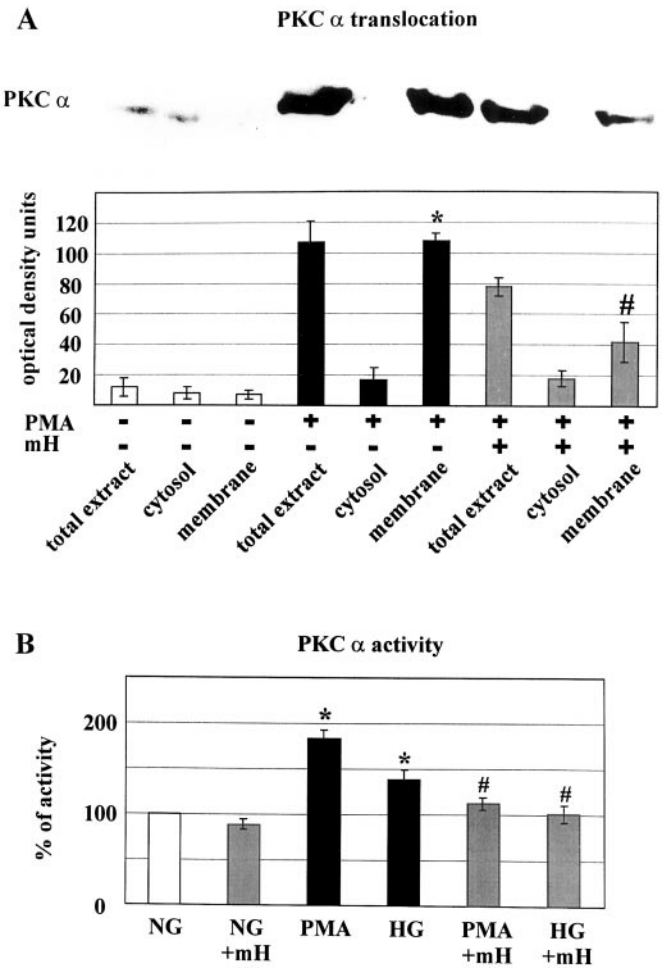

Figure 9. Effects of GAG/mH on PMA-stimulated translocation and PMA- and high glucose-stimulated protein kinase C-α (PKC-α) activity. (A) Translocation of the PKC-α isoform exposed to 0.5 μM PMA. Plasma membrane fractions were prepared and separated by sodium dodecyl sulfate-gel electrophoresis, and Western blotting was performed with PKC isoform-specific antibodies, as described in Materials and Methods. For quantification, the blots from three different experiments were scanned. The graphs show the mean ± SEM of PKC-α present in total cellular extracts and cytosolic and membrane fractions. *$P < 0.05$ *versus* the corresponding unstimulated values; #$P < 0.05$ for the difference between values for GAG-treated cells and the corresponding values for PMA-stimulated cells. (B) Mesangial cells were grown in normal-glucose (NG) or high-glucose (HG) medium or stimulated with PMA in the presence (+mH) or absence of GAG/mH. Mesangial cell PKC-α activity was determined as described in Materials and Methods. Data are given as the mean ± SEM of the percent variation with respect to the 100% control value (normal glucose). *$P < 0.05$ *versus* the corresponding unstimulated values; #$P < 0.05$ for the difference between values for GAG-treated cells and the corresponding values for stimulated cells.

these two methods are not absolutely quantitative procedures, we recently demonstrated the usefulness of RT-PCR for the estimation of TGF-β1 mRNA levels in mesangial cells and, in particular, their inhibition by GAG (31). Third, the widely used Northern blotting procedure confirmed the inhibitory effects of GAG/mH and GAG/DS on glucose-induced TGF-β1 overexpression. Fourth, the dose dependence of the inhibitory effects of both GAG compounds was demonstrated using the RNase protection assay. Together, these data confirm that GAG inhibit TGF-β function by preventing TGF-β1 mRNA overexpression, arguing against the fact that GAG inhibit TGF-β activation, *e.g.*, by inhibition of tissue plasminogen activator formation and subsequent inhibition of latent TGF-β activation to mature TGF-β (32).

However, the data could also be explained by GAG/mH inhibition of any TGF-β1 activity, resulting in interruption of the well known positive feedback mechanism and leading to reduced TGF-β1 mRNA levels (12). To exclude the possibility that GAG bind directly to TGF-β1 protein, thereby neutralizing TGF-β1 activity, we incubated active TGF-β1 with both GAG preparations. Evaluation of the bioactivity, using the conventional mink lung cell proliferation assay, after this coincubation demonstrated no inhibition of cellular TGF-β1 bioactivity, indicating that neither GAG interfered with TGF-β1 receptor binding or with intra- or postreceptor signaling, either by binding to active TGF-β1 and thus blocking binding to TGF-β receptors I and/or II or by interfering with intracellular processes, *e.g.*, with type II receptor serine-threonine kinase. Our results are supported by the recent observation that heparin had no inhibitory effect on TGF-β1 activity (33). In that report, the presence of 3 to 30 μg/ml heparin did not block the mesangial cell synthesis of fibronectin and collagen IV induced by the addition of 6 ng/ml bioactive TGF-β1 protein. Taken together, our results suggest that GAG exert their inhibitory activity on increased *de novo* TGF-β1 formation, without affecting basal TGF-β1 expression. These observations are in line with the finding that GAG/mH treatment has little effect on renal TGF-β1 mRNA levels in nondiabetic animals.

The effects of high glucose concentrations on mesangial matrix production have been attributed to PKC activation (8). Mesangial cell PKC activation by the potent activator PMA resulted in more than two- to fourfold increases in mesangial cell TGF-β1 mRNA levels, which were completely inhibited by treatment with both renoprotective GAG preparations (GAG/mH and GAG/DS) used in this cell culture study and in previous experimental animal studies (19,20). In unstimulated cells, addition of either GAG had no effect on basal TGF-β1 mRNA levels. Because these results indicated that the GAG may exert their inhibitory effects on TGF-β1 overexpression through inhibition of PKC activation, the effects of GAG on PKC isoform translocation were studied. We observed that PMA-stimulated translocation of PKC-α and -β1 was prevented by both GAG preparations used. We never detected PKC-β2 in mesangial cells, in line with previous results demonstrating that PKC-β2 is absent in glomerular cells (34). Although it is generally accepted that translocation of PKC is accompanied by activation, we verified our results for PKC-α by showing that GAG/mH prevented high glucose-induced activation of PKC-α. Our data are intriguing because they indicate that (*1*) GAG may inhibit PKC activation and (*2*) the inhibitory effects of GAG on PKC isoforms demonstrate some specificity (translocation of PKC-δ and -ϵ is not affected). In agreement with our results, it was recently demonstrated, in

peritoneal fibroblasts, that heparin inhibited the glucose-stimulated PKC activity in the membrane fraction (35). It is noteworthy that, as observed for TGF-β1 expression in animals and mesangial cells, GAG treatment had no effect on basal PKC translocation or activity.

Our observation that GAG treatment inhibits only the activated state (induced by PMA or high glucose concentrations) may be explained by the fact that TGF-β1 gene expression is regulated by at least two different transcription factors; one controls basal transcription, whereas the second, *i.e.*, activator protein-1 (AP-1), responds to PMA (36). Our findings are supported by reports on heparin inhibition of AP-1-mediated transactivation induced by PMA (37). Recent data from our laboratory demonstrated that AP-1-stimulated TGF-β1 gene activation may be prevented by GAG treatment (38).

A number of studies have confirmed the renoprotective action of heparins and GAG in experimental diabetic nephropathy (19–21,27,28,39), although one recent report, which confirmed some of the glomerular morphologic effects, revealed a possible harmful effect on renal function, *i.e.*, increased albuminuria (40). The reasons for these different results are probably the high heparin dosage and the type of commercially available heparin used by those authors, as discussed in detail (41). The fact that the structural heterogeneity of heparin and GAG suggests heterogeneous functions is confirmed by our studies showing that different GAG structures affect high glucose-induced mesangial cell TGF-β1 mRNA expression differently (42).

The strategies thus far proposed to inhibit the TGF-β loop are designed to act primarily on TGF-β protein (11–15) or mRNA ready to be translated (16,17). All strategies were effective in preventing fibrosis in kidney and other organs in different models, and no side effects were observed for the treated animals, indicating that short-term blockage of TGF-β is tolerable. However, total nonselective blockage of TGF-β1 synthesis may be deleterious, because TGF-β1 knockout mice die soon after birth as the result of an autoimmune-like disease (43), and loss of responsiveness to TGF-β because of receptor mutations may lead to malignant cell transformation (44). Because these therapies were tested only with short-term protocols, the long-term consequences of these approaches are unknown. Our long-term *in vivo* studies demonstrate that GAG treatment prevents diabetes mellitus-induced overexpression of TGF-β1, with no autoimmune-like disease, excess mortality rate, or occurrence of cancer (19,20). The results may be explained by the fact that GAG inhibit TGF-β1 overexpression without affecting basal TGF-β1 expression.

Heparin can have important side effects, including excessive anticoagulation, although we observed no hemorrhagic death among the animals treated with the modified GAG we used (19,20). Because it has been demonstrated that the renoprotection afforded by heparin-related drugs is not dependent on their anticoagulant activity or degree of sulfation (20,39,45), it should be possible to design GAG molecules or analogs to minimize the possible risks while maintaining the renoprotective activity.

Increased TGF-β1 expression has also been observed for human patients with diabetic nephropathy (5), suggesting that the results obtained in animals are relevant to human disease. Treatment with low-molecular weight heparin reduced albuminuria in both micro- and macroalbuminuric patients with insulin-dependent diabetes mellitus (IDDM) (46,47). Danaparoid, which is a mixture of sulfated GAG consisting mainly of heparan sulfate, decreased proteinuria in a small, double-blind, crossover study of patients with IDDM with albumin excretion rates greater than 300 mg/24 h (48). Sulodexide, which is a formulation composed of the two GAG (80% fast-moving heparin and 20% DS) that were active in preventing diabetic nephropathy in the experimental model described here, was reported to reduce albuminuria in patients with IDDM or non-insulin-dependent diabetes mellitus (49,50), and this effect lasted for several weeks after withdrawal (50). Interestingly, for diabetic patients, the decrease in albuminuria was also documented with oral administration of the GAG sulodexide (51,52), a finding that confirms the possibility of safely and easily administering these agents via the oral route, as previously demonstrated (53).

In conclusion, our results demonstrate that application of GAG/mH prevents (*1*) albuminuria, enhanced mesangial matrix expansion, and increased glomerular and tubular expression of TGF-β1 mRNA in long-term diabetic rats, (*2*) hyperglycemia- and PMA-induced TGF-β1 mRNA and protein overexpression in mesangial cells, and (*3*) PMA- and hyperglycemia-induced mesangial cell PKC translocation and activation. Together with previous results, these data indicate the possibility that GAG therapy may represent a new therapeutic option for the treatment and/or prevention of diabetic nephropathy and possibly other chronic TGF-β-related nephropathies and fibrotic processes.

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