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Renal Connective Tissue Growth Factor Induction in Experimental Diabetes Is Prevented by Aminoguanidine

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The aim of this study was to determine whether aminoguanidine (AG), an inhibitor of advanced glycation, prevents expression of the profibrotic cytokine, connective tissue growth factor (CTGF), as well as accumulation of the previously reported CTGF-dependent matrix protein, fibronectin, in a model of experimental diabetic nephropathy. Diabetic animals were randomly allocated into groups receiving 32 wk of AG or vehicle. Diabetic rats showed increases in CTGF mRNA and protein expression as well as in advanced glycation end-product (AGE) and fibronectin immunostaining, compared with nondiabetic rats. In the diabetic kidney, the increase in CTGF gene and protein expression as well as expression of the extracellular matrix protein fibronectin were prevented by AG. To further explore the relationship between AGEs and mesangial CTGF and fibronectin production, cultured human mesangial cells were exposed *in vitro* to soluble AGE-BSA and carboxymethyl lysine-BSA, and this led to induction of both CTGF and fibronectin. On the basis of our *in vitro* findings in mesangial cells linking AGEs to CTGF expression, the known prosclerotic effects of CTGF, and the ability of AG to attenuate mesangial expansion, it is postulated that the antifibrotic effects of AG in this animal model may be partially mediated by CTGF. (*Endocrinology* 143: 4907–4915, 2002)

ONE OF THE proposed mechanisms whereby diabetes causes nephropathy is through the effects of advanced glycation end-products (AGEs; Ref. 1). These end-products of nonenzymatic glycation of protein and lipid, which are increased in diabetic tissues, can induce the cellular formation of cytokines and growth factors, which may themselves then contribute to diabetic renal disease (2, 3). Furthermore, recent studies using a range of methods to inhibit AGE formation have documented that these agents suppress renal expression of various cytokines, including vascular endothelial growth factor (VEGF) and TGF β (4, 5).

Connective tissue growth factor (CTGF) is being increasingly recognized as a possible contributor to diabetic complications (6, 7). CTGF is a potent inducer of extracellular matrix (8) in various cell types, including mesangial cells (9, 10). Elevated extracellular D-glucose increases CTGF mRNA and protein levels in human and rat mesangial cells *in vitro* (10, 11). Furthermore, CTGF has been reported to be elevated in two different rodent models of diabetic nephropathy (10, 11), and in early (12) and end-stage diabetic nephropathy in humans (13). We recently reported that treatment of human dermal fibroblasts with soluble AGEs induces CTGF mRNA and protein *in vitro* (14), suggesting a possible link between AGE induction of CTGF and end-organ complications in diabetes. Furthermore, AGE-induced expression of the extracellular matrix protein, fibronectin, has been shown in *in vitro* studies to be CTGF dependent (15). Fibronectin has been reported to be increased in the diabetic kidney (16, 17), but its link to AGE accumulation and CTGF expression has not been examined previously.

The aims of the current study were, firstly, to determine whether observed increases in CTGF and fibronectin in the diabetic rodent kidney could be prevented by an inhibitor of AGE formation and whether changes in CTGF relate to pathological changes occurring in diabetic nephropathy. This issue was explored in the context of the known actions of aminoguanidine (AG) on the kidney, which include reduction in mesangial expansion (18, 19). AG, an inhibitor of AGE formation, has previously been reported by our group and others to confer renoprotection when administered after the development of diabetes (19, 20). We now report that AG prevents the induction of CTGF and fibronectin in a rodent model of diabetic nephropathy. Secondly, we explored whether there was a direct relationship between AGEs and mesangial CTGF and fibronectin production by performing *in vitro* studies involving exposure of cultured mesangial cell to carboxymethyl lysine (CML)-containing AGEs.

Materials and Methods

Study protocol 1

This study was carried out over 32 wk, because this duration of diabetes is associated with accumulation of AGEs in the kidney and with renal disease, as assessed by both functional and structural parameters (18). Male Sprague Dawley rats weighing 200–250 g were randomly allocated into control (n = 8) or diabetic groups. The rats were rendered diabetic by iv injection of streptozotocin at 55 mg/kg body weight after overnight fast. Only rats with blood glucose concentrations of at least 20 mmol/liter 1 wk after induction of diabetes were included in the study. Diabetic rats were then further randomized to receive either AG hydrogen carbonate (Fluka Chemica, Buchs, Switzerland) at 1 g/liter in drinking water (18; n = 9) or drinking water without added AG (n = 8).

Abbreviations: AG, Aminoguanidine; AGE, advanced glycation end-product; CML, carboxymethyl lysine; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor.

Throughout the study, all rats were given access to food *ad libitum* (GR2 rat cubes, Clark King and Co., Melbourne, Australia). Diabetic rats received 4 U of ultralente insulin (Ultratard HM, Novo Nordisk A/S, Bagsvaerd, Denmark) every second day to maintain body weight and improve survival over the study period. Rats were caged in groups of three. After 32 wk, rats were weighed and placed in metabolic cages (Iffa Credo, L'Abresele, France) for collection of 24-h urine specimens. Urinary albumin excretion was measured by RIA as previously described (19). The interassay coefficient of variation was 6.5% (n = 48) at a concentration of 180 ng/ml, with the detection limit of the assay being 31.2 ng/ml. Blood was collected concurrently from the tail vein for measurement of plasma glucose by the glucose oxidase technique (21) and for measurement of glycated hemoglobin by HPLC (Bio-Rad Laboratories, Inc., Richmond, CA; Ref. 22). Blood pressure was also measured every 8 wk by tail cuff plethysmography (23). At wk 32, rats were anesthetized with pentobarbital sodium (Nembutal, Bomac, Asquith, Australia), and the kidneys were removed for subsequent *in situ* hybridization and immunohistochemistry. All animal procedures were performed in accordance with ethics protocols determined by the National Health and Medical Research Council of Australia.

In situ hybridization for CTGF

Gene expression of CTGF was determined by *in situ* hybridization using a method described previously (24). Antisense riboprobes for rat CTGF were generated from a human CTGF cDNA (25). A ^{35}S-labeled RNA probe for CTGF was prepared with transcription kit (Promega Corp., Madison, WI). Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Sections (4 μm) were cut onto slides precoated with 3-aminopropyltriethoxysilane and incubated overnight at 37 C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in P buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA], and incubated in 125 μg/ml Pronase E in P buffer for 10 min at 37 C. Sections were then washed twice in 0.1 M sodium phosphate buffer (pH 7.2), postfixed in 4% paraformaldehyde for 10 min, washed twice in 0.1 M sodium phosphate buffer, then rinsed in milliQ water, dehydrated in 70% ethanol, and air dried. Hybridization buffer containing 2×10^4 cpm/μl riboprobe in 300 mM NaCl, 10 mM Tris HCl (pH 7.5), 10 mM Na_2HPO_4, 5 mM EDTA (pH 8.0), 50% deionized formamide, 20 mg/ml yeast RNA, 10% wt/vol dextran sulfate, and 100 mM dithiothreitol was heated to 85 C for 5 min. Twenty-five microliters of this solution were then added to each section and incubated at 60 C overnight in 50% formamide-humidified chambers. As controls for nonspecific signal, sections were incubated with sense riboprobe. Slides were washed in 2× SSC containing 50% formamide prewarmed to 50 C to remove coverslips. Sections were then washed in the above solution for 1 h with shaking at 55 C, rinsed three more times in Rnase buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 0.5 M NaCl], and then incubated with Rnase A (150 μg/ml) for 1 h at 37 C. Sections were later washed in 2× SSC for 45 min at 55 C, dehydrated in graded ethanol, air-dried, and exposed to BioMaxMR autoradiographic film for 3–5 d. After *in situ* hybridization, kidney sections were exposed to x-ray film and then quantitated with the Microcomputer Imaging Device system as previously described. Slides were then dipped in Amersham nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box with desiccant at 4 C for 21–28 d, brought to room temperature, then immersed in Kodak D19 developer (Eastman Kodak, Rochester, NY) washed in 15% acetic acid, and fixed in Ilford Hypan before staining with hematoxylin and eosin.

Immunohistochemistry for CTGF

Immunohistochemical staining was performed as previously described, according to a modified method (26). The CTGF polyclonal antibody used (27) was raised in a New Zealand white rabbit, against full-length purified recombinant human CTGF protein (28). In brief, 20-μm frozen kidney sections were cut on a cryostat at −20 C. Frozen sections were fixed with cold acetone, and endogenous peroxidase was inactivated using 0.1% hydrogen peroxide in PBS. The sections were incubated with protein-blocking agent, and endogenous nonspecific binding for biotin/avidin was blocked using a Biotin/Avidin Blocking Kit (Vector Laboratories, Inc., Burlingame, CA). The kidney sections were incubated for 1 h at room temperature with CTGF antibody at a dilution of 1:200. Biotinylated horse antimouse Ig (Vector Laboratories, Inc.) was used as a second antibody, followed by horseradish peroxidase-conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) substrate.

Immunohistochemistry for AGEs and fibronectin

Immunohistochemical staining for AGEs and fibronectin was performed as previously described (29). In brief, formalin-fixed, paraffin-embedded sections (4 μm) of kidney were rehydrated and treated with 1% H_2O_2/methanol followed by incubation in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, PA) for 20 min at room temperature. Sections were then sequentially incubated with an anti-AGE (30) or anti-fibronectin (DAKO Corp., Carpinteria, CA) antibody for 30 min at room temperature, washed in PBS, and incubated with biotinylated goat antirabbit Ig (DAKO Corp.). This was followed by another PBS wash and incubation with peroxidase-conjugated streptavidin (DAKO Corp.). Peroxidase localization was revealed using diaminobenzidine tetrahydrochloride as the chromogen. The AGE antibody used in this protocol has been described in detail previously (30). This antibody detects advanced glycated proteins including advanced glycated BSA and RNase. Studies to characterize the epitope of this antibody indicate that it detects CML-containing proteins but does not detect native BSA, native Rnase, or the AGE, pentosidine (31).

Histomorphometry

Quantification of immunostaining for CTGF, fibronectin, and AGEs were performed by calculation of the proportion of area occupied by the brown staining in the glomeruli using the Imaging Analysis System (AIS, Imaging Research, Inc. St. Catherines, Ontario, Canada) as described previously (26).

Study protocol 2

Human fetal mesangial cells were isolated after serial sieving from kidneys obtained from termination of pregnancies in the second trimester and were cultured as outgrowths from glomerular isolates. Their identity and purity as mesangial cells was established using previously published criteria (32). Cells were grown in RPMI 1640 medium containing 5 mM glucose (Cytosystems, New South Wales, Australia), and in 10% fetal calf serum in an atmosphere of 5% CO_2 and air. After an overnight incubation in serum-free RPMI medium, near-confluent cells grown in six-well plates between the second and fourth passage were treated with reagents in fresh serum-free medium.

AGE reagents were synthesized by previously described methods, using BSA as the protein substrate (15). BSA (Sigma; RIA grade, fraction V) at 10 mM was coincubated in sterile PBS with 0.5 M D-ribose for 10 d, under aerobic conditions at 37 C. To generate control BSA for comparison with AGE treatments, tubes were prepared with simultaneous incubations under the same conditions without the addition of the D-ribose. Additionally, in parallel preparations, AG at 100 mM, as an inhibitor of formation of products of nonenzymatic glycosylation, was added to the BSA and ribose, and this reagent is termed AG-BSA. In addition the AGE reagent termed N-ϵ carboxymethyl lysine-BSA (CML-BSA) was generated as previously reported (33, 34). In brief, 176 mg BSA was dissolved in sodium phosphate buffer to which 14.3 mg (0.155 M) glyoxylic acid (Sigma) was added, together with 19.8 mg (0.315 M) of the reducing agent, sodium cyanoborohydride (Sigma), all in a total reaction volume of 1 ml, followed by an incubation time of 24 h at 37 C. To generate control BSA for comparison, tubes were prepared with simultaneous incubations under the same conditions without the addition of the glyoxylic acid. All preparations were extensively dialyzed in PBS, using a low molecular weight cut-off membrane (Spectrapor 1, 6–8 kDa, Spectrum Industries, Los Angeles, CA), to remove free AG, reducing agent, and unincorporated sugars.

The AGE content in the preparations was assessed using fluorescence and ELISA. The fluorescence content, measured with a fluorescence spectrometer at 390-nm emission after a 450-nm excitation, in relative fluorescence units per milligram of BSA, was 50.5 ± 2.5 (mean ± SD) for control BSA, 187.9 ± 4.6 for AGE-BSA, and 55.7 ± 5.2 for AG-BSA. By competitive ELISA performed by Alteon Inc. (Ramsey, NJ), using a

synthetic N-ε CML analog as the standard (15), the CML content of the CML-based preparations (picomoles of CML per milligram of BSA ± 95% confidence interval) was 13 ± 1.4 for the CML-BSA, and it was undetectable (<1) for control BSA.

Cells were treated with soluble AGE-BSA, AG-BSA, or control BSA (each at 100 μg/ml BSA) for 72 h. In other experiments, cells were treated for 72 h with 0–600 μg/ml of CML-BSA or control-BSA. After cell treatments, total RNA was isolated and reverse transcribed, and CTGF mRNA and fibronectin mRNA were then measured by quantitative real-time RT-PCR, using specific probes and primers, all as previously described (15). The Δ-Δ method was used to calculate relative changes in mRNA levels of the same transcript, corrected for the housekeeping gene, human β-actin (35).

Statistics

Logarithmic transformation of urinary albumin data was performed to yield a normal distribution for this parameter. The specific contributions of diabetes or treatment in the animal and cell culture studies were assessed by ANOVA using the Statview II Program (Brainpower, Calabasas, CA) on a Macintosh G3 personal computer (Apple, Cupertino CA). Comparisons among groups were performed by Fisher's least significant difference method. A P value less than 0.05 was viewed as statistically significant. All data are shown as mean ± SEM unless otherwise specified.

Results

Study protocol 1

Biochemical and functional parameters. Diabetic rats had reduced weight gain and elevated plasma glucose and glycated hemoglobin levels (Table 1). No difference in any of these parameters was observed in diabetic rats treated with AG when compared with untreated diabetic animals. Systolic blood pressure was modestly elevated in diabetic rats, with no significant effect of AG on this parameter (Table 1).

To determine whether treatment of the diabetic rats with AG prevented AGE formation, immunohistochemistry for AGEs was performed. Compared with nondiabetic controls, AGEs were increased in glomeruli in diabetic rodent kidneys (Table 1). These increases were absent in diabetic rats treated with AG (Table 1). These results indicate that the AG used in the study blocked AGE formation in the renal glomerulus of treated diabetic rats.

CTGF expression. Assessment of CTGF gene expression was initially performed by quantitation of autoradiographs of whole kidney cross-sections after *in situ* hybridization with a radiolabeled CTGF riboprobe. Representative cross-sections in Fig. 1 demonstrate that the CTGF mRNA signal in diabetic rats (Fig. 1B) was more intense and frequent than in nondiabetic controls (Fig. 1A). The signal in diabetic rats was confined predominantly to punctate areas in the renal cortex. The diabetic animals treated with AG (Fig. 1C) had lower CTGF mRNA signal compared with diabetic animals not treated with AG, at a level similar to that seen in the nondiabetic controls. Hybridization using a negative control

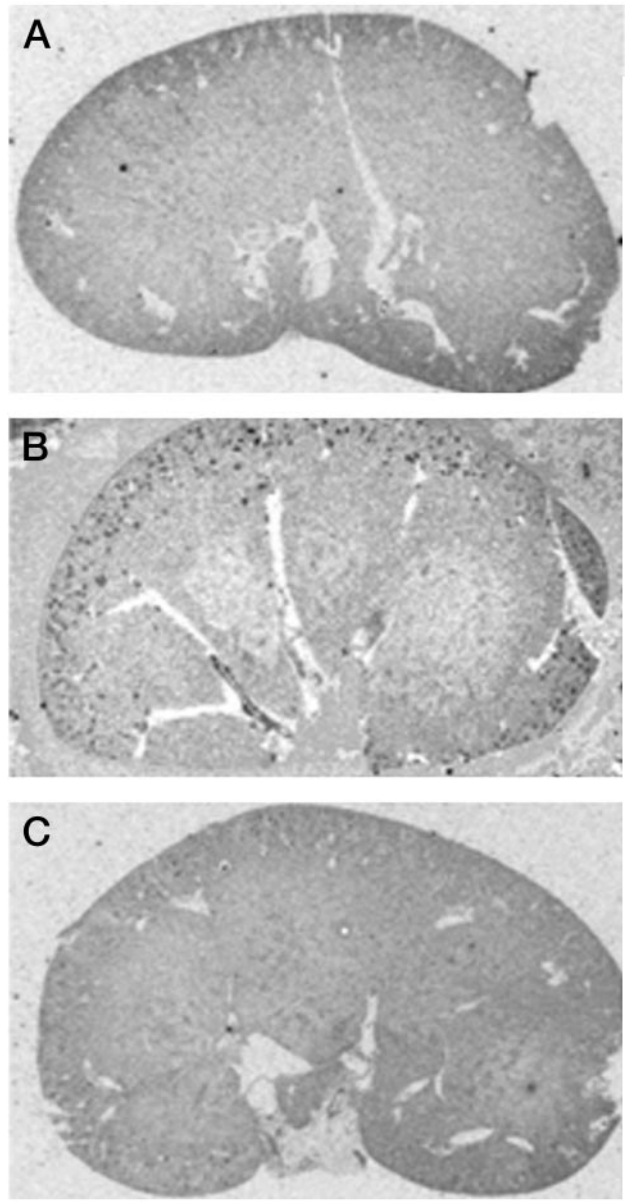

FIG. 1. Effect of diabetes and diabetes treated with AG on renal CTGF mRNA in cross-sections of whole kidney at autoradiography. Punctate signal of the hybridized ^{35}S-labeled CTGF riboprobe is shown. A, Nondiabetic control; B, diabetic; C, diabetic + AG.

TABLE 1. Characteristics of nondiabetic control group, diabetic animals, and diabetic animals treated with AG

Group	n	Body weight (g)	SBP (mm Hg)	Plasma glucose (mM)	HbA_{1c} (%)	AER (mg/24 h)	AGEs (AU)
Controls	8	729 ± 28	116 ± 3	6.3 ± 0.2	4.9 ± 0.2	0.8 ×/÷ 1.1	7.7 ± 0.6
Diabetic	8	347 ± 21[a]	141 ± 8[a]	21.2 ± 1.6[a]	10.2 ± 0.4[a]	13.2 ×/÷ 1.3[a]	13.9 ± 1.3[a]
Diabetic + AG	9	341 ± 12[a]	137 ± 5[a]	20.1 ± 1.9[a]	8.9 ± 0.8	3.0 ×/÷ 1.2[b]	6.1 ± 0.2[c]

Data are shown as mean ± SEM except for albuminuria which are shown as geometric means ×/÷ tolerance factors. SBP, Systolic blood pressure; HbA_{1c}, glycated hemoglobin; AER, albuminuria.

[a] $P < 0.01$ *vs.* control; [b] $P < 0.05$; [c] $P < 0.01$ *vs.* diabetic.

sense riboprobe for CTGF did not show any specific signal (data not shown).

The differences in CTGF mRNA in whole kidney were quantitated in comparable kidney sections by the assessment of the percentage area covered by the CTGF mRNA signal at autoradiography, after *in situ* hybridization with the radiolabeled CTGF antisense riboprobe. As shown in Fig. 2, CTGF mRNA levels in diabetic rats were higher than in nondiabetic controls. The diabetic animals treated with AG had reduced CTGF gene expression when compared with diabetic animals not treated with AG. This level of CTGF gene expression was not significantly different from that seen in control kidney sections.

To determine the structures and cell types that expressed CTGF mRNA, higher power studies were performed after the counterstaining of these kidney sections. By *in situ* hybridization, when compared with nondiabetic control animals (Fig. 3A), CTGF gene expression in untreated diabetic animals was increased mainly in glomeruli (Fig. 3B). The cell types expressing CTGF were spindle shaped, characteristic of mesangial cells. AG treatment of diabetic rats (Fig. 3C) prevented the induction of CTGF gene expression when compared with untreated diabetic rats.

Then, to determine whether the observed differences in CTGF mRNA in the renal cortices were associated with parallel changes in CTGF protein, immunohistochemistry for CTGF was performed. Nondiabetic rat kidneys showed immunostaining for CTGF in glomeruli (Fig. 4A) and in the interstitium of the renal cortex. In comparison with the controls, immunostaining for CTGF was more intense in diabetic rat kidney (9.9 ± 1.3 *vs.* 4.1 ± 0.7% in control). CTGF immunoreactivity was greater in both the glomerulus and the renal interstitium, including areas adjacent to proximal tubules. These patterns of staining were not seen when the same titer of control normal rabbit serum was used in place of the CTGF antiserum. The diabetic rats treated with AG (Fig. 4C) had reduced levels of CTGF protein expression (5.9 ± 1.0%) compared with untreated diabetic animals and a similar level to that observed in control animals (4.1 ± 0.7%).

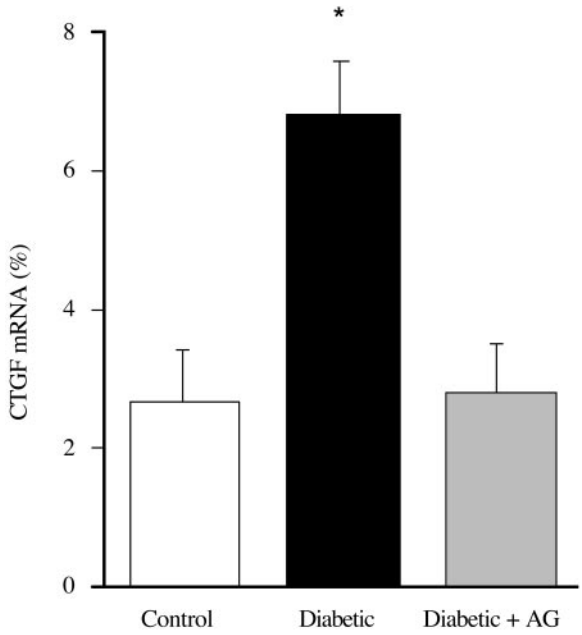

FIG. 2. Quantification of renal CTGF mRNA (percentage renal cortex area) is shown as mean ± SEM. *, $P < 0.05$ *vs.* the nondiabetic control group, and *vs.* AG-treated diabetic animals.

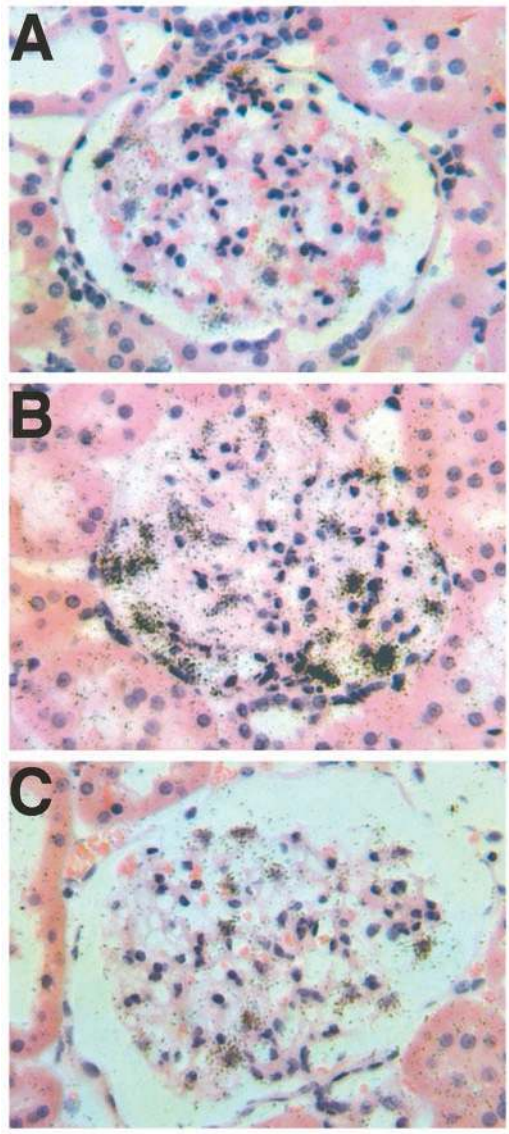

FIG. 3. Effect of diabetes and diabetes treated with AG on renal CTGF mRNA under high power. A, Nondiabetic control; B, diabetic; C, diabetic + AG. Magnification, ×400.

Immunostaining for fibronectin revealed protein predominantly in the glomeruli (Fig. 5A), with minimal expression in the tubulointerstitium in the control kidney. Glomerular fibronectin protein expression was increased in diabetic rats (12.0 ± 0.9% *vs.* control, 8.5 ± 1.0%). Treatment with AG prevented the diabetes-related increase in glomerular fibronectin expression (8.3 ± 1.9%).

Study protocol 2

Because the rodent data presented in the current work suggest that AGEs induce CTGF and fibronectin in mes-

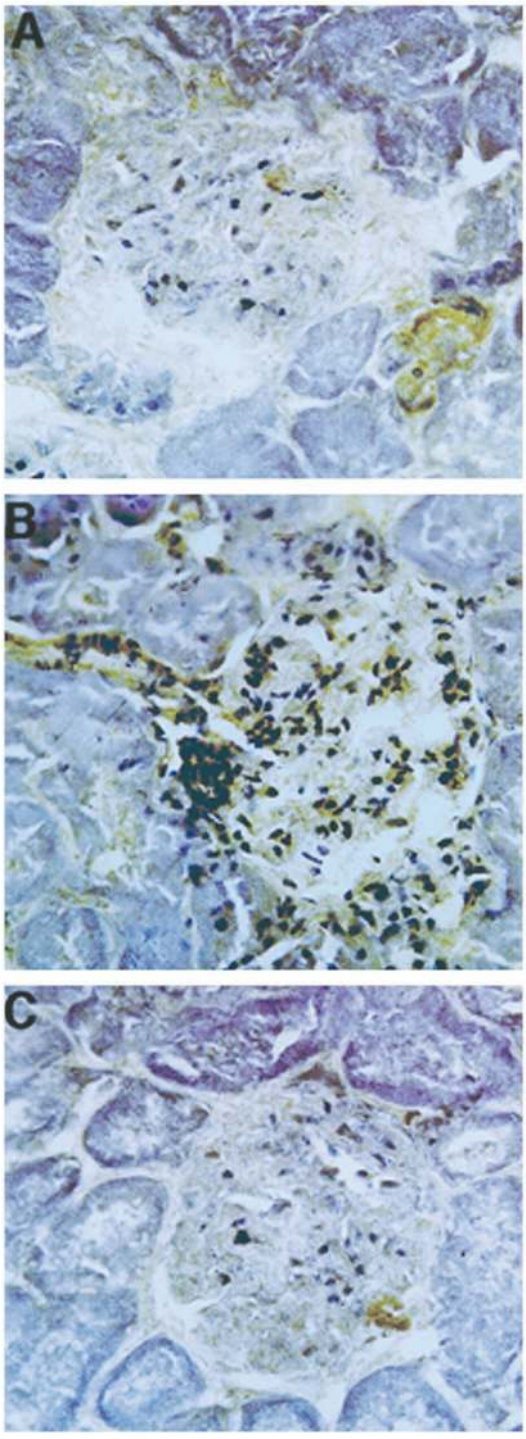

FIG. 4. Effect of diabetes and diabetes treated with AG on renal CTGF protein. Immunohistochemical staining for CTGF in the kidney is shown. A, Nondiabetic control; B, diabetic; C, diabetic + AG. Magnification, ×400.

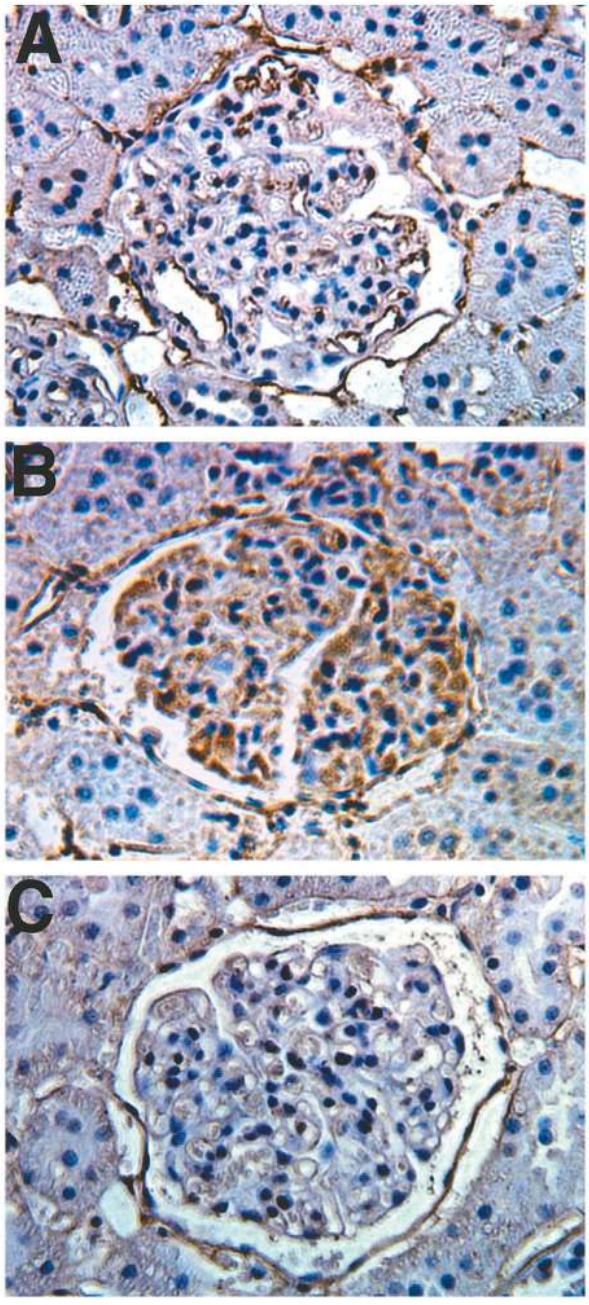

FIG. 5. Immunohistochemical staining for fibronectin in the kidney. A, Nondiabetic control; B, diabetic; C, diabetic + AG. Magnification, ×400.

angial cells, we then tested whether such effects could be observed *in vitro*. Treatment of human fetal mesangial cells in confluent monolayer with the AGEs reagent AGE-BSA caused an up-regulation of both CTGF mRNA and fibronectin mRNA, compared with control-BSA or AG-BSA (Fig. 6A). Because the AGE adduct CML is increased in the rodent diabetic kidney *in vivo* and is prevented by AG treatment of these diabetic animals (Table 1), we then tested whether the specific AGE reagent CML-BSA regulated CTGF and fibronectin in the mesangial cells. Treatment of these cells with CML-BSA caused a dose-dependent induction of CTGF mRNA and fibronectin mRNA (Fig. 6B). These data show that AGEs induce CTGF mRNA and fibronectin mRNA in human mesangial cells *in vitro*, and they support the *in vivo* data implicating AGEs in these observed effects.

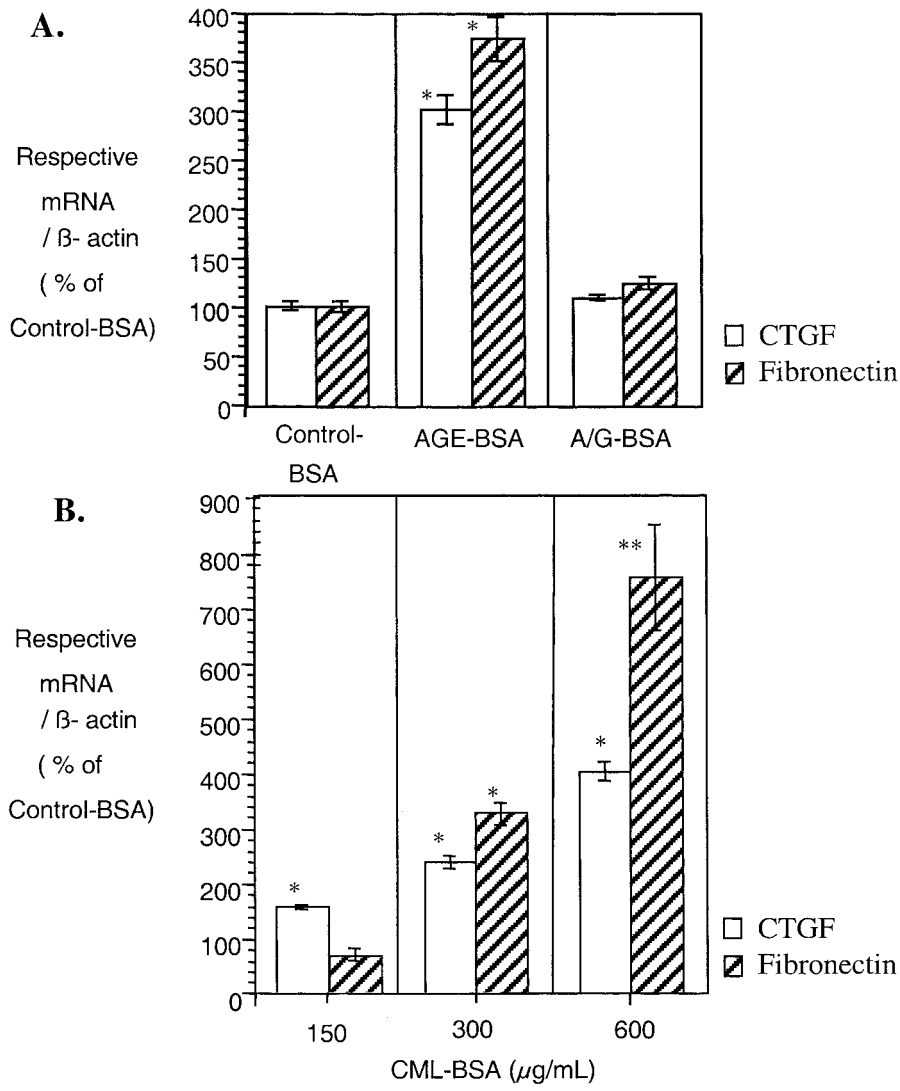

FIG. 6. Treatment of human mesangial cells *in vitro* with AGE-BSA or CML-BSA induces CTGF and fibronectin mRNAs. A, Human mesangial cells in confluent monolayer were treated with control-BSA, AGE-BSA, or AG BSA, each at 100 μg/ml. The induction of CTGF mRNA (*white columns*) and fibronectin mRNA (*shaded columns*) at 72 h, compared with control BSA, were measured by quantitative real-time RT-PCR. The data are shown as means ± SD and are representative of three independent experiments. *, $P < 0.05$ *vs.* control-BSA or AG-BSA for the respective mRNA. B, Human mesangial cells were treated with increasing concentrations of the AGE reagent, CML-BSA, or with increasing control-BSA, each for 72 h. The mRNA levels of CTGF (*white columns*) and fibronectin (*shaded columns*) were measured by real-time quantitative RT-PCR. The data are shown as means ± SD for CML-BSA compared with the same concentration of control-BSA and are representative of two independent experiments. *, $P < 0.05$; and **, $P < 0.01$ *vs.* the same concentration of control BSA for the respective species of mRNA.

Discussion

This study demonstrates that CTGF is induced by diabetes in these rodents. The major finding is that AG treatment of diabetic rats prevents the induction of CTGF at the level of mRNA and protein. We have previously observed that AG-treated diabetic rats have less mesangial expansion (18, 19), which occurs in diabetes predominantly as a result of extracellular matrix accumulation (36). AG treatment prevented in parallel both AGE and CTGF immunostaining in the renal cortices of the diabetic rats. Because the predominant increase in CTGF expression and protein was glomerular in origin, it can be postulated that one mechanism whereby AG is preventing glomerular damage in diabetes is via inhibition of the profibrotic cytokine, CTGF.

There is strong circumstantial evidence to implicate CTGF in contributing to diabetic renal complications. CTGF is known to be induced in renal mesangial cells *in vitro* by high glucose (10, 11), and renal CTGF is increased *in vivo* in experimental (10, 11) and human diabetes (12, 13). A recent study in humans showed that increases in CTGF mRNA levels in diabetic glomeruli correlate with early increases in glomerular collagen type IV expression and with microalbuminuria (12). Moreover, factors implicated in diabetic complications, such as oxidative stress (37), cyclic mechanical strain (10), TGF-β1 (9), and VEGF (38) induce CTGF in a variety of cell types. Of particular relevance to this study is our previous *in vitro* finding demonstrating that AGEs induce CTGF expression, albeit in a nonrenal context (14). We have now presented novel data in mesangial cells showing that both CTGF and fibronectin mRNA are induced by AGE treatment of cells.

The *in vivo* studies involved an immunohistochemical approach to assess renal AGE accumulation using an antibody that predominantly detects CML-containing proteins. Therefore, to further assess whether these AGEs *per se* are linked to CTGF and fibronectin expression, *in vitro* studies were performed that showed that CML-containing AGEs induce both CTGF and fibronectin. These complementary *in vitro* and *in vivo* studies emphasize the likelihood that CTGF plays a central role in diabetes and AGE-associated renal extracellular matrix accumulation, which ultimately results in mesangial expansion. Further evi-

dence linking CTGF expression to renal disease is suggested by a range of studies performed using various potentially renoprotective treatments. ACE inhibitors, angiotensin II antagonists, and statins have been reported to reduce CTGF expression either *in vitro* or in experimental studies, albeit not in a diabetic milieu (39, 40). Clearly, specific methods to inhibit CTGF induction and/or bioactivity *in vivo* are required before causality for CTGF in diabetic renal disease can be definitively determined.

The glomerulus, specifically the mesangial cell, is the main source of CTGF expression in the diabetic kidney (41). Our study showed that the increases in renal CTGF gene expression in diabetes are predominantly but not exclusively localized to the mesangial cells in the glomerulus, and this is consistent with previous reports in diabetes, in which glomeruli were the main renal source of CTGF expression (10). As previously reported (10), CTGF gene expression in nondiabetic animals was very low throughout the kidney in the present study. CTGF produced in mesangial cells may have an autocrine and/or paracrine role. In human renal mesangial cells *in vitro*, soluble recombinant human CTGF potently induced collagens and fibronectin (11), potentially contributing to mesangial matrix expansion. Although CTGF may exert its bioactivity through binding classes of integrin (42, 43) and/or through MAPK pathway activation (44), its exact cellular mechanism of action in mesangial cells is yet to be defined.

TGF-β1 is a potent inducer of CTGF in mesangial cells (9), and the rat and human CTGF promoters contain a unique TGF-β response element, conserved across species (45). In *in vitro* studies in rodent mesangial cells, CTGF was shown to mediate the TGF-β1-induced increase in fibronectin (9), and, in other cell types, CTGF mediates TGF-β1 induced increases in fibronectin (46) and type 1 collagen (47). High glucose induces CTGF *in vitro* in mesangial cells at least partially through increases in TGF-β1 bioactivity (10, 11). Because AGEs can induce TGF-β1 in rodent kidney in both *in vitro* studies as well as *in vivo* studies (48), it is possible that the induction of CTGF in the current study is at least partially occurring through induction of TGF-β1. If this is the case, then the protection from diabetic nephropathy produced by treatment of diabetic mice with neutralizing TGF-β antibodies (49) may be due to prevention of induction of CTGF by TGF-β1. In a recent preliminary report in diabetic mice specifically overexpressing in the myocardium the protein kinase C isoform, β2, up-regulation of CTGF in the heart in association with the development of fibrosis was apparent before an increase in TGF-β was observed (50). These findings provide further evidence that fibrosis, as part of the pathology of diabetic complications, may be partly related to CTGF rather than to TGF-β alone. In contrast to TGF-β1, CTGF is not thought to have an important role in immune modulation (47), and therefore it is potentially a more specific and downstream target for preventing diabetic complications (6).

AG has previously been reported to influence a range of other growth factors, including VEGF, TGF-β, and IGFs (4, 5, 51). However, in contrast to TGF-β, IGF-I has not been shown to directly induce CTGF *in vitro* or *in vivo*. IGF-I potentiates the induction of type IV collagen by CTGF in normal rat kidney cells (52), although the mechanism of this cellular effect has not been defined, and it is not clear whether the observed weak *in vitro* binding of CTGF to IGF-I (28) is responsible. Whether the increased IGF-I and CTGF synergize *in vivo* in their pathological effects in diabetic nephropathy remains to be determined.

AGEs have recently been reported *in vitro* to stimulate fibronectin production, which could be attenuated by coadministration of a neutralizing antibody to CTGF (15). The present study provides evidence that this phenomenon occurs *in vivo*. Indeed, AG prevented the increase in glomerular fibronectin expression observed in the diabetic kidney. Fibronectin has previously been shown to be up-regulated in the diabetic kidney and has been reported to be reduced by renoprotective regimens such as protein kinase C inhibition (53). This reduction in extracellular matrix accumulation is associated with previous quantitative histomorphometric studies that demonstrated reduced mesangial expansion in response to inhibition of renal AGE accumulation with AG (19, 30).

AG is the most well characterized and widely studied inhibitor of AGE formation. That we observed inhibition of CTGF overexpression in parallel with inhibition of AGE accumulation by AG in the diabetic rats suggests that AG is working through inhibition of formation of AGEs to prevent CTGF up-regulation. This is consistent with our *in vitro* finding of induction of CTGF expression by AGEs (14). Furthermore, the novel data presented in the current work showing induction of CTGF mRNA and fibronectin mRNA by AGEs in human mesangial cells is supportive of this concept. AG is also known to have other actions, which include inhibition of nitric oxide synthase (54). We have previously observed in the same rodent model of diabetes that administration of more specific inhibitors of nitric oxide synthase does not reproduce the renal effects of AG (30). Those results and the current *in vivo* study, supported by the *in vitro* data, suggest that AGEs cause renal pathology in this model of diabetes, at least partly through the up-regulation of renal CTGF. Studies using specific CTGF inhibitors are now warranted to determine to what extent CTGF mediates renal pathology caused by AGEs, and by other agents, in diabetes.

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