

# Connective Tissue Growth Factor Plays an Important Role in Advanced Glycation End Product–Induced Tubular Epithelial-to-Mesenchymal Transition: Implications for Diabetic Renal Disease

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Epithelial-to-mesenchymal transition (EMT) of tubular cells contributes to the renal accumulation of matrix protein that is associated with diabetic nephropathy. Both TGF- $\beta$ 1 and advanced glycation end products (AGE) are able to induce EMT in cell culture. This study examined the role of the pro-sclerotic growth factor connective tissue growth factor (CTGF) as a downstream mediator of these processes. EMT was assessed by the expression of  $\alpha$ -smooth muscle actin, vimentin, E-cadherin, and matrix proteins and the induction of a myofibroblastic phenotype. CTGF, delivered in an adenovirus or as recombinant human CTGF (250 ng/ml), was shown to induce a partial EMT. This was not blocked by neutralizing anti-TGF- $\beta$ 1 antibodies, suggesting that this action was TGF- $\beta$ 1 independent. NRK-52E cells that were exposed to AGE-modified BSA (AGE-BSA; 40  $\mu$ M) or TGF- $\beta$ 1 (10 ng/ml) also underwent EMT. This was associated with the induction of CTGF gene and protein expression. Transfection with siRNA to CTGF was able to attenuate EMT-associated phenotypic changes after treatment with AGE or TGF- $\beta$ 1. These *in vitro* effects correlate with the *in vivo* finding of increased CTGF expression in the diabetic kidney, which co-localizes on the tubular epithelium with sites of EMT. In addition, inhibition of AGE accumulation was able to reduce CTGF expression and attenuate renal fibrosis in experimental diabetes. These findings suggest that CTGF represents an important independent mediator of tubular EMT, downstream of the actions of AGE or TGF- $\beta$ 1. This interaction is likely to play an important role in progressive diabetic nephropathy and strengthens the rationale to consider CTGF as a potential target for the treatment of diabetic nephropathy.

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**T**ubulointerstitial fibrosis is a common final pathway that leads to progressive renal injury in a range of conditions, including diabetic nephropathy. Myofibroblasts are considered to be one of the principal effector cells that are responsible for the production and accumulation of matrix that is associated with progressive nephropathy (1,2). Whereas some myofibroblasts migrate to the interstitium as a result of chemokines that are released in response to injury, epithelial-to-mesenchymal transition (EMT) has been implicated in the accelerated fibrogenesis that is seen in diabetic nephropathy (3). In this process, renal tubular cells, in response to injury or local activation, lose their epithelial phenotype and acquire profibrotic features that are characteristic of mesenchymal cells (4). EMT is regulated by several growth factors and cytokines,

including TGF- $\beta$ 1, Fibroblast growth factor, IL-1, EGF, and angiotensin II (5–9). In addition, we previously reported that advanced glycation end products (AGE) also may induce EMT, *via* activation of the receptor for AGE (RAGE), potentially contributing to their profibrotic actions (3). Although the exact mechanism of action remains to be established, RAGE-induced EMT seems to have both TGF- $\beta$ -dependent (3) and -independent elements (10).

Connective tissue growth factor (CTGF) is a 38-kD member of the CCN family of proteins that are involved in matrix remodeling during development and pathologic conditions (11,12). Expression of CTGF is increased in diabetic nephropathy (12) and correlates with the degree of albuminuria (13). Chronic hyperglycemia, TGF- $\beta$ , mechanical strain, and AGE all are able to induce the expression of CTGF in renal tubular cells (12,14–16). An important role for CTGF in diabetic nephropathy is supported further by studies in heterozygous CTGF+/- mice, in which diabetes-induced glomerular basement membrane thickening is attenuated (17). However, the mechanisms that are responsible for the renal actions of CTGF are poorly understood. In rodent models of chronic fibrosis, CTGF is required in addition to TGF- $\beta$  to induce persistent injury (18).

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This has led to the hypothesis that some of the profibrotic effects of TGF- $\beta$  may be mediated by CTGF (19,20), possibly through the TGF- $\beta$ -binding and Smad-responsive elements that are located in its promoter region (19,21).

Although TGF- $\beta$  seems to be the key stimulus for EMT (5), the role of CTGF *per se* in EMT has not been examined. Therefore, the aim of our study was to determine whether AGE and TGF- $\beta$  induce EMT and extracellular matrix (ECM) accumulation partially through a CTGF-dependent pathway and to investigate strategies that directly modulate CTGF expression and action. Finally, the *in vivo* consequences of AGE inhibition on these pathways and, in particular, CTGF expression and *in vivo* assessment of EMT are examined in the diabetic context.

## Materials and Methods

### In Vitro Studies

**Cell Culture.** The well-characterized, normal rat kidney tubular epithelial cell line (NRK-52E) was obtained from the American Tissue Culture Collection (Rockville, MD). Cells were maintained in DMEM that contained 4.5 g/L glucose (Invitrogen, Carlsbad, CA) with 10% FCS at 37°C in a 5% CO<sub>2</sub> atmosphere and passaged twice a week.

**CTGF siRNA Synthesis and Transfection.** siRNA sequences directed to different regions of the CTGF gene were designed using the siRNA selection algorithm of the Whitehead Institute for Biomedical Research (22). siRNA was synthesized using the Ambion siRNA construction kit (Ambion, Austin, TX). For transfection, NRK-52E cells were seeded in 12-well plates in DMEM that contained 10% FCS and allowed to recover for 24 h. Cells were washed twice in PBS before the addition of OptiMEM supplemented with 2% FCS, then incubated for an additional 2 h before transfection. Cells subsequently were transfected with 1 nM CTGF siRNA using Oligofectamine (Invitrogen) (23). After transfection, cells were incubated for 6 h before the initiation of various treatments. Cells were transfected twice more, incubating for 24 h after each transfection. A number of siRNA constructs were tested at a range of concentrations (0.1 to 10 nM) for their ability to reduce endogenous CTGF mRNA expression as measured by real-time reverse transcription-PCR (RT-PCR). After a number of optimization experiments, one siRNA construct, CTGF-286, was found both to be specific and to result in a dose-dependent suppression of CTGF gene expression. The optimum dose was determined to be 1 nM and achieved a 50% reduction in CTGF mRNA in NRK-52E cells.

**Adenoviral and CTGF Treatments.** CTGF sense and empty vector adenoviruses were generated as described previously (12). The sense adenovirus contained the complete human CTGF sequence in the sense direction under the control of the cytomegalovirus promoter (24). The CTGF cDNA was omitted in the empty vector adenovirus that was used as a control for these experiments. Cells were seeded onto six- or 12-well plates in DMEM that contained 10% FCS and allowed to recover for 24 h. Without changing the medium, the same amount of adenovirus for the CTGF-expressing adenovirus and the vector alone ( $2 \times 10^9$  particles) was administered to cultured cells, based on initial adenoviral quantification by spectrophotometer readings, confirmed by adenoviral real-time PCR quantification (25). Confirmation of equivalent infectious particles was by estimation of observed cytopathic effect using tissue culture infectious dose-50 analysis (26). For assessment of the role of active TGF- $\beta$ 1 in CTGF-dependent phenomena, infected cells also were treated with rabbit neutralizing anti-TGF- $\beta$ 1 antibody (10  $\mu$ g/ml; R&D Systems, Minneapolis, MN), a dosage that was shown previously to prevent AGE-BSA- and TGF- $\beta$ 1-dependent EMT (3,27), or with a species-specific isotype control normal rabbit IgG. In separate

experiments, cells were treated with recombinant human CTGF protein at a concentration (250 ng/ml) that was shown previously to be biologically active using untreated cells as a control (28).

**Induction of EMT.** Cells were cultured in the presence of TGF- $\beta$ 1 (10 ng/ml, R&D Systems) or AGE-BSA (40  $\mu$ M), known stimuli of EMT (3). AGE-BSA was synthesized as described previously (3). Untreated cells and cells that were treated with unmodified BSA (40  $\mu$ M) were used as respective controls. Experiments were run for 3 and 6 d. At the completion of the experiment, cells were photographed under a light microscope for morphologic changes (Magnifications:  $\times$ 100 to 400) before harvesting for protein and mRNA.

**Real-Time RT-PCR.** Gene expression of CTGF, collagen IV, fibronectin, TGF- $\beta$ 1, E-cadherin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and vimentin was analyzed by real-time RT-PCR, performed as described previously (3) using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Foster City, CA). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer). For controlling for variation in the amount of DNA that was available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control 18S ribosomal RNA (rRNA; 18S rRNA TaqMan Control Reagent kit, ABI Prism 7700; Perkin-Elmer). Primers and TaqMan probes for the genes of interest (Table 1) and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700; Perkin-Elmer). The amplification was performed with the following time course: 50°C for 2 min and 95°C for 10 min, and 40 cycles of 94°C for 20 s and 60°C for 60 s. Each sample was tested in triplicate. Results were expressed relative to control (untreated) cells, which was arbitrarily assigned a value of 1.

**Immunofluorescence.** NRK-52E cells were grown on coverslips and washed twice with PBS before fixing in ice-cold acetone for 20 min at  $-20^{\circ}\text{C}$ . The cells were rehydrated in PBS with two 10-min washes, and the membranes were permeabilized with 0.1% Triton-X100 for 10 min and blocked in 0.5% BSA/PBS or 10% normal rabbit serum for 30 min for before incubation with primary antibody,  $\alpha$ -SMA (1:100; Clone 1A4; Dako, Cupertino, CA), vimentin (1:100; Dako), or E-cadherin (1:100; Transduction Laboratories, Lexington, KY) for 1 h at room temperature. Nonimmunized mouse serum was used at the same concentration as a negative control. After three washes in PBS, the cells were incubated with the fluorescence secondary antibody ALEXA-488 (rabbit anti-mouse antibody, 1:200; Molecular Probes, Invitrogen) for 1 h at room temperature. Images were captured on a Zeiss 510 Meta laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using LSM 510 software (version 3.2 SP2; Zeiss).

**Western Blot Analysis.** Whole-cell lysates that contained 10 to 50  $\mu$ g of protein were subjected to 10 to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes by semidry transfer (Semi Dry Transfer Cell; BioRad, Hercules, CA). After transfer, all incubations were conducted on a rocking platform at room temperature. The membrane was blocked in 5% skim milk/TBST overnight, then incubated for 1 h with  $\alpha$ -SMA (1:2000; Dako), CTGF (1:1000; Abcam, Cambridge, UK), or E-cadherin (1:2500; Transduction Laboratories). The membrane was washed with TBST and then incubated with a peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (EnVision+; Dako) for 1 h. Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposure to CL-Xposure film (Progen, Darra, Australia).

### In Vivo Studies

**Animals.** To explore further the relationship among CTGF, AGE, and renal EMT, we examined archival material from a previously

Table 1. Probe and primer sequences used for real-time RT-PCR analysis<sup>a</sup>

Probe/Primer	Sequence
Collagen IV	
forward primer 5'-3'	GGCGGTACACAGTCAGACCAT
probe	FAM-CCGCAGTGCCCTAACGGTTGGTC-MGB
reverse primer 5'-3'	GGAATAGCCGATCCACAGTGA
CTGF	
forward primer 5'-3'	TGGCCCTGACCCAACTATGA
probe	FAM-ACTGCCTGGTCCAGAC-MGB
reverse primer 5'-3'	CTTAGAACAGGCGCTCCACTCT
E-cadherin	
forward primer 5'-3'	AACGAGGGCATTCTGAAAACA
probe	FAM-TGCTTGGCCTCAAATCCAAGCCCT-MGB
reverse primer 5'-3'	CACTGTCACGTGCAGAATGTACTG
Fibronectin	
forward primer 5'-3'	CATGGCTTTAGGCGAACCA
probe	FAM-CCCCGTCAGGCTTA-MGB
reverse primer 5'-3'	CATCTACATTCGGCAGGTATGG
$\alpha$ -SMA	
forward primer 5'-3'	GACCCTGAAGTATCCGATAGAACA
probe	FAM-TGCCAGATCTTTTCC-MGB
reverse primer 5'-3'	CACGCGAAGCTCGTTATAGAAG
Vimentin	
forward primer 5'-3'	CCATCAACACCGAGTTCAAGAA
probe	FAM-CCGCACCAACGAGA -MGB
reverse primer 5'-3'	GGCGAAGCGGTCATTCAG

<sup>a</sup>CTGF, connective tissue growth factor; RT-PCR, real time reverse transcription-PCR;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

reported study that explored the utility of the AGE inhibitor alagebrium chloride on diabetic renal disease (29). In brief, 8-wk-old Sprague-Dawley rats were randomized for the induction of diabetes, *via* a single injection of the  $\beta$  cell toxin streptozotocin with citrate buffer injection serving as a control. After 16 wk of study, animals were randomized further to receive the AGE-reducing compound alagebrium chloride at a dosage of 10 mg/kg per d by gavage. After 32 wk of study, the rats were killed and kidneys were removed and fixed in paraformaldehyde for subsequent analyses.

**In Situ Hybridization.** The site-specific expression of collagen IV, CTGF,  $\alpha$ -SMA, and TGF- $\beta$ 1 mRNA was determined by *in situ* hybridization as described previously, with background hybridization controlled for by the inclusion of a sense riboprobe (30).

**Immunohistochemistry.** Immunohistochemical staining for E-cadherin (Transduction Laboratories) was performed according to standard procedures using an avidin-biotin-based system. Briefly, 4- $\mu$ M sections from formalin-fixed kidneys were brought to distilled water, then transferred to PBS. Endogenous peroxidases were blocked in 0.3% hydrogen peroxide in PBS and washed in PBS before blocking in 10% normal rabbit serum to prevent nonspecific binding. Sections then were incubated in the primary antibody overnight at 4°C, washed in PBS, and incubated in a rat anti-mouse biotinylated secondary antibody (Dako) for 30 min. After PBS washes, the addition of streptavidin-conjugated horseradish peroxidase (Vector Laboratories), and further washing in PBS, the signal was developed using diaminobenzidine (DAB; Sigma, St. Louis, MO). Finally, the samples were counterstained in hematoxylin and eosin.

All sections were analyzed for staining using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan) and digitized using

a high-resolution camera (Fujix HC-2000; Fujifilm, Tokyo, Japan). Digitized images then were captured and evaluated using an image analysis system (Imaging Research, St. Catharines, ON, Canada) coupled to an IBM NT computer. Semiquantitative assessment of tubular E-cadherin was performed by determination of the percentage proportion of area of renal cortex section occupied by the brown (diaminobenzidine) staining ( $\times 20$  objective). A total of 10 renal cortex sections per rat kidney ( $n = 8$  rats/group) were analyzed.

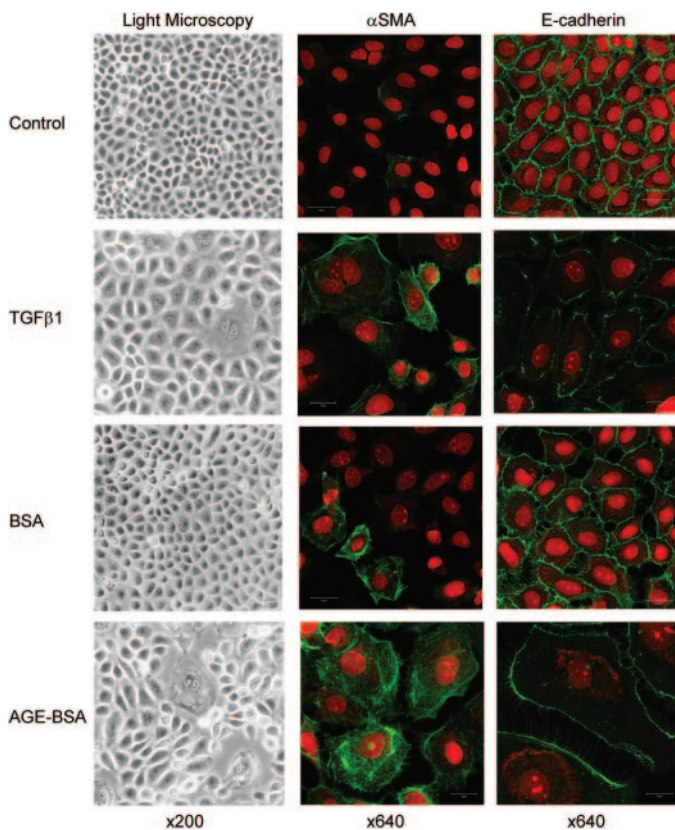
### Statistical Analyses

Values are shown as means  $\pm$  SEM unless otherwise specified. Statview SE (Brainpower, Calabasas, CA) was used to analyze data by ANOVA and compared using the Fisher protected least significant difference *post hoc* test.  $P < 0.05$  was considered significant.

## Results

### In Vitro Studies

**EMT in NRK-52E Cells (6-D Model).** The effects of AGE-BSA and TGF- $\beta$ 1 treatment of NRK-52E cells were assessed by both light microscopy and confocal imaging of immunofluorescence-stained cells. Cells that were treated for 6 d in medium alone exhibited the typical cobblestone morphology of the epithelial cell, associated with strong surface expression of E-cadherin protein (Figure 1). Treatment with TGF- $\beta$ 1 or AGE-BSA led to the induction of morphologic changes, including elongation and hypertrophy, separation from neighboring cells, “front-to-back” polarity (Figure 1), and increased gene and

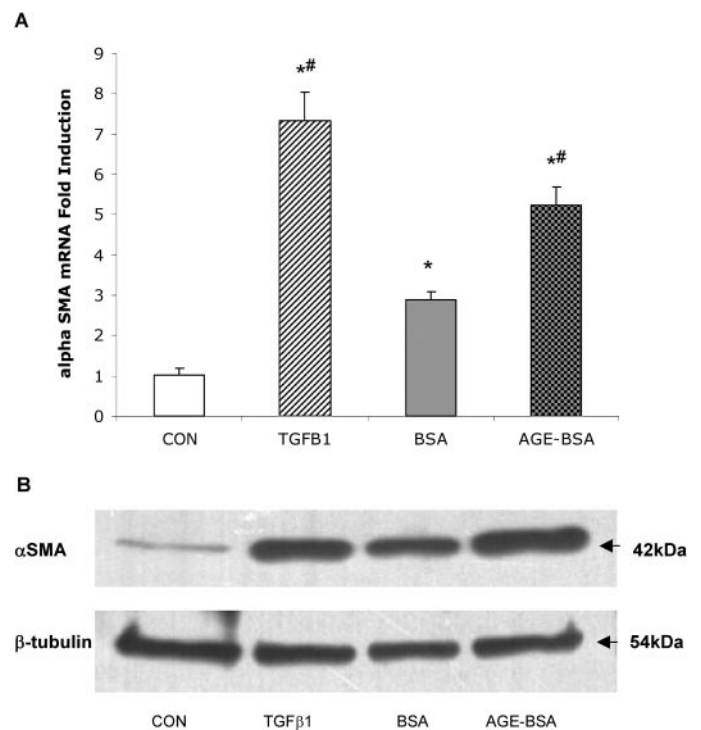


**Figure 1.** TGF- $\beta$ 1 (10 ng/ml) and advanced glycation end product (AGE)-modified BSA (AGE-BSA; 40  $\mu$ M) induce epithelial-to-mesenchymal transition (EMT) in NRK-52E cells as determined by cellular morphology after 6 d of exposure (left), *de novo* expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA; middle), and reduction of E-cadherin expression (right). BSA-treated (40  $\mu$ M) cells show an intermediate phenotype.  $\alpha$ SMA and E-cadherin expression is shown in green. Cells were counterstained with propidium iodide (red) to demonstrate the nuclei.

protein expression of  $\alpha$ -SMA (Figure 2), characteristic of the mesenchymal phenotype. At the same time, gene and protein of the epithelial marker E-cadherin were reduced after TGF- $\beta$ 1 or AGE-BSA treatments, as demonstrated by real-time RT-PCR and Western blotting, respectively (Figure 3). BSA had a modest effect on reducing E-cadherin expression, intermediate between control and AGE-treated cells (Figures 1 and 3). BSA also led to a modest increase in  $\alpha$ -SMA expression, although significantly less than that observed with AGE-BSA or TGF- $\beta$ 1 (Figure 2).

**Induction of CTGF Expression by AGE and TGF- $\beta$ 1.** Both TGF- $\beta$ 1 and AGE-BSA induced the gene expression of CTGF compared with control-treated cells. This effect was maximal at day 3 (Figure 4) but remained significant at day 6 (data not shown). BSA had no effect on the expression of CTGF mRNA at either time point. These results were confirmed at the protein level, by Western analysis of cell lysates (Figure 4).

**EMT in NRK-52E Cells (3-D Model).** Cells that were treated for 3 d with TGF- $\beta$ 1 or AGE-BSA also induced phenotypic changes that are characteristic of EMT, including in-

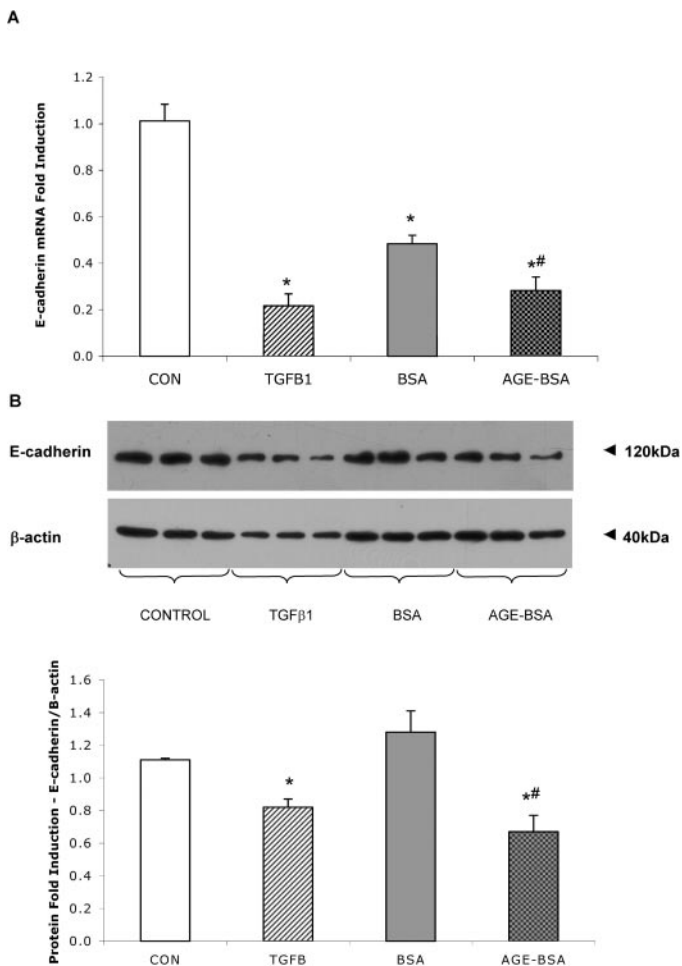


**Figure 2.** TGF- $\beta$ 1 (10 ng/ml), AGE-BSA, and BSA (40  $\mu$ M) induce the gene and protein expression of  $\alpha$ -SMA in NRK-52E cells, as shown by real-time reverse transcription-PCR (RT-PCR; A) and a representative Western blot (B). Each bar represents the mean  $\pm$  SEM of six samples per group. \* $P$  < 0.01 versus control; # $P$  < 0.05 versus BSA.

creased expression of  $\alpha$ -SMA mRNA (control  $1.1 \pm 0.2$ ; TGF- $\beta$ 1  $2.5 \pm 0.4$ ; BSA  $1.1 \pm 0.1$ ; AGE-BSA  $2.7 \pm 0.4$ ) and  $\alpha$ -SMA protein (control  $1.0 \pm 0.0$ ; TGF- $\beta$ 1  $8.9 \pm 0.1$ ; BSA  $1.1 \pm 0.0$ ; AGE-BSA  $2.5 \pm 0.02$ ). Therefore, for the subsequent 3-d intervention studies, expression of  $\alpha$ -SMA protein and/or mRNA was used to establish the presence or absence of EMT.

EMT in NRK-52E cells also was associated with increased expression of ECM components, including fibronectin and collagen IV (3). In this study, cells that were treated with TGF- $\beta$ 1 and AGE-BSA (Figure 5) for 3 d had increased levels of fibronectin and collagen IV mRNA. This difference was less marked at day 6, at which time BSA itself had induced moderate EMT (see above). Consequently, the 3-d time point was used to examine the effect of CTGF on matrix expression that was associated with AGE- and TGF- $\beta$ 1-mediated EMT.

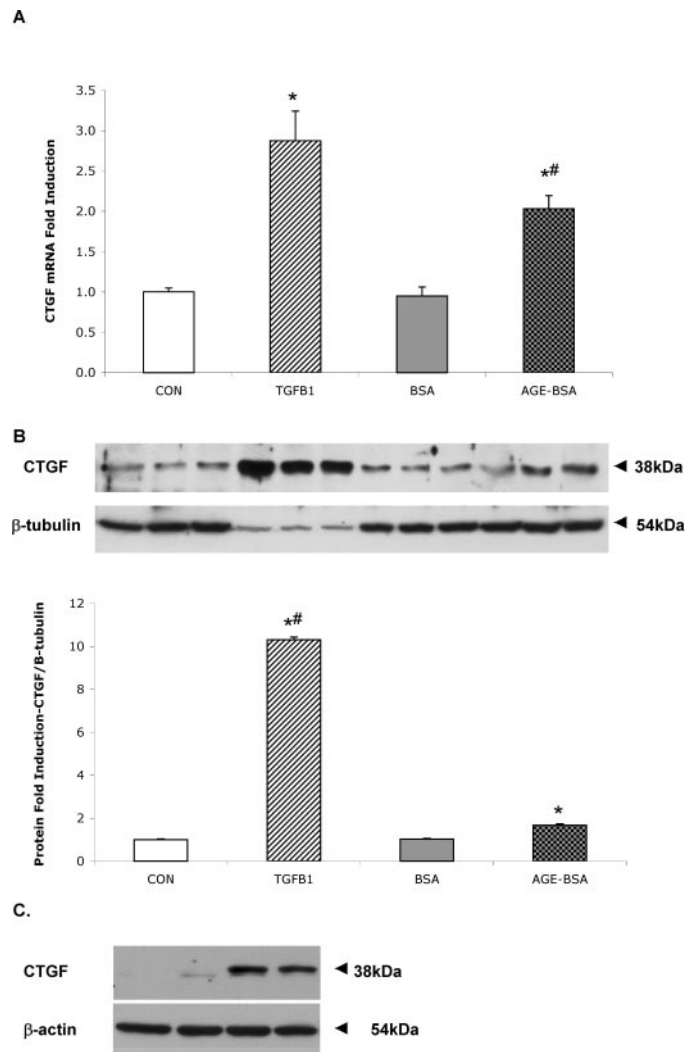
**Induction of EMT by CTGF.** The ability of CTGF to induce EMT independently was assessed first by adenoviral transfection of the CTGF gene and secondly *via* exogenous addition of recombinant CTGF. Infection of NRK-52E cells with the CTGF adenovirus system resulted in a 19- and 14-fold increase in CTGF mRNA at 3 and 6 d, respectively. After 3 and 6 d of incubation in standard media, NRK-52E cells that were infected with CTGF sense adenovirus showed alterations in morphology, including cellular hypertrophy and front-to-back end polarity, characteristic of a mesenchymal phenotype compared with the typical cobblestone appearance of the epithelial cells



**Figure 3.** TGF- $\beta$ 1, AGE-BSA, and BSA reduce the gene and protein expression of E-cadherin in NRK-52E cells, as demonstrated by real-time RT-PCR (A) and a representative Western blot (B). Each bar represents the mean  $\pm$  SEM of six (mRNA) or three (protein) samples per group. \* $P < 0.05$  versus control; # $P < 0.01$  versus BSA.

that were infected with empty vector adenovirus (Figure 6). Gene expression of the mesenchymal marker  $\alpha$ -SMA, which was low in the empty vector-infected cells, was significantly increased by day 3 and more than doubled in CTGF overexpressing cells at the 6-d time point as assessed by real-time RT-PCR (Figure 7A). Similarly, the expression of the mesenchymal marker vimentin was increased in the CTGF sense adenovirus-treated samples by the 6-d time point.

The expression of the epithelial marker E-cadherin also was significantly modified after treatment with CTGF sense adenovirus (Figure 6). This was seen at both the 3-d (Figure 6A) and the 6-d (Figure 6B) time points, with some (larger) cells losing all expression of E-cadherin altogether and other (smaller) cells showing relocalization of E-cadherin expression to the perinuclear region, suggestive of an intermediate phenotype. Western blotting confirmed the loss of E-cadherin protein in cells that were treated with the CTGF sense adenovirus (vector  $1.3 \pm 0.1$ ; CTGF sense  $0.8 \pm 0.0$ ;  $P < 0.05$ ; Figure 7B). However, unlike EMT that was associated with TGF- $\beta$ 1 or AGE-BSA, the total



**Figure 4.** TGF- $\beta$ 1 and AGE-BSA induce the gene and protein expression of connective tissue growth factor (CTGF) in NRK-52E cells, as demonstrated by real-time RT-PCR (A) and representative Western blot analysis of whole-cell lysates (B). (C) Western blot of control and TGF- $\beta$ 1 samples demonstrating expression of CTGF protein. To enable the quantification of CTGF, loading in B was adjusted for TGF- $\beta$ 1 samples to prevent saturation of signal (lanes 4 through 6). Even loading of the control and TGF- $\beta$ 1 samples is shown in C. Each bar represents the mean  $\pm$  SEM of six (mRNA) or three (protein) samples per group. \* $P < 0.01$  versus control; # $P < 0.01$  versus BSA.

gene expression of E-cadherin was increased modestly (Figure 7A). CTGF-induced EMT also was associated with upregulation in the expression of matrix proteins, collagen IV, and fibronectin (Figure 7C).

Co-incubation of adenovirus-infected cells with neutralizing anti-TGF- $\beta$ 1 antibodies had no effect on CTGF-induced changes in the expression of  $\alpha$ -SMA (vector  $1.0 \pm 0.2$ , vector+TGF- $\beta$ 1 antibody  $1.2 \pm 0.1$ , vector+control antibody  $1.2 \pm 0.1$ , CTGF sense  $1.8 \pm 0.1$ , CTGF sense+TGF- $\beta$ 1 antibody  $1.9 \pm 0.2$ , CTGF sense+control antibody  $1.7 \pm 0.1$ ; NS) or in the upregulation of the matrix proteins fibronectin or collagen IV

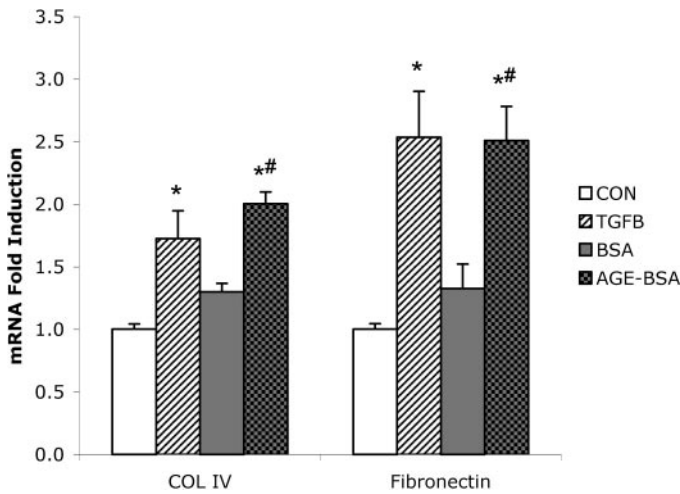


Figure 5. TGF-β1, AGE-BSA, and BSA induce the gene expression of the matrix molecules collagen IV and fibronectin in NRK-52E cells, as demonstrated by real-time RT-PCR. Each bar represents the mean ± SEM of three to six samples per group \*P < 0.01 versus control; #P < 0.05 versus BSA.

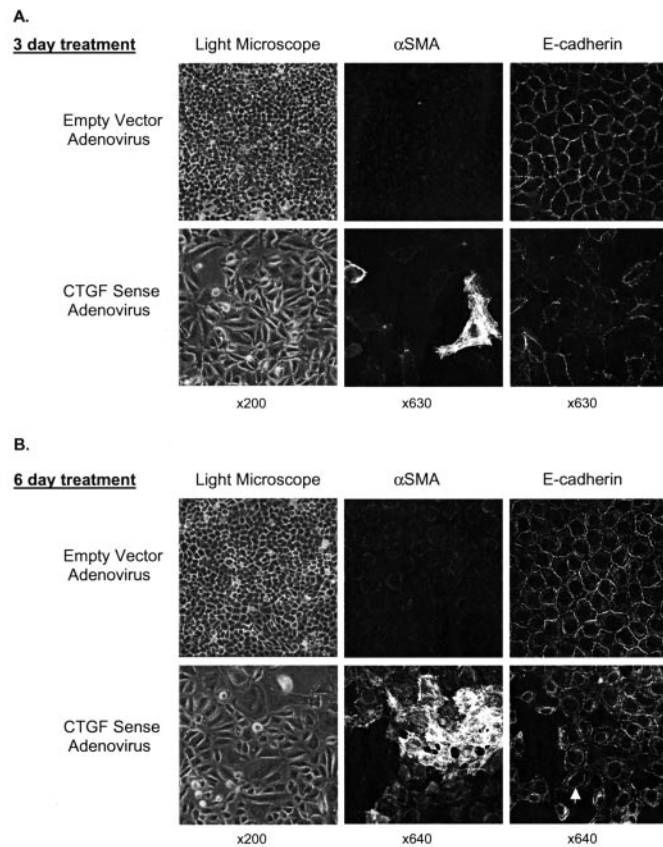


Figure 6. NRK-52E cells that were stimulated with CTGF sense adenovirus for 3 (A) or 6 d (B) undergo EMT, as demonstrated by cellular morphology, *de novo* expression of α-SMA, and reduced expression of E-cadherin at membrane junctions, with relocalization to the perinuclear region (arrow).

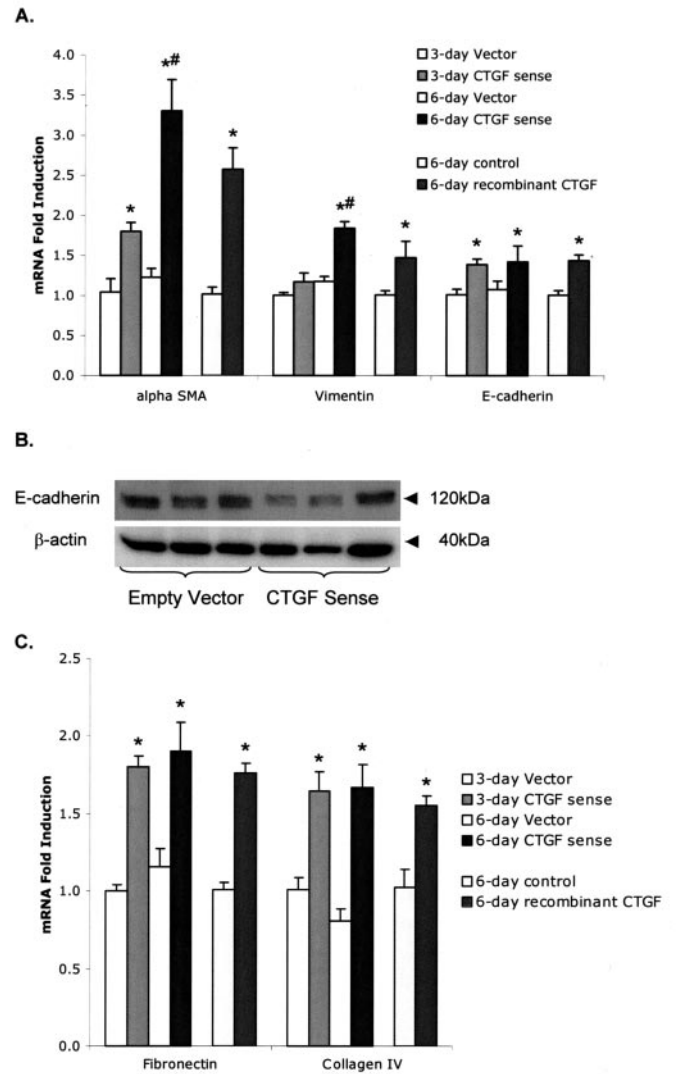


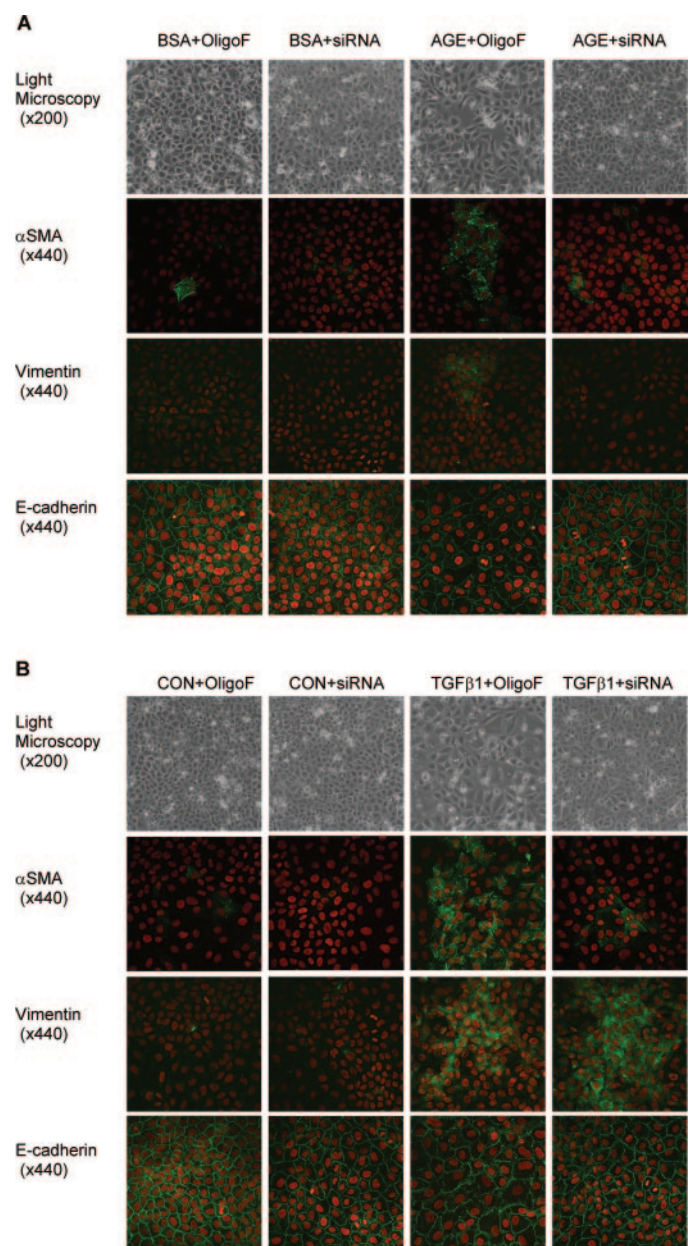
Figure 7. NRK-52E cells that were infected with CTGF sense adenovirus for 3 or 6 d alter the gene expression of the EMT markers α-SMA vimentin, and E-cadherin (A) and the ECM molecules collagen IV and fibronectin (C) in NRK-52E cells, as demonstrated by real-time RT-PCR. (B) NRK-52E cells that were infected with CTGF sense adenovirus for 3 d show decreased expression of E-cadherin protein as assessed by Western blot. Each bar represents the mean ± SEM of six samples per group. \*P < 0.01 versus vector control.

(fibronectin: vector 1.0 ± 0.4, vector+TGF-β1 antibody 1.1 ± 0.3, vector+control antibody 1.2 ± 0.0, CTGF sense 1.8 ± 0.1, CTGF sense+TGF-β1 antibody 1.9 ± 0.1, CTGF sense+control antibody 2.0 ± 0.1 [NS]; collagen IV: vector 1.0 ± 0.7, vector+TGFβ1 antibody 1.2 ± 0.7, vector+control antibody 1.2 ± 0.1, CTGF sense 1.6 ± 0.1, CTGF sense+TGF-β1 antibody 1.9 ± 0.2, CTGF sense+control antibody 1.6 ± 0.1 [NS]). In addition, CTGF sense adenovirus had no effect on the gene expression of TGF-β1 in transfected NRK-52E cells, consistent with previous reports in proximal tubular cell systems (16).

The addition of exogenous recombinant CTGF (250 ng/ml) to NRK-52E cells had a similar effect to that observed in the adenovirus infection system, with the induction of  $\alpha$ -SMA, vimentin (Figure 7A), fibronectin, and collagen IV (Figure 7C) genes after 6 d of incubation. Gene expression of the epithelial marker E-cadherin also was increased by approximately 40% (*versus* untreated,  $P < 0.01$ ).

**Role of CTGF in AGE-Induced EMT.** For determination of whether AGE-induced EMT is mediated by CTGF, cells that were treated with AGE-BSA also were transfected with siRNA to CTGF (CTGF-286). For assessment of the efficacy of the siRNA to knockdown, the gene expression of CTGF was assessed at the 3-d time point using real-time RT-PCR. As expected, treatment with 1 nM CTGF-286 reduced the expression of CTGF in both BSA- and AGE-BSA-treated cells, compared with control cells that were treated with oligofectamine (OligoF) alone (BSA+OligoF  $1.0 \pm 0.1$ , BSA+CTGF-286  $0.6 \pm 0.1$ , AGE+OligoF  $1.4 \pm 0.2$ , AGE+CTGF-286  $1.0 \pm 0.1$ ;  $P < 0.05$  *versus* matching controls). This reduction in CTGF expression was sufficient to prevent the EMT-associated phenotypic changes after treatment with BSA or AGE-BSA (Figure 8A). Immunofluorescence analysis of these samples demonstrated that an increased number of cells stained for the mesenchymal markers  $\alpha$ -SMA and vimentin with AGE-BSA, which subsequently was reduced after treatment with CTGF siRNA (Figure 8A). This reduction in  $\alpha$ -SMA gene expression (BSA+OligoF  $1.0 \pm 0.1$ , BSA+CTGF-286  $0.6 \pm 0.2$ , AGE-BSA+OligoF  $1.9 \pm 0.3$ , AGE-BSA+CTGF-286  $0.9 \pm 0.0$ ; OligoF *versus* CTGF-286,  $P < 0.05$ ) was confirmed using RT-PCR. Notably, expressions of CTGF and  $\alpha$ -SMA were closely correlated ( $R = 0.42$ ), with the greatest reduction in  $\alpha$ -SMA expression associated with the lowest CTGF expression. Confocal microscopy showed a reduction in E-cadherin protein in AGE-BSA-treated cells, which was partially restored in the presence of CTGF-286 siRNA. No change was seen in the expression of E-cadherin at the transcriptional level with the addition of CTGF siRNA (data not shown).

**Role of CTGF in TGF- $\beta$ 1-Induced EMT.** The effect of CTGF-286 siRNA on TGF- $\beta$ 1-mediated EMT changes in NRK-52E cells was assessed as above. The addition of CTGF siRNA attenuated TGF- $\beta$ 1-induced CTGF expression, although it did not return to control levels (control+OligoF  $1.0 \pm 0.0$ , control+CTGF-286  $0.4 \pm 0.0$ , TGF- $\beta$ 1+OligoF  $68.1 \pm 0.1$ , TGF- $\beta$ 1+CTGF-286  $17.2 \pm 0.2$ ; TGF- $\beta$ 1+OligoF *versus* CTGF-286,  $P < 0.01$ ). This reduction in CTGF expression partially restored the morphologic changes that were seen with TGF- $\beta$ 1 treatment but did not completely prevent them (Figure 8B). However, as observed with AGE-BSA-treated cells, CTGF-286 siRNA reduced  $\alpha$ -SMA protein expression in the TGF- $\beta$ 1-treated samples (Figure 8B), which was confirmed by Western blot (control+OligoF  $1.0 \pm 0.1$ , control+CTGF-286  $0.9 \pm 0.1$ , TGF- $\beta$ 1+OligoF  $3.0 \pm 0.1$ , TGF- $\beta$ 1+CTGF-286  $2.3 \pm 0.0$ ; TGF- $\beta$ 1+OligoF *versus* CTGF-286,  $P < 0.0001$ ). The expression of neither vimentin nor E-cadherin was altered with the addition of CTGF-286 siRNA in TGF- $\beta$ 1-treated cells (Figure 8B).



**Figure 8.** EMT in NRK-52E cells associated with BSA and AGE-BSA (A) or control and TGF- $\beta$ 1 treatments (B) was investigated after transfection with CTGF-286; this was assessed by light microscopy and confocal microscopy of cells immunostained for  $\alpha$ -SMA, vimentin, and E-cadherin.  $\alpha$ -SMA, vimentin, and E-cadherin are seen in green. Cell nuclei were counterstained with propidium iodide (red). Magnifications: light microscopy  $\times 200$ ; confocal  $\times 440$ .

### In Vivo Study

As described previously, 32 wk of experimental diabetes in Sprague-Dawley rats was associated with increased renal accumulation of AGE. At the same time, renal gene expression of CTGF, TGF- $\beta$ 1, collagen IV and  $\alpha$ -SMA was increased compared with the control rats (Figure 9). Notably, *in situ* hybridization that was performed on serial sections demonstrated that CTGF and  $\alpha$ -SMA mRNA are induced in the same cells in the

diabetic state (Figure 10A). Treatment with the AGE inhibitor alagebrium chloride resulted in reduced renal AGE accumulation in association with attenuation of changes of diabetic nephropathy (31). Similarly, intervention with alagebrium reduced the gene expression of CTGF, TGF- $\beta$ 1, collagen IV, and  $\alpha$ -SMA to control levels (Figure 9). Immunostaining for E-cadherin protein expression showed a decreased expression in the tubules of diabetic rats compared with controls, which was partially restored by intervention with alagebrium (control  $13.0 \pm 0.5$ , diabetic  $7.1 \pm 0.3$ , diabetic+alagebrium  $9.2 \pm 0.1$ ; diabetic *versus* control,  $P < 0.05$ ; diabetic *versus* diabetic+alagebrium,  $P < 0.05$ ; control *versus* diabetic+alagebrium,  $P < 0.05$ ; Figure 10B).

### Discussion

Our study provides evidence that CTGF plays an important role in promoting EMT. These studies extend the potential stimuli that are relevant to the diabetic milieu that induces

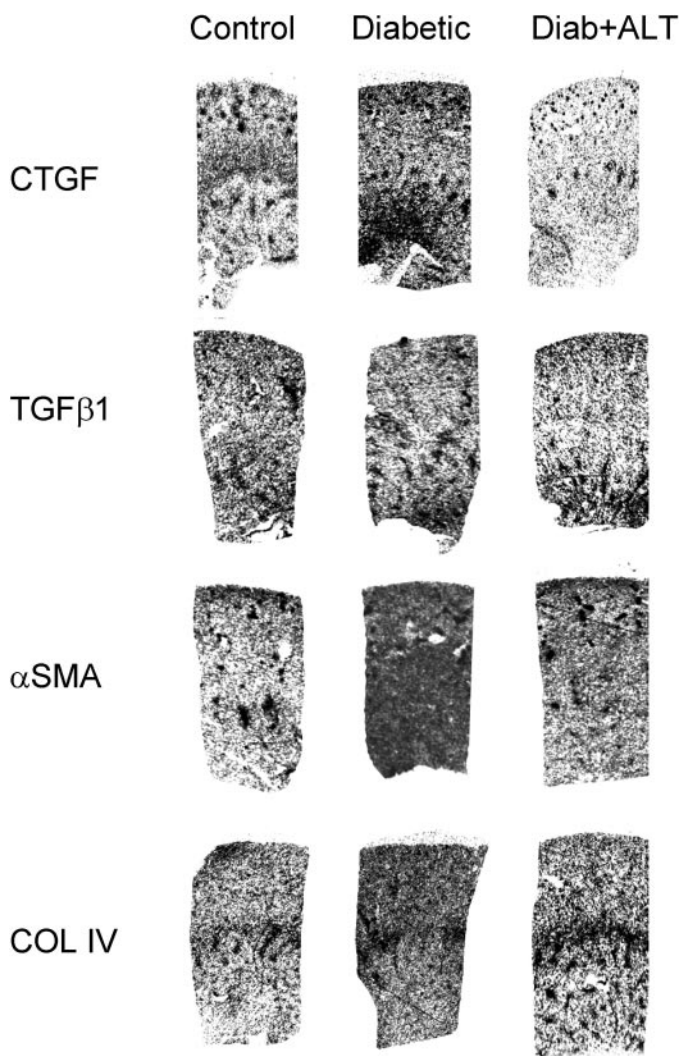


Figure 9. Renal expression of TGF- $\beta$ 1, CTGF,  $\alpha$ -SMA, and collagen IV mRNA, as assessed by *in situ* hybridization. Study groups are control, diabetic for 32 wk, and diabetes + alagebrium treatment (Diab+ALT) from 16 to 32 wk.

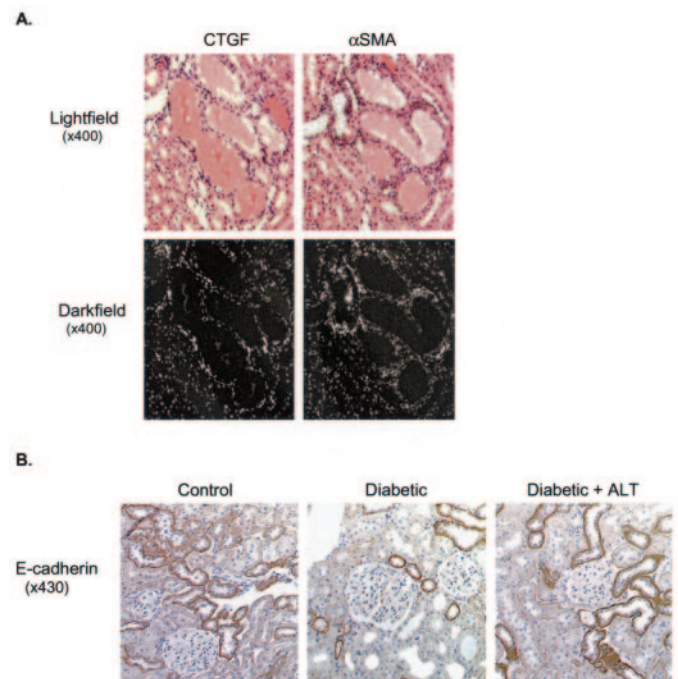


Figure 10. (A) Co-localization of CTGF and  $\alpha$ -SMA in the diabetic tubule as assessed by *in situ* hybridization in serial renal sections. Images are shown in light field (top) or dark field (bottom). (B) E-cadherin protein expression, as assessed by immunohistochemistry. Study groups are control, diabetes for 32 wk, and diabetes+ALT from 16 to 32 wk.

EMT. It was shown previously that TGF- $\beta$ 1 plays an important role in changing the phenotype of renal epithelial cells (5), actions that significantly contribute to the profibrotic actions of this growth factor (2). Our earlier studies also suggested that AGE, which accumulate in the diabetic kidney, are powerful mediators of EMT (3), involving both TGF- $\beta$ 1-dependent and -independent pathways that involve intracellular signaling molecules, such as the Smad (27) and mitogen-activated protein kinase *via* RAGE (3,10). Our study suggests that CTGF represents an additional key component of the pathways that lead to tubular EMT.

In this study, we focused on the pro-sclerotic growth factor CTGF. This protein was examined specifically for a number of reasons. First, on the basis of microarray data from glucose-exposed renal mesangial cells, CTGF was one of the most highly upregulated proteins (32). Second, in contrast to TGF- $\beta$ 1, which has not only profibrotic but also immunomodulatory actions (33), CTGF is a growth factor with more restricted biologic actions and thus may be a more attractive target for antifibrotic therapy in renal disease (17,34). Third, our own studies previously demonstrated upregulation of CTGF in response to AGE in various cell types, including mesangial cells (12). Indeed, in this study, we show that AGE also are potent stimulators of CTGF, as reported for various cell types (35,36).

It remains controversial as to what is the best marker of EMT in the various experiments. In this series of cell culture



experiments, both gene and protein expression and intracellular localization using confocal microscopy were used to characterize and quantify the extent of EMT. In general, the techniques provided a consistent pattern, with most stimuli for EMT indicating upregulation of  $\alpha$ -SMA and vimentin and downregulation of E-cadherin associated with a transition in the epithelial phenotype of the NRK-52E cells to a myofibroblastic phenotype. However, in contrast to TGF- $\beta$ 1 and AGE-BSA, CTGF did not reduce overall E-cadherin gene expression, although E-cadherin protein expression clearly was absent in some mesenchymal-like cells, as shown by confocal microscopy (Figure 6). This loss of E-cadherin protein was confirmed by Western blot of whole-cell lysates. In cells that still expressed E-cadherin strongly, there was a marked alteration of the pattern of expression, from a membrane-associated distribution that is characteristic of epithelial cells to a more perinuclear pattern. This change in E-cadherin distribution was reported previously in response to TGF- $\beta$ , albeit at an earlier time point (37), and is consistent with the relatively long half-life of the molecule in cells (2 to 3 d). The functional significance of this transition remains to be established. However, given the key role of E-cadherin in maintaining the structural integrity and the polarity of epithelial cells, redistribution of E-cadherin may modify adherens junction formation and cell polarity and contribute to the loss of cytoskeletal integrity and the acquisition of mesenchymal phenotype (2,4,38).

Although our study focused on EMT, it is likely that many of the pathways and mediators of EMT also independently contribute to ECM accumulation (2). As has been reported for both AGE (39) and TGF- $\beta$  (33), CTGF also promoted increased expression of various ECM proteins that are implicated in diabetes-associated ECM accumulation in the kidney. In particular, fibronectin and type IV collagen were upregulated in response to both AGE and CTGF. Moreover, their expression in diabetes is reduced by pharmacologic approaches to reduce AGE accumulation (12) or by direct inhibition of CTGF using a genetic manipulation strategy (17). In contrast to a previous study, which did not observe CTGF-induced EMT but only ECM accumulation (24), our study emphasizes that CTGF affects both phenomena that are implicated in renal fibrosis (34). Our study showed not only that CTGF-induced EMT but also that EMT induced by AGE or TGF- $\beta$ 1 can be attenuated by CTGF blockade. However, CTGF-induced EMT was not blocked by neutralizing anti-TGF- $\beta$ 1 antibodies. These findings suggest that CTGF is an important stimulus for downstream mediation of EMT.

Although the most powerful effects were seen with AGE-BSA, modest effects also were observed with BSA. It should be noted that (animal-derived) BSA contains a small degree of AGE modification, albeit less than that observed in AGE-BSA. Therefore, the effects that were observed with BSA partly could be a result of the endogenous AGE modification of BSA preparations. However, these effects were completely attenuated by blockade of CTGF synthesis using siRNA, suggesting that CTGF also has a key intermediate role in this process.

Recent studies, although primarily performed in mesangial cells, suggest that CTGF upregulation in response to AGE is partly TGF- $\beta$ 1 independent, providing an additional rationale to target CTGF specifically (34,40). Our studies support the finding showing that CTGF sense adenovirus was able to induce EMT without altering the gene expression of TGF- $\beta$ 1, consistent with previous reports in proximal tubular cell systems (16). In addition, the failure of neutralizing anti-TGF- $\beta$ 1 antibodies to prevent CTGF-induced EMT suggests that the activation of TGF- $\beta$ 1, as proposed as one of the actions of CTGF (41), does not have an important role in this process.

The relative importance of EMT as a pathway for progressive fibrosis in the diabetic kidney is as yet unknown. Although tubular EMT was demonstrated previously in both rat and mouse models of progressive diabetic nephropathy, as well as in kidneys from patients with longstanding type 1 diabetes (3), most research in diabetic nephropathy generally has focused on the glomerular lesions in this condition. Nevertheless, increasingly, it is appreciated that tubular disease plays an important role in the progression of diabetic nephropathy (42). The identification and co-localization of the various putative mediators and manifestations of renal EMT in the same tubular cells in the diabetic kidney, such as AGE, CTGF, and  $\alpha$ -SMA, further strengthen the likelihood that the EMT pathway also is occurring in the diabetic tubulus. Furthermore, it is possible that many of the molecular and cellular changes, although not identical to those seen in tubular epithelia, also may be occurring in the diabetic glomerulus (12) and important phenotypic changes in podocytes that are associated with local changes in growth mediators in diabetes (43).

## Conclusion

Our study has extended our understanding of the pathways that link diabetes-associated stimuli, such as AGE and TGF- $\beta$ 1, to EMT and provides strong evidence of CTGF's playing a role in this phenomenon. The advent of new strategies to attenuate CTGF action as recently reported in another model of renal injury, ureteric obstruction (44), should allow this hypothesis to be tested specifically in the *in vivo* context of diabetes.

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