Static Pressure Regulates Connective Tissue Growth Factor Expression in Human Mesangial Cells*§

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Connective tissue growth factor (CTGF) is overexpressed in a variety of fibrotic disorders such as renal fibrosis and atherosclerosis. Fibrosis is a common final pathway of renal diseases of diverse etiology, including inflammation, hemodynamics, and metabolic injury. Mechanical strains such as stretch, shear stress, and static pressure are possible regulatory elements in CTGF expression. In this study, we examined the ability of static pressure to modulate CTGF gene expression in cultured human mesangial cells. Low static pressure (40–80 mm Hg) stimulated cell proliferation via a protein kinase C-dependent pathway. In contrast, high static pressure (100–180 mm Hg) induced apoptosis in human mesangial cells. This effect was reversed by treatment with CTGF antisense oligonucleotide but not with transforming growth factor β1-neutralizing antibody or protein kinase C inhibitor. High static pressure not only up-regulated the expression of CTGF, but also the expression of extracellular matrix proteins (collagen I and IV, laminin). This up-regulation of extracellular matrix proteins was also reversed by treatment with CTGF antisense oligonucleotide. As judged by mRNA expression of a total of 1100 genes, including apoptosis-associated genes using DNA microarray techniques, recombinant CTGF protein induced apoptosis by down-regulation of a number of anti-apoptotic genes. Overexpression of CTGF in mesangial cells by transient transfection had similar effects. Taken together, these results suggest that high blood pressure up-regulates CTGF expression in mesangial cells. High levels of CTGF in turn enhance extracellular matrix production and induce apoptosis in mesangial cells, and may contribute to remodeling of mesangium and ultimately glomerulosclerosis.

Connective tissue growth factor (CTGF)1,2 represents the latest addition to the list of growth factors implicated in the pathogenesis of renal fibrosis (3, 4). CTGF mRNA has been shown to be overexpressed in the extracapillary and severe mesangial proliferative lesions of crescentic glomerulonephritis, IgA nephropathy, focal, and segmental glomerulosclerosis and diabetic nephropathy (5). In anti-Thy1 nephritis, an animal model of acute glomerulonephritis, CTGF mRNA was shown to be up-regulated in both mesangial cells and podocytes (6). CTGF mRNA up-regulation in sclerotic glomeruli and fibrotic interstitium was also found in a chronic hypertension model in rats (uninephrectomized spontaneously hypertensive rats) (6). Hence, substantial evidence both in human disease as well as in experimental models of kidney disease suggests an important role of CTGF in renal diseases. Nonetheless, we have just begun to understand the regulation of CTGF expression in renal diseases. In particular, the role of high blood pressure in regulating CTGF expression has not yet been elucidated.

TGF-β is thought to be the major pathogenic factor in the development of renal fibrotic disorders. However, the development and progression of renal sclerosis is determined by complex interactions of many factors, including growth factors and direct hemodynamic action. In human skin fibroblasts, CTGF mRNA is specifically induced by TGF-β but not by platelet-derived growth factor, epidermal growth factor, or basic fibroblast growth factor (6). Recent reports also suggest a possible role for hepatic growth factor, interleukin-1β, interleukin-4, tumor necrosis factor-α, and vascular endothelial growth factor (VEGF) in regulation of CTGF (6, 7). Thus far, TGF-β has been shown to be the strongest inducer of CTGF expression in a variety of cells derived from different organs (8). In fact, Grotendorst et al. (8) found a unique TGF-β-responsive element within the promoter sequence of the CTGF gene, where point mutation of this responsive element abolished the regulatory effect of TGF-β on CTGF gene expression. More importantly, in anti-Thy1 nephritis, TGF-β and CTGF are expressed in coordinate fashion, suggesting the important role of TGF-β in regulating CTGF expression in vivo (3, 6).

Hypertension has been shown to independently accelerate the progression of chronic renal failure in humans irrespective of the pathogenesis of the renal disease (9, 10). The potential role of local hemodynamic changes on the progression of glomerulosclerosis and renal fibrosis has also been extensively studied in animal models. In patients with hypertension and diabetic nephropathy, the autoregulation of glomerular pressure is impaired, resulting in exposure of the capillary bed to systemic blood pressure fluctuation reaching unphysiologically high levels of local pressure. In normal tissue, this peak level of pressure is absorbed by the elasticity of the mesangium in the kidney glomeruli. The increased capillary pressure causes the mesangial area, including the mesangial cells to spread and expand. At the same time, the increased pressure also compresses the cells in a tangential direction. Chronic exposure to...
high blood pressure eventually leads to chronic expansion and remodeling of the mesangial area with accumulation of extracellular matrix and finally glomerulosclerosis. Numerous locally produced factors, including TGF-β and PDGF have been implicated in this mechanically induced mesangial expansion and remodeling. However, it is impossible to separate the effect of stretch and tangential compressive strain by pressure, in vivo. Therefore, to study these differential effects of mechanical strain on mesangial cells, we have utilized in this study an in vitro model in which only static pressure was applied to mesangial cells in culture without stretch (11–14). Cyclic stretching increases production and release of various vasoactive substances and growth factors in cultured cells, including TGF-β, which in turn up-regulates CTGF mRNA expression (15–20). So far, the role of static pressure in regulating CTGF expression in mesangial cells has not been elucidated. We now provide direct evidence that high static pressure up-regulates CTGF levels in CTGF-mediated apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents—Human renal mesangial cells were obtained (21). Cells were cultured in MCDB 131 (Clonetics) and from passages 2 to 4 from three different kidneys were used for experiments. All experiments were performed after 24 h of incubation in 1% fetal bovine serum. TGF-β1-neutralizing antibody was obtained from (R&D Systems). Treatment with TGF-β1 (Roche Molecular Biochemicals) for 48 h reduced cell viability of human mesangial cells in a concentration-dependent manner (1–10 ng/ml), and pretreatment with the neutralizing antibody (10 ng/ml) completely prevented it. On the other hand, treatment with PDGF-BB (Roche Molecular Biochemicals) (5 ng/ml) stimulated cell proliferation in human mesangial cells, but pretreatment with the neutralizing antibody never prevented it. The specificity and efficacy of the neutralizing antibody is available on the Web from R&D Systems. Chelerythrine was purchased from LC laboratories.

Exposure of Mesangial Cells to Constant Static Pressure—The pressure-loading apparatus was set up as previously described (11–14). The culture plates and dishes were placed in the pressure chamber containing 5% CO₂ and the chamber was completely sealed using clamps. The inner pressure was raised using compressed helium gas, and the chamber was kept at 37 °C in a thermal incubator. Whereas the helium gas was being pumped into the sealed chamber, no air in the sealed chamber was released, so that the partial pressures of the gases contained in the chamber such as oxygen, nitrogen oxide, and carbon dioxide were kept constant in accordance with Boyle-Charles’s law. In fact, there were no significant changes in pH and partial pressure of oxygen in culture medium throughout the experiments (up to 48 h). Mesangial cells were cultured under conditions to mimic the possibility of stretch and spreading under high static pressure.

Preparation of Antisense and Scrambled CTGF Oligonucleotide—16-mer CTGF antisense phosphorothioate oligonucleotide (5’-TACTGGCGGCGGTCACT-3’) containing the initial ATG translation start site was synthesized and purchased from Amersham Pharmacia Biotech as described previously (22). An oligonucleotide containing a scrambled nucleotide sequence (5’-GGTCATCGTGC-3’) was used as control. The synthetic oligonucleotides were added directly to the cell culture medium (final concentration, 20 μg/ml).

Cell Viability—Cell viability was evaluated using an MTT assay kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions (23). After exposure to increased static pressure (40–160 mm Hg), cells were incubated for an additional 4 h in the presence of MTT reagent under atmospheric pressure. Cells were then lysed with lysis buffer provided in the kit, incubated overnight at 37 °C, and absorbance was measured at A690 nm to A550 nm.

Nuclear Morphology—Both floating and trypsinized adherent mesangial cells were collected, washed with phosphate-buffered saline, fixed with fresh 10% paraformaldehyde for 30 min, and incubated in Hoechst 33258 (Sigma) at room temperature for 30 min (final concentration, 30 μg/ml) (23). Nuclear morphology was examined using fluorescence microscopy with standard excitation filters. To calculate the percentage of apoptotic cells, all cells from four randomly selected microscopic fields were counted at 400 × magnification.

DNA Fragmentation—Tdt-mediated dUTP biotin nick end-labeling (TUNEL) was performed with the In Situ Cell Death Detection Kit from Roche Molecular Biochemicals according to the manufacturer’s instructions (23). To calculate the percentage of TUNEL-positive cells, all cells from four randomly selected microscopic fields were counted at 100 × magnification. DNA fragmentation was measured using the Cell Death Detection ELISA PLUS kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Western Blot Analysis—Cell lysates (20 μg) were subjected to 12.5% percentage gel SDS-polyacrylamide gel electrophoresis (Ready Gel, Bio-Rad), transferred to polyvinylidene difluoride membranes (Bio-Rad), and incubated with anti-CTGF antibody at 1:250 dilution for 1 h as previously described (24). Equal amounts of protein loading were confirmed by Coomassie Brilliant Blue staining before blotting. The membranes were visualized using an ECL kit (Amersham Pharmacia Biotech). Semi-quantitative analyses of the blots were performed using the public domain IMAGE 1.60 program for Apple Macintosh from the National Institutes of Health.

Plasmid Constructs—To overexpress the CTGF protein in human mesangial cells, a mammalian expression vector (pCMV-CTGF) containing the complete open reading frame of the CTGF gene, driven by the CMV promoter, was constructed (23, 24). Mesangial cells transfected with pCMV vector alone were used as controls. Transient transfection was performed using Superfect reagent (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. Transfection efficiency was evaluated by fluorescence microscopy in cells co-transfected with plasmid containing the green fluorescence protein gene (pEGFP-C1; CLONTECH). The average transfection efficiency using 1 μg of pEGFP-C1 and 1 μg of pCMV-CTGF in 1 × 10⁵ human mesangial cells was calculated to be about 30%.

DNA Microarray—The DNA microarray hybridization experiments were performed using IntelliGene DNA chips (codes X101, X102, X103) (Takara, Tokyo, Japan) according to the manufacturer’s protocol. The protocol and the complete listing of genes on Intelligent DNA chips are available on the Web. The DNA arrays were scanned using ScanArray (GS Lumonics), and the data were analyzed using the Quant Array (BM BIO, Tokyo, Japan). Results of important genes are shown in Figs. 4 and 7, and all results are provided in the supplemental material.

Statistics—Statistical analysis of the data was performed using analysis of variance followed by Fisher’s test. p < 0.05 was considered statistically significant.

RESULTS

Low Static Pressure Stimulates Cell Proliferation, but High Static Pressure Reduced Cell Viability—Exposure of mesangial cells to low static pressure (80 mm Hg) for 48 h significantly increased the number of viable cells as compared with cells exposed to atmospheric pressure (Fig. 1A). This increase was inhibited by chelerythrine (CHE, 0.6 μM), a selective protein kinase C inhibitor, but not by CTGF antisense oligonucleotide (AS, 20 μg/ml), scrambled oligonucleotide (SC, 20 μg/ml), or TGF-β1-neutralizing antibody (nAb, 10 μg/ml) (Fig. 1B). In contrast, exposure of human mesangial cells to high static pressure (140 and 180 mm Hg) significantly reduced cell viability as compared with cells exposed to atmospheric pressure (Fig. 1A), and this effect was reversed by treatment with CTGF antisense oligonucleotide but not by scrambled oligonucleotide, protein kinase C inhibitor, or TGF-β1-neutralizing antibody at the same concentrations (Fig. 1C).

High Static Pressure Induces Apoptosis via CTGF—To investigate the mechanisms of high static pressure-induced reduction in cell viability, we performed nuclear staining of mesangial cell culture using Hoechst nuclear stain. As shown in Fig. 2A, exposure to high static pressure (140 mm Hg) significantly increased the number of apoptotic cells. DNA fragmentation analysis (Fig. 2B) and TUNEL staining (Fig. 2C) confirmed that the reduced cell number is due to the increase in the number of apoptotic cells. This effect was inhibited by treatment with CTGF antisense oligonucleotide (20 μg/ml). Control experiments using scrambled oligonucleotide (20 μg/ml) or TGF-β1-neutralizing antibody (10 μg/ml) had no effect (Fig. 2, A–C), suggesting that CTGF directly mediates the high static pressure-induced apoptosis in these cells.

High Static Pressure Induces CTGF—To clarify whether...
high pressure induced CTGF expression at protein level as well, we performed Western blot analysis of CTGF protein expression in human mesangial cells exposed to 80 or 140 mm Hg static pressure for 48 h. As shown in Fig. 3, exposure to high static pressure (140 mm Hg), but not to low static pressure (80 mm Hg), significantly increased CTGF protein expression. This effect was significantly reduced by treatment with CTGF antisense oligonucleotide (20 μg/ml) but not by scrambled oligonucleotide (20 μg/ml) or TGF-β1-neutralizing antibody (10 μg/ml) (Fig. 3B). Treatment with protein kinase C inhibitor Chelerythrine up to 0.6 μM also had no effect (data not shown).

**High Static Pressure Induces Mesangial Cell Matrix Production**—To investigate the effects of pressure-induced CTGF overexpression on matrix production, human mesangial cells were incubated in the presence and absence of CTGF antisense oligonucleotide for 24 h. Again, scrambled oligonucleotide was used as control. The expression of extracellular matrix mRNA was analyzed using Intelligene DNA chips. The signal intensity of the DNA arrays between treated and non-treated samples was adjusted to that of glyceraldehyde-3-phosphate dehydrogenase or β-actin. The signal intensity of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase or β-actin was unchanged in treated versus non-treated samples and was arbitrarily set to 1 for use as a control. As compared with cells exposed to atmospheric pressure, exposure to 140 mm Hg static pressure up-regulated not only CTGF mRNA but also collagen types I and IV and laminin mRNA expression in human mesangial cells (Fig. 4A). On the other hand, mRNA expression of TGF-α, TGF-β1, TGF-β2, and TGF-β3 was never changed by high static pressure (Fig. 4A). Under this condition (140 mm Hg), addition of CTGF antisense oligonucleotide (20 μg/ml) to the culture medium down-regulated CTGF, collagen types I and IV and laminin mRNA but never modulated that of fibroblast growth factor, hepatocyte growth factor, vascular endothelial growth factor, and platelet-derived growth factor (Fig.
sure were treated with recombinant CTGF (10 ng/ml) using Intelligene DNA chips. Cells grown in atmospheric pressure of human mesangial cells, we performed DNA microarray experiments to investigate the mechanism by which CTGF induces apoptosis in human mesangial cells (Fig. 6, A–C). Transfection of human mesangial cells with recombinant CTGF protein significantly reduced cell viability with increased DNA fragmentation and TUNEL-positive cells. In addition, transient transfection of human mesangial cells with an expression vector containing the complete open reading frame of the human CTGF gene driven by the CMV promoter (pCMV-CTGF) resulted in CTGF overexpression and significant reduction of cell viability, as judged by Western blot analysis. Fluorescence intensity was normalized to that of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase or β-actin were used as control. As shown in Fig. 7A, recombinant CTGF up-regulated mRNA expression of several extracellular matrix protein. Moreover, several anti-apoptotic genes, including BCL2-like2, BCL2A1, DAD1, apoptosis inhibitor 1, apoptosis inhibitor 2, and survivin were considerably down-regulated (up to 5-fold), and several caspase genes (caspases 1, 3, 7, 8, and 9) were up-regulated (up to 1.7-fold) in human mesangial cells treated with recombinant CTGF protein.

CTGF Induces Apoptosis in Human Mesangial Cells—To elucidate the direct effects of CTGF on cell survival, human mesangial cells were treated with recombinant human CTGF (0.05–10 μg/ml) for 48 h. As shown in Fig. 5 (A–C), treatment of mesangial cells with recombinant CTGF protein significantly reduced cell viability with increased DNA fragmentation and TUNEL-positive cells. In addition, transient transfection of human mesangial cells with an expression vector containing the complete open reading frame of the human CTGF gene driven by the CMV promoter (pCMV-CTGF) resulted in CTGF overexpression and significant reduction of cell viability, as well as increases in DNA fragmentation and TUNEL-positive cells (Fig. 6, A–C). Transfection of human mesangial cells with the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase or β-actin were used as control. As shown in Fig. 7A, recombinant CTGF up-regulated mRNA expression of several extracellular matrix protein. Moreover, several anti-apoptotic genes, including BCL2-like2, BCL2A1, DAD1, apoptosis inhibitor 1, apoptosis inhibitor 2, and survivin were considerably down-regulated (up to 5-fold), and several caspase genes (caspases 1, 3, 7, 8, and 9) were up-regulated (up to 1.7-fold) in human mesangial cells treated with recombinant CTGF protein.

DISCUSSION

In the present study, we provide for the first time evidence that high static pressure induces up-regulation of CTGF expression and that this up-regulated CTGF expression is involved in overproduction of extracellular matrix and apoptosis in human mesangial cells. This is based on the following observations: (i) exposure to high static pressure increased CTGF mRNA and protein expression in human mesangial cells; (ii) exposure to high static pressure induced apoptosis in human mesangial cells, and this effect was reversed by treatment with CTGF antisense oligonucleotide; (iii) exposure to high static pressure as well as treatment with recombinant CTGF protein increased mRNA expression of extracellular matrix protein in human mesangial cells, and this effect was inhibited by treatment with CTGF antisense oligonucleotide; (iv) treatment with...
recombinant CTGF protein or transient overexpression of the CTGF gene induced apoptosis in human mesangial cells; and (v) treatment with recombinant CTGF protein down-regulated anti-apoptotic genes.

Circumstantial evidence suggests an important role of CTGF in the development of glomerulosclerosis in patients with diabetic nephropathy (3, 5). Although the mechanism by which CTGF affects renal function in vivo is not yet clear, Murphy et al. (22) recently demonstrated that high glucose stimulates mesangial CTGF expression and matrix production by inducing TGF-β. Hypertension and hyperglycemia are well known independent pathogenic factors in the development of glomerulosclerosis in diabetes patients. Indeed, the present study provides the evidence that CTGF expression in cultured mesangial cells is regulated by static pressure. Increased CTGF activity in turn increased extracellular matrix production and induced apoptosis in these cells, suggesting the important role of CTGF overexpression induced by high static pressure in mesangial expansion, mesangial matrix accumulation, remodeling, and, ultimately, glomerulosclerosis. Therefore, it is conceivable that high blood pressure may also affect the renal function via CTGF pathway similar to hyperglycemia. The increase in proliferation of human mesangial cells induced by exposure to low static pressure (80 mm Hg) was abolished by treatment with the specific protein kinase C inhibitor chelerythrine (14). These results were in line with recent studies showing the inhibitory effect of protein kinase C inhibitors in proliferation of rat mesangial cells and rat vascular smooth muscle cells (13, 25). In contrast, our results showed that increased CTGF expression and apoptosis in human mesangial cells exposed to high static pressure is protein kinase C-independent, contrary to the effect of high glucose-induced CTGF expression in rat mesangial cells, which is protein kinase C-dependent (26). Interestingly, Fan et al. (27) reported that activation of protein kinase C inhibited CTGF induction. A recent report by Suzuma et al. (7) indicated that vascular endothelial growth factor (VEGF) induced up-regulation of CTGF expression in bovine retinal capillary cells. This effect was mediated primarily by phosphatidylinositol 3-kinase activation, whereas involvement of protein kinase C and ERK pathways were only minimal. These results suggest heterogeneity in the regulation of CTGF by a variety of signaling pathways in different cell types.

CTGF has been shown to be mitogenic to NRK cells and chemotactic to NIH3T3 cells (1) and to induce connective tissue cell proliferation and extracellular matrix synthesis in skin fibroblasts (reviewed in Ref. 2). On the other hand, CTGF is also expressed at very high levels in non-proliferating prolifera-
ating cell nuclear antigen-negative smooth muscle cells in atherosclerotic lesions in human (28). In human chondrosarcoma, proliferating cell nuclear antigen expression is negatively correlated with CTGF expression (29). Taken together, these findings support a pro-apoptotic function of CTGF in a variety of human cells. Indeed, we showed that transient overexpression of CTGF in human aortic smooth muscle cells (24) and human breast cancer cells (MCF-7) (23) as well as treatment of these cells with recombinant human CTGF protein induced apoptosis (23, 30). The recombinant CTGF protein used in the present study was able to stimulate cell proliferation of normal rat kidney cells in a dose-dependent manner. However, this same CTGF preparation has no mitogenic effect on rat or human mesangial cells, or rat or human vascular smooth muscle cells. Thus far, the reasons for these opposite effects of CTGF on different cell type are not clear. In our hands, transient overexpression of CTGF by transfection with the CTGF gene construct appears to be more effective than using purified recombinant CTGF protein in inducing apoptosis. Recently, Kubota et al. (31) found that overexpressed CTGF protein was predominantly localized intracellularly in COS-7 cells transfected with similar construct and that intracellular CTGF acts as an anti-mitogenic factor in these cells by modulating the cell cycle. Taken together, these observations corroborate our findings and suggest multiple functions for CTGF depending on the location and the cell types where CTGF is found.

To gain insight into the biological role of CTGF in human mesangial cells, we used DNA microarrays containing 1100 genes, including apoptosis-associated genes to analyze CTGF-regulated genes in human mesangial cells. As shown in Fig. 7, recombinant CTGF protein failed to up-regulate the 1100 genes contained in the DNA microarrays by more than 2.0-fold except extracellular matrix protein. In contrast, several anti-apoptotic genes were down-regulated by up to 5-fold, and several caspase genes were up-regulated by up to 1.7-fold in human mesangial cells treated with recombinant CTGF. Recombinant CTGF protein used in this study has also been shown to induce apoptosis in MCF-7 cells and human vascular smooth muscle cells by down-regulating bcl2 expression and increasing caspase 3 activity (24, 30). Anti-apoptotic genes such as apoptosis inhibitor protein and bcl2 are known to inhibit caspase 3 activity. A specific inhibitor of caspase 3 such as Z-Asp(Ome)-Glu(Ome)Val-Asp(Ome)-FMK (32) was also able to prevent CTGF-induced apoptosis in human mesangial cells. Hence, down-regulation and diminished inhibitory effect of inhibitor protein and bcl2 in human mesangial cells induced by high static pressure or CTGF overexpression may increase caspase 3 activity and mediate the apoptotic effect of high static pressure via the CTGF-dependent pathway.

Recently, Hahn et al. (33) reported that the regulation of CTGF expression in mesangial cells was mediated by changes in actin cytoskeleton. It is conceivable that static pressure may also modulate cytoskeleton and thus modulate CTGF expression. Mechanical stimuli such as shear stress, cyclic stretch,
and static pressure could induce different alterations of the cell surface, and activate different signaling pathways, but the precise mechanism remains to be determined. For example, stretch increased 1,4,5-inositol trisphosphate concentration only transiently (34), but static pressure induced sustained high levels of 1,4,5-inositol trisphosphate production (13). Stretch also rapidly increased intracellular calcium concentration, but shear stress and static pressure did not (13, 35). These differences in response to mechanical stimuli may explain why high static pressure increased sustained high level CTGF mRNA expression and cyclic stretch only induced transient overexpression of CTGF mRNA in rat mesangial cells (20).

In conclusion, high static pressure appears to induce CTGF expression in human mesangial cells. The precise mechanism by which high static pressure regulates CTGF expression and apoptosis remains to be elucidated. Nevertheless, our results clearly show that hypertension regulates extracellular matrix production and apoptosis in human mesangial cells via CTGF and thus may play a more substantial role in accelerating the development of glomerulosclerosis than previously thought. Our results also suggest that CTGF could serve as a potential therapeutic target for the prevention and treatment of hypertension-induced mesangial expansion, remodeling, and sclerotic process in kidney diseases.

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