Expression of connective tissue growth factor in human renal fibrosis

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Expression of connective tissue growth factor in human renal fibrosis. Chronic renal failure may occur in etiologically diverse renal diseases and can be caused by hemodynamic, immunologic and metabolic factors. Initial damage may evoke irreversible scarring, which involves production of a number of proinflammatory and fibrogenic cytokines, including platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). Connective tissue growth factor (CTGF), a cytokine of the family of growth regulators comprising cef10, cyr61, CTGF and nov, has recently been described in association with scleroderma and other scarring conditions. We investigated CTGF mRNA expression in 65 human renal biopsy specimens of various renal diseases by in situ hybridization. In control human kidney CTGF mRNA was mainly expressed in visceral epithelial cells, parietal epithelial cells, and some interstitial cells. Connective tissue growth factor was strongly up-regulated in the extracapillary and severe mesangial proliferative lesions of crescentic glomerulonephritis, IgA nephropathy, focal and segmental glomerulosclerosis and diabetic nephropathy. An increase in the number of cells expressing CTGF mRNA was observed at sites of chronic tubulointerstitial damage, which correlated with the degree of damage. In the tubulointerstitial area the majority of the CTGF mRNA positive cells coexpressed α-smooth muscle actin, and were negative for macrophage markers. Our results indicate that CTGF may be a common growth factor involved in renal fibrosis.

Fibrosis is the final common pathway for almost all forms of renal disease that progress to end-stage renal failure including immunologically mediated glomerulonephritis and tubulointerstitial nephritis, hemodynamic disorders, metabolic diseases, and hereditary diseases [1]. Histologically, such scarring consists of glomerulosclerosis, tubulointerstitial fibrosis, as well as vascular hyalinosis and sclerosis. Whatever the primary disturbance, further mediators are required to cause renal scarring characterized by cell proliferation and accumulation of matrix constituents [2]. An important role in this process was shown for the cytokines platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), as indicated by in vivo modulation of the activity of these cytokines [3]. Blocking of the activity of PDGF or TGF-β was shown to prevent extracellular matrix expansion and glomerulosclerosis [4–7]. Connective tissue growth factor (CTGF) is a cysteine-rich member of a new family of growth regulators, which are comprised of cef10, cyr61, CTGF and nov [8–12]. Connective tissue growth factor was originally cloned from human umbilical vein endothelial cells (HUVEC) [8]. Subsequently, skin fibroblasts in scleroderma lesions were found to overexpress CTGF [13]. By using a differential cloning technique, a cDNA clone identical to CTGF was isolated from a human atherosclerotic aorta cDNA library [14], in which expression was found to be 50- to 100-fold increased as compared to normal arteries [14]. Connective tissue growth factor was shown to induce kidney fibroblast proliferation and extracellular matrix synthesis [15]. Effects of TGF-β on fibroblasts were found to be partially mediated by CTGF [16].

The present study proposes the possible involvement of CTGF in renal fibrosis. As a first step in the elucidation of its possible role in renal sclerosis, we investigated the expression of human CTGF mRNA in human biopsy specimens of various renal diseases by in situ hybridization.

METHODS

Human kidney specimens

Sixty-five specimens from control and diseased human kidneys were studied. Kidney samples were obtained from patients undergoing diagnostic evaluation at the Academic Medical Center of the University of Amsterdam. Control human kidney specimens (N = 5) were taken from normal portions of nephrectomy specimens of patients who underwent surgery because of localized renal tumors. Specimens from diseased kidneys were obtained by percutaneous renal biopsy from the patients with various renal diseases. The histologic diagnoses are listed in Table 1. These included minimal change nephrotic syndrome (MCNS), IgA nephropathy (IgAN), idiopathic membranous nephropathy (MN), focal glomerulosclerosis (FGS), crescentic anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, lupus nephritis (LN), diabetic glomerulosclerosis (DM), membranoproliferative glomerulonephritis (MPGN), chronic rejection of transplanted kidneys, acute postinfectious glomerulonephritis, and nephrosclerosis. The classification of LN was based on the WHO...
Denhardt’s solution, 0.1% Triton X, 200 mM ethanol, dissolved in 100 mM Tris-HCl, pH 8.0 (Boehringer-Mannheim, Germany). One microgram of linear template DNA was used in each labeling reaction. The labeling reaction was performed using an RNA Labeling Kit (Boehringer-Mannheim) and digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany). The digoxigenin-11-UTP was synthesized using a DIG RNA Labeling Kit (Roche Diagnostics). The labeled RNA was purified using a Nunc RNeasy Mini Kit (Invitrogen, Carlsbad, CA, USA) and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**Immunohistochemistry**

Serial formalin-fixed paraffin sections were deparaffinized with xylene, rehydrated, and washed with phosphate-buffered saline (PBS). After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide and free protein binding sites with normal goat serum, the tissue was incubated with primary murine monoclonal antibodies, for α-smooth muscle actin (α-SMA, 1A4; DAKO) or for CD68 (PG-M1; DAKO) during two hours at room temperature. Immunoreactivity for CD68 was enhanced by microwave oven heating of sections in 10 mM sodium citrate, pH 6.0, for 10 minutes. Immunoreactivity for CD68 was enhanced by microwave oven heating of sections in 10 mM sodium citrate, pH 6.0, for 10 minutes. Immobilized mouse antibodies were detected by the immunalkaline phosphatase anti-alkaline phosphatase method (DAKO), or a streptavidin-biotin-immunoperoxidase technique using the strept ABC complex/HRP kit (DAKO). The specificity was checked by omission of primary antibodies and use of non-immune mouse IgG as negative control.

**Immunocytochemistry-in situ hybridization double labeling**

In situ hybridization in combination with immunohistochemistry was performed on the same section to simultaneously detect CD68 or α-SMA and CTGF mRNA. Sections were first hybridized with DIG-RNA probe and transcripts were detected. After washing with PBS, endogenous biotin present in renal tissue was blocked with 1 mg/ml streptavidin (Zymed, San Francisco, CA, USA) and 0.1 mg/ml d-biotin (Sigma) in two successive steps. Then tissues were stained with α-smooth muscle actin or CD68 monoclonal antibody. Microwave pretreatment enhanced the immunoreactivity for CD68. Presence of CTGF mRNA was visualized with NBT/BCIP (purple-black) and α-SMA or CD68 were stained with diaminobenzidine (brown). The results of the double staining experiments showed staining patterns and staining intensities similar to those obtained in simultaneously performed single staining experiments.

**Correlation between connective tissue growth factor mRNA expression and tubulointerstitial injury**

The correlation between interstitial CTGF expression and chronic tubulointerstitial injury was assessed in 45 cases. Cases of acute renal injury (6 cases of LN, and 7 cases of crescentic ANCA-associated glomerulonephritis) were excluded. Another seven cases were excluded because these lacked adequate tissue for quantification of the interstitium. Tubulointerstitial expression of the CTGF gene was determined by counting the CTGF mRNA
positive cells within the whole cortical area of each biopsy. At least twenty fields of 0.044 mm² were assessed and the average number of positive cells in 0.1 mm² was calculated. Tubulointerstitial injury was classified into four groups, according to the extent of cortical interstitial fibrosis and of tubular atrophy and degeneration: (1) normal; (2) involvement up to 25% of the cortex; (3) involvement of 26 to 50% of cortex; and (4) extensive damage involving more than 50% of the cortex. This is comparable to the chronic change score in the Banff classification of kidney transplant pathology [18].

Culture of glomerular visceral epithelial cells

For this study an established cell-line of glomerular visceral epithelial cells (GVEC) was used, derived from Sprague-Dawley rat glomeruli as has been described elsewhere [19–21]. This cell line was found to express a ganglioside reported to be specific for visceral and not parietal epithelial cells of the glomerulus [22]. The cell line was cultured and maintained in K1 medium containing hormone mix (all ingredients from Sigma), 100 U/ml penicillin (Gibco, Gaithersburg, MD, USA), 100 µg/ml streptomycin (Gibco) and 5% NuSerum (Collaborative Research Inc., Bedford, MA, USA) on a collagen gel (Vitrogen 100; Collagen Corp, CA, USA) in a humidified 5% CO₂—95% air incubator, as described [19]. Experiments were performed with GVEC at passage numbers 25 to 30.

RNA extraction and Northern blot analysis

Total RNA was isolated from renal cortical tissues of nephrectomy specimens and from cultured GVEC by the TRIzol method (Life Technologies, MD, USA).

Fifteen micrograms of total RNA were size separated by electrophoresis on a 0.22 m formaldehyde-1% agarose gel, transferred to a nylon membrane, (Boehringer-Mannheim), and U.V. cross-linked. Before transfer to a nylon membrane, ethidium-stained gels were visualized by ultraviolet illumination to determine the position of 28S and 18S ribosomal RNA and to assess the integrity of the RNA. Prehybridization and hybridization was performed in DIG Easy Hyb (Boehringer-Mannheim), according to the manufacturer's instructions. The Spearmann rank correlation coefficient was used for analysis of correlation.

RESULTS

Connective tissue growth factor expression in control kidney tissue

In the glomeruli of control human kidney, CTGF mRNA was expressed mainly by visceral epithelial cells. CTGF transcripts were also detected in some parietal epithelial cells (Fig. 1A). Some interstitial cells in the peritubular and periglomerular areas also showed CTGF expression (Fig. 1B). To determine the cellular origin of the CTGF mRNA positive cells in the tubulointerstitial area, tissue sections were double-labeled for α-SMA and CTGF mRNA and also for CD68 and CTGF mRNA. The majority of the CTGF mRNA positive cells coexpressed α-SMA and did not express CD68 antigen. CD68 positive cells also did not coexpress CTGF mRNA (Fig. 1 C, D). These cells resemble interstitial fibroblasts, but we could not completely exclude the possibility that CTGF mRNA might be expressed in endothelial cells of peritubular capillaries. All sections hybridized with a sense probe were negative and binding of the antisense probes to these cells was prevented by preincubation with RNase.

Localization of connective tissue growth factor transcripts in diseased kidney tissue

Connective tissue growth factor expression was found to be normal as compared to control or only marginally increased in glomerular diseases characterized by non-inflammatory lesions and proteinuria, such as MCNS and MN, and in acute postinfectious exsudative glomerulonephritis. In contrast, CTGF expression was found to be markedly increased in inflammatory glomerular and tubulointerstitial lesions, associated with cellular proliferation and matrix accumulation. These lesions include IgA nephropathy, chronic transplant rejection, crescentic glomerulonephritis, FGS, lupus nephritis (WHO class IV) and MPGN.

Glomerular lesions. In three out of five MCNS and three out of seven MN without glomerulosclerosis no up-regulation was observed as compared to that in control renal tissue samples. In the other two cases of MCNS and in the remaining four cases of MN without glomerulosclerosis, CTGF mRNA expression was slightly increased in visceral epithelial cells (Fig. 2). In disorders with extracapillary proliferative lesions, a significant increase of CTGF mRNA was noted in the glomeruli. In the cellular crescents of crescentic glomerulonephritis, IgA nephropathy, and diffuse lupus nephritis CTGF expression was strongly increased (Fig. 3 A, B). According to immunostaining for CD68 and α-SMA, macrophages and myofibroblasts are not the main components of the crescents (Fig. 3 C, D). This finding suggests that in these crescents CTGF is mainly expressed by proliferating epithelial cells. In the few remaining cells in fibrocellular crescents, CTGF mRNA expression was still high. Connective tissue growth factor expression was also increased in other extracapillary lesions, including segmental sclerosis with adhesion to Bowman’s capsule observed in FGS (Fig. 4) and MN with fibrosis. Although CTGF expressing cells were mainly epithelial cells, severe mesangial proliferative lesions in IgAN, diabetic diffuse lesions, and diffuse
lupus nephritis also expressed CTGF (Fig. 5 A, B). In contrast, CTGF was not up-regulated in postinfectious endocapillary proliferative nephritis in spite of the numerous infiltrating neutrophils (Fig. 5 C, D).

**Tubulointerstitial area.** Uninjured portions of renal cortical interstitial tissues were generally indistinguishable from the interstitial patterns of sparse CTGF mRNA expression described above in control kidneys. In the case of MCNS, FGS and MN without interstitial fibrosis, CTGF mRNA positive cells were rare in the tubulointerstitial area (Figs. 2 and 4). In contrast, increased

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**Fig. 1.** Demonstration of connective tissue growth factor (CTGF) mRNA in control human kidney by in situ hybridization. CTGF mRNA is expressed in visceral and parietal epithelial cells (A), and in some interstitial cells (B). Double-labeling is shown for α-SMA (brown) and CTGF mRNA (purple-black) (C), and for CD68 (brown) and CTGF mRNA (purple-black) (D). In tubulointerstitial areas with slight fibrosis the majority of interstitial CTGF mRNA positive cells coexpress α-smooth muscle actin (×250).

**Fig. 2.** In idiopathic membranous nephropathy, expression of connective tissue growth factor (CTGF) mRNA is slightly increased in podocytes but not present in the mesangial and tubulointerstitial area. This section was counterstained with PAS without hematoxylin (×200).

**Fig. 4.** Renal biopsy from a patient with focal glomerulosclerosis (FGS). PAS staining (A), and connective tissue growth factor (CTGF) mRNA (B). CTGF expression is increased in segmental sclerotic lesions with adhesion to Bowman’s capsule (×125).
Fig. 3. Renal biopsy from a patient with crescentic glomerulonephritis. PAS staining (A), connective tissue growth factor (CTGF) mRNA (B), CD68 (C), and α-smooth muscle actin (D). In the cellular crescent, CTGF mRNA is mainly expressed by proliferated epithelial cells (×200).

Fig. 5. In situ hybridization for connective tissue growth factor (CTGF) mRNA reveals CTGF upregulation in severe mesangial proliferative lesions of diabetes mellitus (DM) nephropathy diffuse lesion (A) and IgA nephropathy (B). In contrast, CTGF is not up-regulated in acute postinfectious glomerulonephritis (C, PAS stain; D, CTGF; ×200).
Fig. 6. Connective tissue growth factor (CTGF) expression in tubulointerstitial fibrosis. Renal biopsy of a case showing a focal zone of tubular atrophy and interstitial fibrosis (lower part of photograph), with adjacent areas of well-preserved tubular architecture. (A, PAS stain) Serial sections of the same biopsy show that CTGF mRNA is expressed strongly in the zones of interstitial fibrosis as compared to its expression in normal appearing adjacent tubular parenchyma (B). The distribution of CTGF is similar to that of α-SMA staining (C) (×80).

Fig. 7. Connective tissue growth factor (CTGF) expression in periglomerular fibrosis in a case of focal glomerulosclerosis (FGS) with hypertension (A, PAS stain; B, CTGF mRNA; and C, α-smooth muscle actin stain on serial sections). CTGF expression is increased in the periglomerular lesion and in lesions of proliferated visceral and parietal epithelial cells. CTGF is expressed by α-smooth muscle actin positive cells in the periglomerular interstitial fibrosis (×200).
numbers of CTGF mRNA positive cells were identified within the tubulointerstitial fibrotic areas in chronic transplant rejection, and in the chronic interstitial damage in the context of glomerulonephritis. Within these lesions, the distribution of CTGF mRNA positive cells was similar to that of α-SMA staining (Fig. 6). Also in periglomerular fibrosis, CTGF mRNA was strongly up-regulated in α-SMA positive cells (Fig. 7). A statistically highly significant correlation between the extent of tubulointerstitial fibrosis and the number of CTGF mRNA positive cells per surface area was demonstrated (Fig. 8) using the Spearman rank correlation coefficient \( r = 0.849; P < 0.001 \).

**Northern blot analysis**

By Northern analysis, a 2.4 Kb CTGF transcript was detected in RNA extracted from control human kidney (Fig. 9A) and from cultured podocytes (Fig. 9B).

**DISCUSSION**

A widely accepted paradigm in concepts of development of renal fibrosis is that TGF-β up-regulation is a main factor responsible for fibrotic changes and scarring in response to renal injury [3]. Its importance in glomerulosclerosis has been established by in vivo studies and by analysis of glomerular cells in culture [24–29]. Inhibition of overexpression of TGF-β by antisense oligonucleotides [30] or decorin [6, 7] suppressed the accumulation of extracellular matrix in experimental glomerulonephritis induced by anti-Thy-1.1 antibody. Transforming growth factor-β is a pleiotropic factor with many effects also not directly related to fibrosis and scarring, including immunosuppressive and anti-inflammatory activities [31]. Intervention by blocking TGF-β may therefore have conflicting effects. Identification of a factor downstream of TGF-β that is directly involved in the accumulation of matrix might provide a better target for intervention.

Studies by Grotendorst and Oemar have demonstrated that CTGF is a novel growth factor that can be up-regulated in fibroblasts and vascular smooth muscle cells by TGF-β [14, 32, 33]. It is a chemoattractant and mitogen for fibroblasts [8] and it also induces extracellular matrix deposition [15]. Connective tissue growth factor significantly increased type I collagen and fibronectin production, as well as expression of α5 integrin by a normal kidney fibroblast cell line [15]. In vivo subcutaneous injection of CTGF induced connective tissue cell proliferation and matrix deposition [15]. Moreover, the induction of anchorage-independent growth of fibroblasts by TGF-β appears to be mediated by a CTGF-dependent pathway [16]. It thus appears that CTGF may mediate at least part of the fibrosis inducing activity of TGF-β.

In the present study, using DIG-labeled RNA in situ hybridization on paraffin sections of human renal biopsies, we have observed that a strong increase of CTGF mRNA expression is a general phenomenon in proliferative and fibrotic renal lesions. In the glomeruli of control human kidney samples taken from nephrectomy specimens with renal cell carcinoma we detected baseline expression of CTGF in visceral and parietal epithelial cells. This is in agreement with detection of the 2.4 Kb CTGF mRNA transcript by Northern analysis of control kidney tissue, as well as of cultured podocytes which thus appear to express CTGF under basal conditions.

In the glomerular lesions CTGF was not increased in postinfectious endocapillary GN, and also not or only slightly increased in MCNS and MN. In all other types of lesions characterized by cellular proliferation, CTGF expression was found to be up-regulated. The cell type expressing CTGF and the degree of up-regulation varied with the type of lesion. Since TGF-β has
been documented to be increased in a variety of chronic forms of renal injury and TGF-β was found to induce expression of CTGF in various cell types [14, 32, 33] including mesangial cells and podocytes (Y. Ito, manuscript in preparation), CTGF may have a role, downstream of TGF-β, in a final common pathway leading to fibrosis in etiologically diverse diseases. In addition to TGF-β, other stimuli may enhance CTGF expression. For instance, CTGF mRNA expression was found to be increased in the absence of TGF-β up-regulation in a monkey renal epithelial cell line, both after exposure to calcium-oxalate-monohydrate crystals and after scrape wounding of monolayers [34, 35].

With respect to the source of fibrogenic growth factors in the kidney, macrophages and mesangial cells have thus far received most attention. As far as CTGF is concerned, our results suggest that leukocytes do not significantly express this growth factor. Connective tissue growth factor expression in the mesangium was up-regulated in mesangial proliferative lesions, mainly in diabetic nephropathy and mesangial proliferative lesions in IgA nephropathy, but less so than CTGF expression in the epithelial lesions of other glomerulopathies. CTGF mRNA expression was strongly increased in particular in extracapillary proliferative lesions, capsular adhesions, and periglomerular fibrosis. Three different cell types including interstitial fibroblasts, mesangial cells, and epithelial cells have been shown to express CTGF mRNA. It should be noted that these cells all share a mesodermal origin. Endothelial CTGF expression in glomeruli was not prominent. However, CTGF can be expressed by endothelium from other vessels, like human umbilical vein [8] and atherosclerotic arteries [14].

Tubulointerstitial injury is an important parameter in the assessment of renal damage because it correlates well with the decline in renal function and long-term progression [36, 37]. Many potentially fibrogenic factors, including TGF-β, have been implicated in tubulointerstitial injury [38–41]. Our present data show a strong increase at the sites of chronic tubulointerstitial damage of the number of cells expressing CTGF mRNA, which significantly correlates with the degree of tubulointerstitial damage. In the tubulointerstitial area, macrophages and fibroblasts are the most important source of TGF-β [2]. Transforming growth factor-β [42] and PDGF-B [43] were shown to induce transformation of fibroblasts to myofibroblasts (α-smooth muscle actin positive cells), which are major contributors to matrix deposition in the tubulointerstitial area. The presence of myofibroblasts within the renal interstitium correlates with the extent of tubulointerstitial scarring and functional outcome in clinical [44, 45] and experimental glomerulonephritis [46, 47]. In both control and pathological conditions, we observed that the majority of CTGF positive cells in the tubulointerstitial area were myofibroblasts, as defined by coexpression of α-smooth muscle actin, and not macrophages. This suggests both autocrine and paracrine inductions of this factor by TGF-β.

In summary, a local increase of CTGF expression may play a role in the development and progression of glomerulosclerosis and tubulointerstitial fibrosis. The relative importance of CTGF in these different processes will have to be established by manipulation of CTGF levels in the presence or absence of other fibrogenic cytokines.

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APPENDIX

Abbreviations used in this article are: α-SMA, α-smooth muscle actin; ANCA, anti-neutrophil cytoplasmic antibody; CTGF, connective tissue growth factor; DEPC, diethyl pyrocarbonate; DIG-UTP, digoxigenin-labeled uridine-triphosphate; DM, diabetes mellitus; FGS, focal glomerulosclerosis; GVEC, glomerular visceral epithelial cells; HE, hematoxylin and eosin stain; HUVEC, human umbilical vein endothelial cells; IgAN, IgA nephropathy; LN, lupus nephritis; MCNS, minimal change nephrotic syndrome; MN, idiopathic membranous nephropathy; NBT/BCIP, nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate; NTP, nucleotidetriphosphate; PAS, periodic acid-Schiff stain; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β.

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