Connective tissue growth factor in renal development and injury
Ito, Y.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Expression patterns of connective tissue growth factor and of TGF-β isoforms during glomerular injury recapitulate glomerulogenesis

Yasuhiko Ito, Roel Goldschmeding, Hirotake Kasuga, Nike Claessen, Masahiro Nakayama, Yukio Yuzawa, Akiho Sawai, Seiichi Matsuo, Jan J Weening, Jan Aten


doi:10.1152/ajprenal.00120.2009
http://ajprenal.physiology.org/content/299/3/F545.full.html
Supplemental material for this article can be found at:
http://ajprenal.physiology.org/content/suppl/2010/06/30/ajprenal.00120.2009.DC1.html

Used with permission of the American Physiological Society.
ABSTRACT

TGFβ1, -β2, and -β3 are involved in control of wound repair and development of fibrosis. Connective tissue growth factor expression is stimulated by all TGFβ isoforms and is abundant in glomerulosclerosis and other fibrotic disorders. CTGF is hypothesized to mediate profibrotic effects of TGFβ1 or to facilitate interaction of TGFβ1 with its receptor, but its interactions with TGFβ isoforms in nonpathological conditions are unexplored so far. Tissue repair and remodeling may recapitulate gene transcription at play in organogenesis. To further delineate the relationship between CTGF and TGFβ, we compared expression patterns of CTGF and TGFβ isoforms in rat and human glomerulogenesis, and in various human glomerulopathies. CTGF mRNA was present in the immediate precursors of glomerular visceral and parietal epithelial cells in the comma- and S-shaped stages, but not in earlier stages of nephron development. During the capillary loop and maturing glomerular stages and simultaneous with the presence of TGFβ1, -β2 and -β3 protein, CTGF mRNA expression was maximal and present only in differentiating glomerular epithelial cells. CTGF protein was also present on precursors of mesangium and glomerular endothelium, suggesting possible paracrine interaction. Concomitant with the presence of TGFβ2 and -β3 protein, and in absence of TGFβ1, CTGF mRNA and protein expression was restricted to podocytes in normal adult glomeruli. However, TGFβ1 and CTGF were again coexpressed, often with TGFβ2 and -β3, in particular in podocytes in proliferative glomerulonephritis and also in mesangial cells in diabetic nephropathy and IgA nephropathy. Coordinated expression of TGFβ isoforms and of CTGF may be involved in normal glomerulogenesis and possibly in maintenance of glomerular structure and function at adult age. Prolonged overexpression of TGFβ1 and CTGF is associated with development of severe glomerulonephritis and glomerulosclerosis.
INTRODUCTION

Connective tissue growth factor (CTGF or CCN2) is member of the CCN family of structurally related proteins (11, 14, 21). The CCN proteins contain an N-terminal secretory signal peptide and four structural domains: an insulin-like growth factor binding domain (domain 1); chordin-like cysteine-rich domain with similarity to von Willebrand Factor type c domain (domain 2), which is connected via a protease-sensitive hinge region to a thrombospondin type I repeat (domain 3); and finally a C-terminal cystine knot (domain 4) (1, 11). CCN proteins are involved in regulation of essential cell functions as adhesion, migration, mitogenesis, differentiation and survival (13, 29). As such, CCN family members have been implicated a.o. in control of wound repair, development of fibrosis, and tumorigenesis (20, 30, 44). CTGF in particular has been reported to be overexpressed in fibrosis of diverse organs (22, 34, 40, 42). We demonstrated that CTGF mRNA is strongly upregulated in human renal fibrosis and in rat experimental proliferative glomerulonephritis (23, 24).

TGF-β is a key component in control of wound repair and in development of fibrosis (7, 10). CTGF mRNA expression was observed to be associated with differential expression of the three closely related isoforms of TGF-β in the course of the anti-Thy-1.1 glomerulonephritis model (24). All three TGF-β isoforms are equally able to induce upregulation of CTGF mRNA in both cultured mesangial cells and glomerular visceral epithelial cells (24). In vitro effects of TGF-β1 on matrix synthesis by fibroblasts and mesangial cells do at least partially require the presence of CTGF (9, 30, 67). These findings suggest that CTGF may be involved in tissue repair in response to glomerular injury, possibly downstream of TGF-β. Tissue repair may recapitulate developmental programs at play in organogenesis and comparison of these processes may yield insight in the nature of common regulatory factors. In development of the kidney in mammals, the ureteric bud is induced by the metanephric blastema to develop from the mesonephric duct. Growth of the ureteric duct comprises both elongation of the duct and (asymmetric) branching. In turn, the metanephric blastema is induced by the invading ureter branch tips to undergo nephrogenesis involving mesenchymal cell condensation and transition into polarized epithelium forming a vesicle. The vesicle undergoes patterning to form subsequently comma-shaped and S-shaped bodies. Capillary sprouts as well as vasculogenic precursors of endothelial cells are recruited into the cleft that is formed by the proximal curvature of the S-shaped body. The proximal and distal parts of the S-shaped body differentiate into the glomerular epithelium and into the proximal and distal tubular epithelium, respectively.

Glomerulogenesis proceeds through the capillary loop stage and the maturing stage to the adult stage during which mesangial progenitor cells migrate into the glomerulus, the glomerular capillary network is shaped and the podocytes fully differentiate. The tips of the distal tubule and the ureteric duct fuse and further growth and transformation within the tubular compartment leads to formation of Henle’s loop (50, 51). In early stages of metanephric development in mice, the three TGF-β isoforms are
Glomerular co-expression of CTGF and TGFβs

expressed by the branching ureteric duct epithelium and TGF-β2 localization is restricted to the basal side of a subset of these epithelial cells (43). During further ramification, expression of all TGF-β isoforms decreases in the tubular epithelium and becomes apparent in the surrounding mesenchyme (43). By in situ hybridization TGF-β1 mRNA was found in the developing ureteric duct epithelium as well as in mesenchymal cells in the nephrogenic zone (17). In addition, TGF-β1 mRNA was reported to be present in early nephron structures, including condensates, comma-shaped bodies, S-shaped bodies, and immature glomeruli. In subsequent stages of nephron development, TGF-β1 mRNA was observed in the distal part of the nephron tubules but not in maturing glomeruli or proximal tubules (17). No detailed information on expression of TGF-β2 or TGF-β3 in developing glomeruli has been reported so far.

Mice that are completely deficient in TGF-β1 (31) or lack expression of the TGF-β type II receptor (41) die at midgestation due to defects in vasculogenesis and hematopoiesis, thereby precluding information on the possible role of TGF-β1 in nephrogenesis. In developing rat metanephroi in vitro, addition of TGF-β1 was found to inhibit ureteric duct growth and thereby nephron endowment (17, 48). TGF-β1 does not appear to inhibit epithelial patterning in nephron development significantly once the epithelial vesicle is formed in these cultures (17). In contrast, neutralizing anti-TGF-β1 does inhibit formation of the glomerular capillary network when injected in rats between days 3 and 5 after birth (32). In metanephric explant cultures, TGF-β2 was shown to be secreted by ureteric bud epithelial cells and to stimulate at low concentrations nephrogenesis in cooperation with leukemia inhibitory factor and fibroblast growth factor 2 (45). In this experimental setup also TGF-β1 and TGF-β3 were shown to have nephrogenic capacity (45). TGF-β2−/− mice display varying renal phenotypes, ranging from complete renal agenesis to a dilated pelvis (49). Absence of the ureteric bud-derived TGF-β2 is associated with strongly decreased numbers of branch tips as well as of glomeruli in cultures of renal explants, possibly due to the loss of TGFβ2-dependent functional mesenchyme as an active inducer of branching morphogenesis (56). Interestingly, accelerated ureteric branching morphogenesis and an associated increase in nephron number was observed in kidneys of TGF-β2+/− mice by Sims-Lucas and colleagues. Apparently, when TGFβ2 is present at reduced levels the metanephric mesenchyme can survive and induce branching but the previously described TGFβ2-dependent negative feedback regulation of branching morphogenesis is alleviated (18, 56). The mean glomerular volumes were smaller in TGF-β2−/− mice but renal histopathology was otherwise not observed (56).

CTGF has been detected in the kidney during murine embryogenesis (27, 57), but CTGF expression during glomerulogenesis has not been reported so far. Since the TGF-β isoforms may be involved not only in early metanephric development but also in glomerulogenesis and since they are potent inducers of CTGF expression in wound repair and in development of fibrosis, we investigated CTGF expression in glomerulogenesis in relation to the expression of TGF-β1, TGF-β2 and TGF-β3. In addition, expression of these factors was compared in various adult human glomerulopathies with distinct patterns of injury, proliferation and remodeling.
MATERIALS AND METHODS

Rat kidney specimens
Ten-weeks-old female Wistar rats and timed pregnant Wistar rats were purchased from Charles River (Broekman Instituut BV, Someren, The Netherlands). Pups at days 1 (n=2), 2 (n=2), 5 (n=3), and 9 (n=3) after birth were anaesthetized and subsequently killed by decapitation. Adult rats (n=3) were anaesthetized and subsequently killed by bleeding via aorta puncture. Kidneys were rapidly excised and part of the tissue was snap-frozen in liquid nitrogen and stored at −80 °C; another part of the tissue was fixed during 16 h in 10% buffered formalin and embedded in paraffin. Kidneys from another group of 9-days-old rat pups were put into RNAlater immediately after excision (Ambion, Huntingdon, UK). All rats were control animals used in another study for which the committee for experimental animal procedures of the University of Amsterdam approved all applied procedures.

Human kidney specimens
Archival human renal tissue specimens were included in this study. Ten specimens of fetal kidneys of 15- to 22-weeks gestational age were obtained from tissue examined after therapeutic termination of pregnancy at the Osaka Medical Center and Research Institute for Maternal and Child Health. Similarly, 5 specimens of fetal kidneys of 17- to 23-weeks gestational age were obtained from tissue examined at the Academic Medical Center, University of Amsterdam. Samples of mature kidneys were from patients undergoing diagnostic evaluation at the Nagoya University School of Medicine, the University Medical Center Utrecht, or the Academic Medical Center at the University of Amsterdam and were studied after completion of the diagnostic procedures. Control mature human kidney specimens were taken from tumor-free, macroscopically normal portions of nephrectomy specimens of patients (n=9) who underwent surgery because of localized renal tumors. Kidney specimens from patients with various renal diseases (n=109; Table 1) were obtained by percutaneous renal biopsy. Research was performed according to the Code for Proper Secondary Use of Human Tissues (2004) of the Dutch Federation of Medical Research Associations (www.federa.org) and the AMC Research Code (2010). The medical ethical committees of the Osaka Medical Center and Research Institute for Maternal and Child Health and the Nagoya University School of Medicine approved the applied procedures.
Glomerular co-expression of CTGF and TGFβs

Table 1  Adult subjects examined by immunohistochemistry

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CTRL; tumor-free parts of kidneys with localized, polar carcinoma)</td>
<td>9</td>
</tr>
<tr>
<td>Minimal change nephrotic syndrome (MCNS)</td>
<td>11</td>
</tr>
<tr>
<td>Focal glomerulosclerosis (FGS)</td>
<td>5</td>
</tr>
<tr>
<td>Idiopathic membranous glomerulopathy (MGP)</td>
<td>11</td>
</tr>
<tr>
<td>IgA nephropathy (IgA NP)</td>
<td>38</td>
</tr>
<tr>
<td>Crescentic glomerulonephritis</td>
<td>17</td>
</tr>
<tr>
<td>Diabetic mellitus nephropathy (DM NP)</td>
<td>15</td>
</tr>
<tr>
<td>Others (lupus nephritis, membranoproliferative glomerulonephritis, myeloma kidney)</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
</tr>
</tbody>
</table>

**Generation of CTGF riboprobe**

A 542 bp cDNA fragment of rat CTGF (Genbank gi5070343 496-1037) was amplified by PCR using 5’-ATTTAGGTAAGCTATAGAAGAGGCGTGTGCACTGCCAAAGAT-3’ and 5’-TAATACGACTCACTATAGGGAGAGCAGCCAGAAAGCTCAAACTTGA-3’ as sense and antisense primers, of which the underlined regions include SP6 and T7 RNA polymerase binding sequences, respectively. The amplicon was cloned and in vitro transcription of the purified insert was performed using SP6 or T7 RNA polymerases and digoxigenin (DIG)-conjugated UTP (Roche, Almere, The Netherlands) to produce DIG-labeled sense or antisense riboprobes. The specificity of the DIG-labeled CTGF antisense riboprobe was examined by Northern blotting of total RNA, isolated from kidneys of 9-days-old rats, and from established lines of glomerular visceral epithelial cells (GVEC) derived from rat, mouse, and human kidneys (28, 35, 46, 58).

**In situ hybridization (ISH) and immunohistology double staining**

Six-μm-thick sections of formalin-fixed, paraffin-embedded tissue were dewaxed, rehydrated, incubated with 20 μg/ml proteinase K (Life Technologies, Breda, The Netherlands) in PBS at 37 °C for 15 minutes, treated with 0.2% glycine in PBS, washed in PBS, and postfixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for 20 minutes. After prehybridization for 1 h at 70 °C, hybridization with 250 ng/ml CTGF DIG-labeled riboprobe in hybridization buffer (50% formamide, 5X SSC, 1% blocking reagent (Roche), 5 mM EDTA, 0.1% Tween-20, 0.1% CHAPS, 0.1 mg/ml heparin, 1 mg/ml yeast tRNA) was performed at 70 °C for 16 hours. Slides were briefly rinsed in 2X SSC, and subsequently washed twice in 2X SSC containing 50% formamide, for 15 min each at 65 °C, followed by washing in PBS, 0.1% Tween-20 (PBS/T). After blocking non-specific binding sites by 2% blocking reagent in PBS/T, sections were incubated for 2 h with alkaline phosphatase-conjugated sheep anti-DIG antibodies (Roche) diluted to 1:1500 in blocking buffer. After washing in PBS/T and in 0.1 M Tris, pH
Glomerular co-expression of CTGF and TGFβs

9.5, 0.1 M NaCl, 0.05 M MgCl₂, and 0.05% Tween-20 (NTM/T), alkaline phosphatase activity was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate, toluidine salt ready made solution (Roche), diluted to 1:50 in NTM/T. After overnight incubation at 20 °C, sections were rinsed with distilled water. Subsequently, immunohistochemistry was performed for collagen type IV to provide a counterstain that does not interfere with the cellularly localized ISH signals. Briefly, endogenous peroxidase activity was inactivated, non-specific protein binding sites were blocked with 10% normal goat serum in PBS, and sections were incubated for 1 hour with rabbit anti-collagen type IV (Euro-Diagnostica, Arnhem, The Netherlands), diluted to 1:100 in PBS. After washing, sections were incubated with poly-HRP goat anti-rabbit IgG (Powervision, Immunovision Technologies) for 10 min, followed by washing in PBS. HRP activity was detected using 3,3’-diaminobenzidine and hydrogen peroxide. Sections were dehydrated and mounted with Pertex.

**Polyclonal and monoclonal CTGF specific antibodies**

Polyclonal antibodies were raised in rabbit against an 11-amino acid peptide, i.e. CEADLEENIKK, corresponding to amino acid residues 242-252 of human CTGF (GenBank accession number P29279), 241-251 of mouse CTGF (GenBank P29268), and 240-250 of rat CTGF (GenBank AAD39132). The CEADLEENIKK sequence is located in between domain 3 and domain 4 of CTGF and does not occur in other members of the CCN family of structurally related proteins. Rabbit IgG antibodies were affinity purified to the linear peptide. A human IgG1 monoclonal antibody (FG-3019) and a mouse IgG1 monoclonal antibody (FG-3145) that bind distinct epitopes in domain 2 of CTGF (61) were kindly supplied by FibroGen (FibroGen Inc., South San Francisco, CA). FG-3019 was produced in Medarex mice (Medarex, Princeton, NJ) by immunization with recombinant human CTGF. The recombinant human CTGF was produced in a baculovirus expression system (FibroGen). The specificity of the antibodies was analyzed by Western blotting of cell lysates of mouse NIH/3T3 or rat NRK-49F fibroblasts (obtained from ATCC, Manassas, VA, USA) that were cultured for 24 hr with or without recombinant human TGF-β1 (R&D Systems). In addition, ELISA to recombinant fragments of CTGF and to full length recombinant CTGF was performed for the rabbit anti-CEADLEENIKK.

**Immunohistochemistry**

For immunohistochemistry on rat kidney, 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded tissue. After dewaxing and rehydration of the sections, the slides were incubated with protease XXIV (Sigma) at 4 U/ml in 0.1 M phosphate buffer, pH 7.8, for 3 min at 37 °C. After washing in water, the slides were heated in 0.04 M citrate, 0.12 M phosphate, pH 5.8, for 3 min at 100 °C by microwave. Subsequently, the slides were washed in PBS, and endogenous peroxidase activity was blocked by incubation in 1% H₂O₂ in PBS for 20 min. After washing, and blocking of non-specific protein binding sites by incubation in normal goat serum (Dako, Glostrup, Denmark), the sections were incubated with FG-3019 anti-CTGF, at 1 μg/ml in PBS/5% BSA for 16 h at 4 °C. After
washing, sections were subsequently incubated with rabbit anti-human IgG (diluted to 1:5000 in PBS/10% normal rat serum; Dako), washed, and incubated with undiluted poly-HRP goat anti-rabbit IgG (PowerVision; ImmunoVision Technologies, Springdale, AR). HRP activity was detected using 3,3’-diaminobenzidine as substrate. Sections were counter-stained with methyl-green and were mounted with pertex.

For immunohistochemistry on human kidney, 4-µm-thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinated and rehydrated. The sections were incubated in 0.3% H₂O₂ in methanol to block endogenous peroxidase activity. Antigen retrieval was performed by heating in 10 mM Tris-HCl and 1 mM EDTA, pH 9.0, for 10 min at 100 °C. After washing, the sections were incubated with FG-3145 anti-human CTGF at 1 µg/ml in Antibody Diluent (ImmunoLogic, Klinipath, Duiven, The Netherlands) for 16 hours at 4 °C, washed, and incubated with poly-HRP goat anti-mouse IgG (BrightVision, ImmunoLogic). HRP activity was detected using 3,3’-diaminobenzidine as substrate. Sections were counter-stained with methyl-green and were mounted with pertex.

In addition, immunohistochemistry on human and rat kidneys was performed on cryostat sections. Four-µm-thick sections were fixed in methanol for CTGF immunohistochemistry or in acetone for TGFβ1, TGFβ2, and TGFβ3 immunohistochemistry. Endogenous peroxidase activity was inhibited with 0.1% NaN₃ and 0.3% H₂O₂, and non-specific protein binding sites were blocked with normal goat serum. The sections were incubated with rabbit polyclonal IgG anti-CEADLEENIKK antibody for 16 hours at 4 °C. Other sections were incubated with rabbit polyclonal IgG anti-TGFβ1, anti-TGFβ2, or anti-TGFβ3 (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hours at room temperature. The polyclonal anti-TGFβ1, anti-TGFβ2, and anti-TGFβ3 antibodies were raised against peptides that map at or near the carboxy terminal ends corresponding to amino acid residues 328-353 (GenBank accession number P01137), 352-377 (P08112), and 350-374 (P10600), respectively. The antibodies are expected to bind both the bioactive and the latent conformations of the TGFβ isoforms (36). HRP-labeled polyclonal goat anti-rabbit IgG antibodies (EnVision System, Dako), were applied as secondary reagent. Finally, enzyme activity of horseradish peroxidase was detected using 3,3’-Diaminobenzidine Tetrahydrochloride liquid system (Dako). Negative controls were performed by pre-absorbing the polyclonal anti-CTGF antibodies with the free CEADLEENIKK peptide and by replacing first step antibodies for species-matched Ig. Mesangial CTGF protein expression was analyzed and semiquantitatively classified into five groups: 0, no staining; 1, weak staining; 2, segmental or diffuse weak staining; 3, diffuse staining; and 4, strong diffuse staining. For each glomerulus, the “mesangial CTGF protein expression score” was assessed and the average of the score in each patient was calculated.

To compare localization of CTGF and TGF-β isoforms with that of the glomerular cell types in the developing kidney, we performed IHC on consecutive sections for synaptopodin as marker of glomerular visceral epithelial cells (5, 6) using mab G1D4 (mouse IgG1; Progen, Heidelberg, Germany), for CD31 as marker of endothelial cells (39) using mab JC/70A (mouse IgG1; Dako), and for α-smooth muscle actin (αSMA) as marker of fetal and activated mesangial cells (2, 39) using mab 1A4 (mouse IgG2a; Dako). In addition,
expression of the EDA-domain-containing, ‘cellular’ splice variant of fibronectin (EDA-FN) (12) and expression of collagen type IV were studied, using mab IST-9 (mouse IgG1; Accurate Chemical & Scientific Corporation, Westbury, NY) and rabbit anti-collagen type IV polyclonal antibody (Chemicon International, Temecula, CA), respectively. Binding of these reagents was detected with HRP-conjugated goat anti-mouse IgG antibodies or HRP-conjugated goat anti-rabbit IgG antibodies (EnVision System, Dako). Negative controls were performed by replacement of the first-step antibody with incubation buffer only or with isotype- and species-matched antibodies. HRP activity was detected using 3,3’-Diaminobenzidine Tetrahydrochloride liquid system (Dako) or 3-amino-9-ethyl-carbazole (Dako).

**Confocal Laser Scanning Microscopy**

To examine the localization of CTGF during normal human glomerulogenesis in detail, two-color immunofluorescence histology was performed on methanol-fixed 4-μm-thick sections that were pre-incubated with 10% normal goat serum. Sections were incubated with rabbit anti-CEADLEENIKK antibody for 16 hours at 4 °C, followed by rhodamine-labeled donkey anti-rabbit F(ab’)2 antibodies (Chemicon International) absorbed with normal human serum. Subsequently, monoclonal anti-synaptopodin, anti-αSMA, or anti-CD31 antibodies were applied for 30 minutes. Then sections were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse F(ab’)2 antibodies (Zymed Laboratories, San Francisco, CA) absorbed with normal human serum. After the final wash with TBS, all sections were mounted with medium containing p-phenylenediamine. Confocal laser scanning microscopy was performed using a Bio-Rad MRC-1024 scan head (Bio-Rad, Hercules, CA) attached to a microscope E800 (Nikon, Tokyo, Japan), applying double excitation with a 488 nm bandpass filter for FITC emission, and a 568 nm longpass filter for rhodamine emission. Both images were adjusted to the full dynamic range. Subsequently, FITC- and rhodamine-derived images were corrected for cross-talk and merged using the Laser Sharp Acquisition Software (Bio-Rad, Tokyo, Japan) and a look-up table to convert FITC signals to green, rhodamine signals to red, and overlapping areas to yellow.

**Statistical analysis**

Data of the mesangial CTGF score are not normally distributed and are therefore presented as median values and ranges. Statistical analysis was performed using the Kruskal-Wallis test and groups were compared using the Dunn’s multiple comparison test (GraphPad Software, La Jolla, CA).
RESULTS

**Characterization of CTGF-specific antisense riboprobe and antibodies**

Northern blotting demonstrated binding of the DIG-labeled CTGF antisense riboprobe to a 2.4 kb RNA, which corresponds to the reported size of CTGF mRNA. As expected, more probe was bound to RNA isolated from cells that had been exposed to TGF-β1, compared to RNA from control cell cultures (Figure 1A).

![Figure 1](image)

**Figure 1.**

Northern blotting (A) using a DIG-labeled antisense riboprobe reveals CTGF mRNA in glomerular visceral epithelial cell lines of human (lanes 1, 2), mouse (lanes 3, 4) and rat (lanes 5, 6) origin, that were cultured without (lanes 1, 3, 5) or with TGF-β1 (5 ng/ml for 24 hr; lanes 2, 4, 6), as well as in rat kidney (lane 7). Western blotting (B) using rabbit anti-CEADLEENIKK shows the presence of CTGF protein in the supernatant of TGF-β1-stimulated NIH/3T3 cells under both non-reducing (lanes 2, 3) and reducing conditions (lanes 5, 6). CTGF protein is not detected in the supernatant of NIH/3T3 fibroblasts that were cultured without TGF-β1 (lanes 4, 7). Both rabbit anti-CEADLEENIKK peptide and monoclonal human FG-3019 bind recombinant human CTGF in a doublet (lanes 1 and 8, respectively), which was also detected in lysate of TGF-β1-stimulated rat NRK-49F cells using FG-3019 (lane 9); samples in lanes 1, 8 and 9 were prepared under reducing conditions.

Western blot analysis revealed binding of both rabbit anti-CEADLEENIKK polyclonal IgG and human FG-3019 to proteins of 38 kD and 35 - 36 kD, corresponding to the size of CTGF under reducing and non-reducing conditions, respectively (Figure 1B). Both rabbit anti-CEADLEENIKK and FG-3019 did bind recombinant human CTGF in a doublet, possibly due to heterogeneous glycosylation of the recombinant CTGF, which was also detected in the NRK-49F cell lysate using FG-3019. In addition to the full size protein, 10 kD proteins were detected by rabbit-anti-CEADLEENIKK in some samples; these proteins may be degradation products of CTGF. Pre-incubation
of rabbit anti-CEADLEENIKK with the peptide that was used as immunogen blocked binding to CTGF; pre-immune rabbit serum and normal human serum demonstrated only weak background binding (not shown). ELISA confirmed binding of rabbit anti-CEADLEENIKK to the purified recombinant C-terminal fragment of CTGF, containing domains 3 and 4, as well as to full length recombinant CTGF, and absence of binding to the N-terminal fragment of CTGF, containing domains 1 and 2 (Supplemental Figure 1; the online version of this article contains supplemental data).

**CTGF mRNA and protein expression during glomerulogenesis in the rat**

In the first week after birth, all stages of glomerular development can be examined in the rat kidney. ISH with the CTGF antisense riboprobe revealed CTGF mRNA expression in the proximal part of comma- and S-shaped bodies containing the immediate precursors of visceral and parietal glomerular epithelial cells (Figure 2A, arrowhead and short arrow, respectively). CTGF mRNA expression was highest in later stages of glomerular development, i.e. in the capillary loop stage and in the maturing stages (Figures 2 A, long arrow, and 2C, respectively), where it is mainly observed in the periphery of the glomeruli, indicating preferential expression by glomerular epithelial cells. Throughout renal development CTGF mRNA was expressed in the media of arcuate (Figure 2C, insert) and interlobular arteries and could also be found in endothelial cells of the large venules. Also in the vessel walls of the afferent arterioles CTGF mRNA was observed (Figure 2C). In adult glomeruli, CTGF mRNA expression was detected in podocytes and occasionally in glomerular parietal epithelial cells (Figure 2E). In all stages of development, tubular epithelial cells of all compartments of the nephron expressed CTGF mRNA at most to a minor extent in a diffuse pattern. As controls, ISH with an oligo-dT probe yielded positive staining of virtually all cells in the tissue under examination, ISH with a von Willebrand Factor antisense probe resulted in staining of only endothelia of the large vessels (not shown). ISH with the control CTGF sense probe did not yield staining (Supplemental Figure 2).

CTGF protein was very weakly stained using FG-3019 in only some epithelial cells in the comma- and S-shaped bodies (Figure 2B). In glomeruli of the capillary loop stage and in the maturing stage, CTGF protein was detected mostly in the periphery of the glomeruli, suggesting presence of CTGF in differentiating parietal and visceral epithelial cells (Figure 2D). In adult glomeruli, CTGF protein was frequently present in podocytes (Figure 2F). At days 1 and 2 after birth, strong staining for CTGF was observed in a subset of tubular epithelial cells (Figure 2B). Staining for CTGF in tubules was less abundant at days 5 and 9 (Figure 2D). Some faint staining for CTGF was found in large vessel walls. Epithelial cells in capillary loop and maturing stage glomeruli were also stained with the rabbit anti-CEADLEENIKK antibody, directed against the C-terminal part of CTGF, whereas staining of the tubular portion of the nephron was not detected. In contrast, rabbit anti-CEADLEENIKK, but not FG-3019, did bind to structures in association with the developing cortical collecting ducts in the medullary ray area (Supplemental Figure 3). Incubations with normal human serum instead of FG-3019 on paraffin sections or
Glomerular co-expression of CTGF and TGFβs

Figure 2
Expression of CTGF mRNA (A, C, E: in situ hybridisation, blue signal) and CTGF protein (B, D, E: immunostaining using FG-3019, brown signal) in rat kidneys at ages of 1 day (A, B), 9 days (C, D), and 2 months (E, F). Counterstains are either type IV collagen immunostaining (A, C: brown signal) or methyl-green (B, D, F). Original magnifications: ×200 (A-F), or ×400 (inset in C).

CTGF mRNA is detected in comma- and S-shaped bodies (A: arrowhead and short arrow, respectively), in the capillary loop stage (A: long arrow), in maturing glomerular stages (C), and in adult glomeruli (E); CTGF mRNA is also present in afferent arterioles (C) and in the media of arcuate (C, insert) and interlobular arteries. CTGF protein is very weakly stained in only some epithelial cells in comma- and S-shaped bodies, and in capillary loop stages (B: arrowhead, short arrow, and long arrow, respectively). CTGF protein is readily detected in maturing and adult glomeruli (D and F, respectively). Subsets of tubular epithelial cells stain strongly for CTGF in neonatal kidney (B); tubular staining intensity is less in kidneys at day 9 (D).
with pre-immune normal rabbit serum instead of rabbit anti-CEADLEENIKK on cryostat sections did not yield any staining, indicating that detection of the primary antibodies by the secondary reagents was specific (Supplemental Figure 3).

**Expression of CTGF mRNA and protein in fetal and control adult human kidney**

Kidneys from 15- to 23-weeks-old human fetuses typically contain ureteric buds, metanephric blastema, and glomeruli of all stages of development. CTGF mRNA was very weakly detected in comma- and S-shaped glomerular stages (Figure 3A) and strongly in the early and late capillary loop stages (Figures 3A, 3B, 3D) mainly in parietal and visceral epithelial cells. In the subsequent maturing stages (Figure 3A), CTGF mRNA was predominantly present in the transitional area between parietal and glomerular visceral epithelials (Figure 3A). In control adult glomeruli, CTGF mRNA was detected in some podocytes (see Figure 7). CTGF protein expression was not observed in the renal vesicles and in the S-shaped body stage of the fetal kidneys when using either mouse FG-3145 or rabbit anti-CEADLEENIKK (not shown). In contrast, CTGF was present in the capillary loop stage glomeruli as detected with FG-3145 (Figure 3C, 3E) or with rabbit anti-CEADLEENIKK (Figure 4A, 5, 6), and to a lesser extent in maturing glomeruli (Figure 4B, 5, 6), and also in adult glomeruli (Figure 4C, 5, 6) and in distal parts of nephrons (not shown).

In the capillary loop stage, CTGF expression was strongest at the basal part of the glomerular visceral epithelial cells and was also present in a mixed cell cluster at the root of the glomerulus (Figure 3E, 4A). Synaptopodin staining was restricted to the basal part of the glomerular visceral epithelial cells (Figure 4D), which display a columnar shape and have not yet formed foot processes in this stage. CD31 positive endothelial cells were present with αSMA-positive mesenchymal cells in small groups at the root of the immature glomerulus (Figures 4G and 4J). In addition, CD31-positive endothelial cells could be found in a layer in juxtaposition to the GVEC, thereby forming immature glomerular capillaries; CD31 staining of these endothelial cells was restricted to their luminal aspect (Figure 4G). Glomerular EDA-fibronectin was abundant in this stage of nephrogenesis in a similar distribution pattern as observed for type IV collagen, being localized at the basal portion of the podocytes, and in between the clustered endothelial and mesenchymal cells as well as along the Bowman’s capsule (Figures 4M, 4P).

In the maturing glomerulus stage, the number of endothelial cells and mesangial precursor cells is strongly increased and the glomerular capillary network is formed. CTGF was detected in a scattered pattern along the capillary wall and in the mesangial area, where αSMA staining was positive (Figures 4B, 4K). Synaptopodin-positive GVEC and CD31-positive endothelial cells were found in the periphery of the glomerular tuft (Figures 4E, 4H). Fibronectin EDA and type IV collagen were present in the mesangial area and type IV collagen also in Bowman’s capsule (Figures 4N, 4Q).
Figure 3
Expression of CTGF mRNA (A, B, D: in situ hybridization, blue signal; no counterstain) and CTGF protein (C, E: immunostaining using FG-3145, brown signal; methyl green counterstain) in glomeruli at the capillary loop stage and at the maturing stage in human fetal kidneys. CTGF mRNA is very weakly detected in comma- and S-shaped bodies (A), and strongly in glomerular epithelial cells in capillary loop stages (A, B, D). CTGF protein is detected predominantly in visceral epithelial cells and weakly in mesangial and/or endothelial cells in capillary loop stage glomeruli (C, E). Bar sizes: 100 μm (A), 25 μm (B – E).
Figure 4
Protein expression of CTGF (rabbit anti-CEADLEENIK; A, B and C), synaptopodin (D, E and F), CD31 (G, H and I), αSMA (J, K and L), fibronectin EDA (M, N and O) and type IV collagen (P, Q and R) in human glomeruli at the capillary loop stage (A, D, G, J, M and P), the maturing stage (B, E, H, K, N and Q), and at the adult stage (C, F, I, L, O and R) detected by immunostaining with hematoxylin counterstain. Original magnifications: ×400 (A, D, G, J, M, P), ×250 (B, E, H, K, N, Q), ×100 (C, F, I, L, O, R).
At the capillary loop stage (A), CTGF is expressed in the basal portion of the podocytes and in a mixed cell cluster at the root of the glomerulus. In maturing glomeruli (B), CTGF protein expression shows a more extended distribution, as podocytes and mesangial cells have migrated to the periphery. In the normal adult glomerulus (C), CTGF protein is mainly expressed by podocytes. Fibronectin-EDA is expressed from the capillary loop stage to the maturing glomerulus stage in association with presence of αSMA-positive cells in the mesangial area.
In the adult glomerulus, CTGF is mainly expressed by podocytes (Figure 4C). Synaptopodin is localized in the foot processes of podocytes along the glomerular basement membrane (Figure 4F). In the adult kidney, mesangial CTGF expression decreased in association with diminished expression of αSMA and fibronectin EDA (Figures 4C, 4L, 4O), whereas type IV collagen is abundantly present in the GBM (Figure 4R).

Double immunostaining for CTGF and synaptopodin, αSMA, or CD31 was performed to examine the localization of CTGF in detail by confocal laser scanning microscopy (Figure 5). In the capillary loop stages, CTGF protein was observed in the basal part of the podocytes, in αSMA-positive cells and in CD31-positive endothelial cells. In the maturing glomerulus stages, CTGF protein was detected in podocytes and αSMA-

![Figure 5](image)

**Figure 5**
Double immunofluorescence immunostaining for protein expression of CTGF (rabbit anti-CEADLEENIKK; red) and synaptopodin, αSMA or CD31 (all green) during human glomerulogenesis analysed by confocal laser scanning microscopy; double-positive areas are depicted in yellow. Original magnifications: ×400 (capillary loop stage), ×250 (maturing stage), ×100 (adult stage).

In the capillary loop stages, CTGF is expressed by synaptopodin-positive podocytes, αSMA-positive cells and CD31-positive endothelial cells. In maturing glomerulus stages, CTGF is detected in podocytes and αSMA-positive mesangial cells, but not in CD31-positive endothelial cells. In normal adult glomeruli, CTGF expression is restricted to the podocytes; the single red staining indicates CTGF-positive podocyte cell bodies, apical from the synaptopodin-positive terminal foot processes.
positive mesangial cells, but not in CD31-positive endothelial cells. In the normal adult glomeruli, CTGF protein expression was confined to the podocytes. In particular, CTGF single staining can be observed in the cell body of the podocytes, apical of the synaptopodin-positive area that is adjacent to the basolateral membrane of the podocyte foot processes (Figure 5). These distribution patterns could be confirmed for podocytes also by conventional light microscopy using double immunostaining (Supplemental Figure 4).

![Image of immunostaining results](image.png)

**Figure 6**
Protein expression of CTGF (rabbit anti-CEADLEENIKK), TGFβ1, TGFβ2, and TGFβ3 during human glomerulogenesis detected by immunostaining with hematoxylin counterstain. Original magnifications: ×400 (capillary loop stage), ×250 (maturing stage), ×100 (adult stage).

In the capillary loop stages, CTGF protein is expressed in the basal portion of the podocytes and in a mixed cell cluster within the glomerular tuft. TGFβ1, TGFβ2 and TGFβ3 are detected in the basal portion of the podocytes. Also TGFβ1 is weakly detected in the mixed cell cluster. In the maturing glomerular stages, the expression pattern of each TGFβ isofrom is similar to that of CTGF. In the normal adult glomerulus, TGFβ2 and TGFβ3 as well as CTGF are mainly expressed by podocytes. TGFβ1 is only detected in the tubules.
**Expression of TGFβ1, TGFβ2 and TGFβ3 proteins in human fetal and adult kidney**

In order to investigate the possible relation between expression of CTGF and that of the TGFβ isoforms during glomerulogenesis, sections of human fetal and adult kidneys were stained for these proteins.

In the capillary loop stage, staining for TGFβ1, TGFβ2 and TGFβ3 was present in the basal portion of the GVEC (Figure 6), similar to that for CTGF (Figures 3E, 4A, 5, 6). TGFβ1 and CTGF protein were also weakly detected in the mixed cluster of mesangial and endothelial cell precursors in the developing glomerular tuft (Figures 5, 6). TGFβ2 showed weak staining in the capillary loop stage (Figure 6).

In the maturing glomerulus stage, the expression patterns of all TGFβ isoforms were similar to that of CTGF (Figure 6), which was expressed by differentiating podocytes and αSMA-positive mesangial cells (Figures 4B, 5).

---

**Figure 7**

PAS staining and expression of CTGF mRNA (in situ hybridization, blue signal; no counterstain) and CTGF protein (immunostaining using FG-3145, brown signal; methyl green counterstain) in renal biopsy specimens from patients with MCNS, diabetic glomerulosclerosis, and crescentic glomerulonephritis. CTGF expression is increased mainly in podocytes in both mesangial and extracapillary proliferative lesions. Original magnifications: ×400.
In the normal adult glomerulus, TGFβ2 and TGFβ3 (Figure 6), as well as CTGF (Figures 4C, 5, 6), were mainly expressed by podocytes (Figure 6). In contrast, TGFβ1 was not or only weakly detected (Figure 6). CTGF was faintly detected in distal tubules and/or connecting segments in one third of adult kidney tissues. TGFβ1, -β2, and -β3 were variably detected in tubular epithelial cells in adult kidneys. TGFβ1 staining was localized at the apical sites of a subset of tubular epithelial cells.

**Expression of CTGF and TGFβ1, -β2, and -β3 in human glomerulopathies**

In the non-inflammatory glomerular diseases examined, i.e. minimal change nephropathy (Figure 7) and idiopathic membranous glomerulopathy (not shown), CTGF mRNA and protein expression were not up-regulated in the mesangial area. However, in circa 50% of biopsies of MCNS (5/11) and MGP without glomerulosclerosis (6/11) CTGF expression was increased in podocytes and was detected in a continuous pattern along the glomerular capillary wall (Figure 8A; rabbit anti-CEADLEENIKK). In the remaining subjects of MCNS and MGP, no up-regulation of CTGF was observed in epithelial regions as compared to control renal tissue samples. In MCNS and MGP, staining for the TGFβ isoforms was not different from that in normal control kidneys.

CTGF expression was increased in inflammatory glomerular and tubulo-interstitial lesions, associated with cell proliferation and matrix accumulation. Increased CTGF mRNA expression was predominantly observed in podocytes (Figure 7). Concomitantly, CTGF protein was also strongly detected in podocytes both with FG-3145 (Figure 7) and with rabbit anti-CEADLEENIKK (Figure 8A, 8B). In addition, CTGF protein was detected in the mesangial proliferative lesions in IgA nephropathy (Figure 8A), diabetic nephropathy (Figure 8B), and lupus nephritis (WHO class IV, Figure 8B) with rabbit anti-CEADLEENIKK (Figure 8) but not with FG-3145 (Figure 7).

Although CTGF was abundant in these proliferative lesions, it was not detected in adjacent obsolescent glomeruli and completely sclerotic glomerular lesions, such as in diabetic nodular lesions (not shown). CTGF was strongly expressed in tubulo-interstitial fibrotic areas (not shown).

In case of mild mesangial proliferation increased presence of CTGF, TGFβ2 and TGFβ3, but not of TGFβ1, was observed (mild proliferative IgA nephropathy, Figure 8A). In contrast, expression of CTGF and of all isoforms of TGFβ was strongly increased in severe proliferative lesions (crescentic glomerulonephritis, Figures 7, 8B). In all kidney diseases examined, TGFβ1 expression was increased to a larger extent in tubulo-interstitial fibrotic areas than in glomerular lesions (not shown).

By semi-quantitative analysis, the “mesangial CTGF protein expression score” as determined by immunostaining with rabbit anti-CEADLEENIKK was significantly higher in IgA nephropathy and diabetic glomerulosclerosis than in non-proliferative renal diseases and control kidneys (Figure 9). In addition, in mesangial lesions of IgA nephropathy CTGF expression was much higher when proteinuria exceeded values of 1g/day than in those of IgANP patients with proteinuria of less than 1g/day.
Glomerular co-expression of CTGF and TGFβs

A

MCNS  mild proliferative IgA NP  moderate proliferative IgA NP

CTGF

TGF-β1

TGF-β2

TGF-β3

B

DM NP  Lupus N  Crescentic GN

CTGF

TGF-β1

TGF-β2

TGF-β3
The expression of CTGF is increased in fibrotic disorders in a large variety of organs in association with simultaneous overexpression of TGFβ. Indeed, the well-known profibrotic factor TGFβ is the most potent inducer of CTGF expression to date. The presence of extracellular CTGF is required for effectuation of several of the profibrotic effects of TGFβ, as indicated by blocking studies in experimental models both in vivo and in vitro (9, 20, 34, 67). Moreover, CTGF and TGFβ synergistically stimulate development of fibrosis upon administration in vivo (34). In this respect, two alternative but not exclusive hypotheses on the action of CTGF have emerged. First, CTGF may modulate signaling upon binding to various cell surface receptors (15, 33, 52, 59, 64). Second,
Glomerular co-expression of CTGF and TGFβs

CTGF may directly bind TGFβ and subsequently enhance binding of TGFβ to its signaling receptor (1). Here, we have confirmed co-expression of CTGF and TGFβ isoforms in several types of glomerular injury and have in addition shown a similar orchestrated co-expression during nephron development. We demonstrated that sustained expression of CTGF mRNA and CTGF protein is present in the renal glomeruli from the S-shaped stage onward and that the glomerular CTGF expression is in all stages associated with colocalized expression of at least one of the TGFβ family members. To our knowledge, this is the first time that such simultaneous and colocalized expression of CTGF and TGFβ is described in the absence of development of fibrosis. Interaction of the multidomain CTGF with various ligands may affect its detection, possibly explaining the different subcellular staining patterns of the antibodies directed against either domain 2 (FG-3019, FG-3145) or domain 3-4 (rabbit anti-CEADLEENIKK). Physiologic CTGF cleavage products may occur at various sites as well. CTGF protein was abundant in a punctate pattern in a subset of tubular epithelial cells in newborn rat kidneys, as detected using the anti-domain 2, but not with the anti-domain 3-4 antibodies. Since CTGF mRNA was hardly expressed by these cells, this observation suggests that CTGF may be endocytosed by tubular epithelial cells. Indeed, CTGF can bind and modulate signaling via LRP scavenging receptors (33, 52) and is degraded in endosomes (16). The in vivo dynamics of CTGF turnover and processing require more investigation.

The overall expression patterns of CTGF during glomerulogenesis in rat and man correspond with each other. Further analysis revealed that in the capillary loop stage CTGF mRNA and protein are predominantly expressed by synaptopodin-positive glomerular visceral epithelial cells; at this stage, CTGF protein is also present on αSMA-positive precursors of mesangial cells and CD31-positive precursors of glomerular endothelial cells. Paracrine effects of podocyte-derived CTGF on precursors of the other glomerular cell types may be envisaged, as has been demonstrated for VEGF-A (19). In maturation, CTGF protein expression became restricted to podocytes in adult glomeruli. Expression of TGFβ1 was high in the capillary loop stage and subsequently diminished to undetectable levels. Inversely, TGFβ2 was weakly detected in the capillary loop stage and was clearly present in fully matured podocytes at the adult age. Expression of TGFβ3 was observed in podocytes at all glomerular stages. Previously, we showed that TGFβ1, TGFβ2 and TGFβ3 may all induce CTGF gene transcription in mesangial and podocyte cell lines to a similar extent (24). All TGFβ isoforms are capable to stimulate production of extracellular matrix proteins in renal fibroblasts, tubular epithelial cells and mesangial cells in vitro (68). Upon forming a complex with TGFβ receptor type I and type II, each of the three TGFβ isoforms may cause activation of the Smad2 and Smad3 signaling intermediates, that subsequently bind Smad4 and translocate to the nucleus as a transcription activating complex (55). Induction of CTGF gene transcription by TGFβ in fibroblasts and mesangial cells in vitro involves signaling through Smad, MAPkinase, PKC and Ets1 dependent pathways (8, 38).
Expression of Smad2 and Smad3, the phosphorylated active pSmad2, and the common mediator Smad4 is widespread in early stages of nephron development, decreases with maturation, but remains present in podocytes in adult kidneys (4). Interestingly, Wu and colleagues described an increased production of TGFβ2 and its requirement to induce cell-cycle arrest and differentiation in a model of podocyte maturation in vitro; the levels of TGFβ1 did not change (63). In contrast, addition of higher concentrations of either TGFβ1 or TGFβ2 induced apoptosis of podocytes in this model (63).

Recent studies by Sims-Lucas and colleagues using TGFβ2 homozygous and heterozygous mutant mice also suggest that a critical and possible narrow range of TGFβ2 concentrations is required for normal branching morphogenesis and nephrogenesis (56).

Our data, indicating the continuous presence of TGFβ2 in fully differentiated podocytes under normal conditions in vivo, support the in vitro observations by Wu and colleagues. These observations suggest that the TGFβ – Smad signaling pathway is active in podocytes at all stages of development and may be involved in the apparently constitutive CTGF expression in these cells. Homozygous CTGF knockout mice die shortly after birth because of respiratory failure due to defective endochondral ossification and associated skeletal abnormalities (25, 26). To what extent CTGF contributes to maintaining normal glomerular form and function is therefore not clear at present.

The early stages of glomerular development, in which coexpression of TGFβ1 and CTGF was observed, are characterized by processes like cell migration, proliferation, and matrix production that are also common in proliferative glomerulopathies and that are known to be induced by TGFβ1 and CTGF in various cell types in vitro (8, 9, 21, 29, 37, 47). In agreement with earlier reports (3, 39), αSMA expression was common in the mesangial area in the capillary loop stage. In addition, strong expression of the EDA-containing fibronectin splice variant was present. Interestingly, the presence of the fibronectin EDA domain is crucial for myofibroblastic phenotype induction by TGFβ1 (53), a common phenomenon upon renal tissue injury. CTGF promotes migration of mesangial cells in an in vitro model for wound repair (9). We observed that transformation of mesangial cells into αSMA expressing myofibroblast-like cells was induced by TGFβ1, but not affected by CTGF. However, CTGF was shown to be able to induce synthesis of fibronectin, type I collagen and type IV collagen by mesangial cells (9). In an experimental model of tubulointerstitial nephritis, antisense blocking of CTGF expression resulted in reduced expression of EDA-fibronectin (66).

The results of the present study support the concept that overexpression of CTGF and TGFβ1 contributes to development of renal fibrosis in adults. CTGF mRNA is up-regulated in proliferative lesions of human and experimental glomerulonephritis, in particular in extracapillary proliferative lesions, confirming previous reports (23, 24). Here, we extend these observations by detailing CTGF expression at the protein level. We further explored the relation between glomerular CTGF and TGFβ protein expression in several types of human glomerulopathies. The mesangial CTGF expression score was significantly increased in diabetic glomerulosclerosis and in
IgA nephropathy, in particular in subjects with high proteinuria. In these lesions, CTGF mRNA is predominantly expressed by podocytes, which are therefore probably the main source of full length CTGF protein. In accordance, both N-terminal and C-terminal domains of CTGF were detected in podocytes. Interestingly, predominantly C-terminal CTGF was detected in the mesangial area, warranting further investigation of possible CTGF cleavage and differential availability of CTGF fragments in these glomerulopathies. In non-proliferative glomerular diseases, such as MCN and MGP, the mesangial CTGF expression score was not different from that in control kidneys. Yokoi and colleagues recently demonstrated in podocyte-specific CTGF-transgenic mice that CTGF overexpression per se does not cause glomerulopathy in otherwise normal mice. Importantly, streptozotocin-induced diabetes resulted in more severe diabetic nephropathy in the CTGF-transgenic mice than in their wild-type littermates (65). SMAD3-dependent signalling is involved in development of diabetic glomerulosclerosis (60), in line with the widely appreciated role of TGFβ in renal fibrosis (7, 10). Accordingly, high expression of all TGFβ isoforms was present in severe proliferative lesions, including diabetic glomerulosclerosis. TGFβ1 expression was less in mild mesangial proliferative lesions and in non-proliferative glomerulopathies. More than half of the subjects with MCNS or MGP showed no expression of TGFβ1 in the glomerulus whereas relative strong expression of TGFβ2 and TGFβ3 was present in podocytes. These findings are in agreement with those from several experimental models in which also all TGFβ isoforms were present in only the most severe cases of glomerulonephritis (24, 54, 62).

In summary, our findings suggest that coordinated expression of TGFβ isoforms and of CTGF may be involved in normal glomerulogenesis and possibly in maintenance of glomerular structure and function at adult age. Prolonged overexpression of TGFβ1 and CTGF is associated with development of severe glomerulonephritis and glomerulosclerosis.

ACKNOWLEDGEMENTS

We thank Miss Mio Tsuda and Miss Yurilko Fujitani for their technical assistance, Dr Noelynn Oliver (FibroGen, South San Francisco, CA) for providing recombinant CTGF and monoclonal anti-CTGF antibodies, Dr Karin G.F. Gerritsen (Dept of Internal Medicine, University Medical Centre Utrecht, Utrecht, The Netherlands) for performing ELISA to CTGF. The present address of Jan J. Weening is at TerGooi Hospitals, Blaricum, The Netherlands.

GRANTS

Supported by a grant from the Dutch Kidney Foundation (C96.1545) and by the Aichi Kidney Foundation, Nagoya, Japan.
DISCLOSURE

R. Goldschmeding received financial support from FibroGen for research projects and consultancy.

REFERENCES


Glomerular co-expression of CTGF and TGFβs


49. Sanford LF, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, and Doetschman T. TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. Development 124: 2659-2670, 1997.


Glomerular co-expression of CTGF and TGFβs


Supplemental Figure 1
Protein-A affinity purified rabbit IgG anti-CEADLEENIKK binds the C-terminal half of CTGF (C-CTGF, containing domains 3 and 4) as well as full length CTGF (CTGF-W, consisting of domains 1 – 4) and does not bind to the N-terminal half of CTGF (N-CTGF, containing domains 1 and 2). The CEADLEENIKK peptide is located in between domains 3 and 4.
Recombinant human CTGF-W, C-CTGF and N-CTGF were purified from cultures of CHO cells that had been transfected with the respective expression vectors. For ELISA, CTGF-W, C-CTGF and N-CTGF were each coated at 40 nM. Dilution series of rabbit anti-CEADLEENIKK were examined by ELISA for binding to CTGF and its fragments; shown are the results of rabbit anti-CEADLEENIKK assayed at 0.4 μg/ml.
Supplemental Figure 2

Expression of CTGF mRNA was detected by in situ hybridization using a DIG-labeled antisense riboprobe in formalin-fixed paraffin sections of rat kidneys at ages of 9 days (A), 1 day (C) and 5 days (E). Bound DIG is detected by immunostaining with alkaline phosphatase-conjugated sheep anti-DIG antibody and nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as substrate, yielding a blue precipitate. Incubation with the control DIG-labeled sense riboprobe on serial sections (B, D, E) does not yield any staining. Following the ISH procedure, immunohistochemistry was performed for collagen type IV to provide a counterstain that does not interfere with the cellularly localized ISH signals. Binding of the rabbit anti-collagen type IV was detected with poly-HRP goat anti-rabbit IgG and 3,3’-diaminobenzidine and hydrogen peroxide as substrate, yielding a brown precipitate. CTGF mRNA is detected in glomerular epithelial cells in capillary loop stage and maturing stage glomeruli. Bar sizes: 200 μm (A, B), 50 μm (C – F).
Supplemental Figure 3
Expression of CTGF protein in a rat kidney of 9 days old. Immunostaining using FG-3019 (specific for domain 2 in the N-terminal part of CTGF) on formalin-fixed paraffin section (A) and rabbit anti-CEADLEENIKK (specific for an epitope in the C-terminal part of CTGF, containing domains 3 and 4) on methanol-fixed cryostat section (B) shows presence of CTGF protein in capillary loop stage and in maturing stage glomeruli with both methods. Different staining patterns are apparent for the tubular compartment. FG-3019 binds a subset of tubular epithelial cells in a punctuate pattern (A), whereas rabbit anti-CEADLEENIKK binds to developing cortical collecting ducts in the medullary ray area (B). Replacement of the anti-CTGF antibodies with either normal human serum on paraffin sections (not shown) or pre-immune normal rabbit serum on cryostat sections (C) did not yield any binding of the enzyme-conjugated secondary step reagents. Bar size: 100 μm.

Supplemental Figure 4
Double immunostaining for protein expression of CTGF (rabbit anti-CEADLEENIKK; red) and synaptopodin (blue) in a glomerular capillary loop stage (A) and in an adult glomerulus (B) of human kidneys. Original magnifications: ×400.
At the capillary loop stage, CTGF is expressed by synaptopodin-positive epithelial cells and also by precursors of mesangial and glomerular endothelial cells. In the normal adult kidney glomerular CTGF expression is less abundant and restricted to podocytes. Supplemental Figure 4 confirms data presented in Figure 5, applying light microscopy of double-stained sections instead of confocal laser scanning fluorescence microscopy.