Expression of CCN1 (CYR61) in developing, normal, and diseased human kidney

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Departments of 1Medicine and Clinical Science and 2Urology, and 3Congenital Anomaly Research Center, Kyoto University Graduate School of Medicine, Kyoto; 4Department of Nephrology, Osaka Satseikai Nakatsu Hospital, Osaka; 5Children’s Renal Unit, University of Bristol, Bristol, United Kingdom; 6Department of Biochemistry, National Cardiovascular Research Institute, Osaka, Japan

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Sawai K, Mukoyama M, Mori K, Kasahara M, Koshikawa M, Yokoi H, Yoshioka T, Ogawa Y, Sugawara A, Nishiyama H, Yamada S, Kuwahara T, Saleem MA, Shiota K, Ogawa O, Miyazato M, Kangawa K, Nakao K. Expression of CCN1 (CYR61) in developing, normal, and diseased human kidney. Am J Physiol Renal Physiol 293: F1363–F1372, 2007. First published August 15, 2007; doi:10.1152/ajprenal.00205.2007.—CCN1 (cysteine-rich protein 61; Cyr61) is an extracellular matrix-associated signaling molecule that functions in cell migration, adhesion, and differentiation. We previously reported that CCN1 is induced at podocytes in rat anti-Thy-1 glomerulonephritis, a well-known model of reversible glomerular injury, but its expression and significance in the human kidney remain totally unknown (Sawai K, Mori K, Mukoyama M, Sugawara A, Suganami T, Koshikawa M, Yahata K, Makino H, Nagae T, Fujimaga Y, Yokoi H, Yoshioka T, Yoshimoto A, Tanaka I, Nakao K. J Am Soc Nephrol 14: 1154–1163, 2003). Here we report that, in the human kidney, CCN1 expression was confined to podocytes in normal adult and embryonic glomeruli from the capillary loop stage. Podocyte CCN1 expression was decreased in IgA nephropathy, diabetic nephropathy, and membranous nephropathy, whereas it remained unchanged in minimal change disease and focal segmental glomerulosclerosis. Downregulation of CCN1 was significantly greater in diseased kidneys with severe mesangial expansion. CCN1 protein was also localized in the thick ascending limb of Henle’s loop, distal and proximal tubules, and collecting ducts, which was not altered in diseased kidneys. In vitro, recombinant CCN1 protein enhanced endothelial cell adhesion, whereas it prominently inhibited mesangial cell adhesion. CCN1 also completely suppressed mesangial cell migration, suggesting its role as a mesangial-repellent factor. In cultured podocytes, CCN1 markedly induced the expression of cyclin-dependent kinase inhibitor p27Kip1 as well as synaptopodin in a dose-dependent manner and suppressed podocyte migration. These data indicate that CCN1 is expressed in podocytes, can act on glomerular cells to modulate glomerular remodeling, and is downregulated in diseased kidneys, suggesting that impairment of CCN1 expression in podocytes may contribute to the progression of glomerular disease with mesangial expansion.

podocyte; glomerular visceral epithelial cell; cysteine-rich protein 61; mesangial cell; synaptopodin

Cysteine-Rich Protein 61 (Cyr61; also known as CCN1) is a secreted, 42-kDa, angiogenic protein belonging to the family of CCN proteins, which contains Cyr61 (CCN1), connective tissue growth factor (CTGF; CCN2), nephroblastoma overexpressed (Nov; CCN3), and Wnt-induced secreted proteins (WISP)-1, -2, and -3 (CCN4, 5, and 6, respectively) (3, 19, 28). CCN proteins are associated with extracellular matrix, interact with various integrins, and mediate a variety of biological actions including cell adhesion, migration, and differentiation, and induce angiogenesis both in vitro and in vivo (2, 3, 13, 19, 28). Among them, CCN1 is essential for vessel bifurcation during development, and most CCN1-null mice suffer embryonic death between embryonic (E) days E11.5 and E14.5 due to vessel malformation in the placenta and within embryos (22). In adults, CCN1 is suggested to be involved in skin wound healing (4) and adaptation to cardiovascular stress such as ischemia and pressure overload (11), but its role in the kidney remains undefined.

We have recently reported that CCN1 is prominently induced at podocytes during glomerular regeneration in rat anti-Thy-1 glomerulonephritis (Thy-1 GN) (32), a well-known model of reversible glomerulonephritis. CCN1 expression in podocytes was potentiated by transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (32). CCN1 was also expressed in the proximal straight tubules, where its expression was not altered during Thy-1 GN. Conditioned medium from CCN1-overexpressing cells inhibited mesangial cell migration without affecting cell proliferation (32). From these results, we speculated that CCN1 secreted from podocytes might enhance glomerular repair in Thy-1 GN by protecting the glomerular capillary lumen from being occluded by migrating mesangial cells. However, the expression and the pathophysiological significance of CCN1 in human kidneys remain to be elucidated.

In this study, we investigated CCN1 expression in fetal and adult human kidneys, as well as in various glomerular diseases. Furthermore, we studied the effects of CCN1 on mesangial cells and podocytes in vitro. Our data indicate that CCN1 expression in podocytes is decreased in diseased kidneys with mesangial expansion, suggesting that impairment of CCN1 expression may contribute to the progression of various glomerular diseases.
and with severe mesangial expansion (Group I). Table 1 summarizes details of the analyzed materials. Patients with IgA nephropathy (IgAN), 29 with type 2 diabetic nephropathy (DN), 7 with minimal change nephrotic syndrome (MCNS), 9 with focal segmental glomerulosclerosis (FSGS), and 8 with membranous nephropathy (MN). Diagnosis was confirmed by pathological evaluation of specimens, such as light microscopy, electron microscopy, and immunofluorescence staining. No patients received steroids or immunosuppressive drugs before biopsy. Ten human fetal kidneys (estimated gestational age ranging from 16 to 20 wk) were obtained fresh from tissues examined after therapeutic abortion at the Congenital Anomaly Research Center, Kyoto University Graduate School of Medicine. For normal controls, tissues obtained from seven patients afflicted with localized neoplasm using uninvolved portions of surgical specimens were used. Histopathological examination of control tissues excluded any glomerular diseases. This study was approved by the Ethics Committee of Kyoto University Graduate School of Medicine, and informed consent was obtained in accordance with protocols approved by the committee.

After resecting, the samples were fixed in Dubsq-Brazil solution. Table 1 summarizes details of the analyzed materials. Patients with IgAN were further classified as those with mild mesangial expansion (group I) and with severe mesangial expansion (group II), defined as a mesangial area <30% (group I) and >30% (group II) of total glomerular area, respectively. Clinical parameters of IgAN between two groups are summarized in Table 2.

Immunohistochemical analysis. Immunohistochemical analysis was performed as previously described with some modifications (32, 33). In brief, deparaffinized 3-μm kidney sections were treated with autoclave heating, and specimens were incubated with 1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) in PBS for 20 min, washed three times with PBS, and incubated with 10% normal donkey serum in PBS for 10 min at room temperature. Goat antibody against the COOH terminus of human Cyr61 (CCN1; sc-8561, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-human Wilms’ tumor-1 (WT1) antibody (sc-192, Santa Cruz Biotechnology) was diluted 1:50 in PBS containing 1% BSA (1% BSA/PBS) and incubated for 1 h at room temperature. After blocking of endogenous phosphatase with 2 mM levamisole, the sections were incubated with alkaline phosphatase-conjugated donkey anti-goat or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) in 1% BSA/PBS for 30 min at room temperature. The sections were processed with NBT-BCIP (Roche Diagnostics, Mannheim, Germany) in alkaline buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl2) and counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan).

For double staining, the sections were pretreated with 3% hydrogen peroxide for 15 min at room temperature, incubated with peroxidase-conjugated secondary antibody against rabbit IgG (Jackson ImmunoResearch), and processed with 3,3′-diaminobenzidine tetrahydrochloride (Kanto Chemical, Tokyo, Japan). Nonimmune goat or rabbit serum was used as negative control.

Histological and morphometric analysis. Staining intensity of CCN1 and WT1 in kidney samples was examined by two independent investigators, and the score was averaged. At least six glomeruli per biopsy were evaluated at high-power magnification, and the intensity was semiquantified by a rating of 0–3 (most prominent). The number of CCN1- and WT1-positive cells per glomerular cross section was quantified as described elsewhere (33). Mesangial area was computer analyzed by measuring the periodic acid-Schiff (PAS)-positive area in cross sections of glomeruli scanned from vascular poles, using an automatic image analyzer (KS400, Carl Zeiss Vision, Munich, Germany) (35). To analyze CCN1 expression in cultured human podocytes, cells were fixed with 3.7% formaldehyde for 20 min followed by permeabilization with 0.1% Triton X-100 for 20 min at room temperature. After being rinsed with PBS, primary antibody anti-Cyr61 (CCN1; sc-8561, Santa Cruz Biotechnology) was applied for 60 min at room temperature. Antigen-antibody complexes were visualized using FITC-conjugated secondary antibody (705–095-147, Jackson ImmunoResearch), and processed with 3,3′-diaminobenzidine tetrahydrochloride (Kanto Chemical, Tokyo, Japan). Nonimmune goat or rabbit serum was used as negative control.

Purification of recombinant CCN. Conditioned media of SP9 cells (Invitrogen, Carlsbad, CA) infected with baculovirus driving the

Table 1. Clinical parameters of the patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Gender (M/F)</th>
<th>Age, yr</th>
<th>sCr, mg/dl</th>
<th>BUN, mg/dl</th>
<th>Urinary Protein, g/g Cr</th>
<th>Ccr, ml/min</th>
</tr>
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<tbody>
<tr>
<td>Normal adult human kidney (control)</td>
<td>12</td>
<td>2/10</td>
<td>55±6</td>
<td>0.70±0.04</td>
<td>14.8±1.2</td>
<td>0.06±0.02</td>
<td>100±11</td>
</tr>
<tr>
<td>Nephrectomy</td>
<td>7</td>
<td>2/5</td>
<td>68±5</td>
<td>0.79±0.04</td>
<td>16.9±1.3</td>
<td>0.04±0.01</td>
<td>95±23</td>
</tr>
<tr>
<td>Minor glomerular abnormalities</td>
<td>5</td>
<td>0/5</td>
<td>36±8</td>
<td>0.58±0.04</td>
<td>11.8±1.6</td>
<td>0.08±0.03</td>
<td>103±9</td>
</tr>
<tr>
<td>Human fetal kidney</td>
<td>10</td>
<td>5/5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IgAN</td>
<td>33</td>
<td>16/17</td>
<td>37±3</td>
<td>0.98±0.10*</td>
<td>15.6±1.4</td>
<td>0.86±0.16</td>
<td>84±5</td>
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<tr>
<td>MCNS</td>
<td>7</td>
<td>3/4</td>
<td>39±3</td>
<td>0.77±0.07</td>
<td>13.7±1.8</td>
<td>10.2±2.76</td>
<td>96±9</td>
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<tr>
<td>FSGS</td>
<td>9</td>
<td>6/3</td>
<td>57±2</td>
<td>1.23±0.14†</td>
<td>26.8±5.7</td>
<td>2.17±0.79</td>
<td>50±8*</td>
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<tr>
<td>MN</td>
<td>8</td>
<td>3/5</td>
<td>59±1</td>
<td>0.73±0.07</td>
<td>12.3±1.6</td>
<td>2.18±0.48</td>
<td>80±6</td>
</tr>
<tr>
<td>DN</td>
<td>29</td>
<td>22/7</td>
<td>59±2</td>
<td>1.35±0.12†</td>
<td>24.2±2.0†</td>
<td>6.14±0.96‡</td>
<td>55±9‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.005, and ‡P < 0.0005 vs. control.

Table 2. Clinical parameters of IgAN patients with mild (group I) and severe (group II) mesangial expansion

<table>
<thead>
<tr>
<th>Gender (M/F)</th>
<th>Age, yr</th>
<th>sCr, mg/dl</th>
<th>BUN, mg/dl</th>
<th>Urinary Protein, g/g Cr</th>
<th>Ccr, ml/min</th>
<th>Mesangial Area (% of Total Glomerular Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n = 21)</td>
<td>11/10</td>
<td>30±2</td>
<td>0.85±0.06</td>
<td>13.5±1.2</td>
<td>0.58±0.13</td>
<td>96±4</td>
</tr>
<tr>
<td>Group II (n = 12)</td>
<td>5/7</td>
<td>42±5</td>
<td>1.28±0.20</td>
<td>19.1±3.2</td>
<td>1.42±0.31</td>
<td>62±9</td>
</tr>
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</table>

P value, group I vs. group II

| NS          | NS      | P < 0.05   | P < 0.05   | P < 0.0001             |

Values are means ± SE. IgAN were classified into 2 groups defined as those with mesangial area <30% (group I) and >30% (group II) of total glomerular area, respectively. NS, not significantly different.
synthesis of CCN1 were used as a source for purification, as previously described (13). Full-length mouse CCN1 cDNA was generated by RT-PCR using total RNA from C57BL/6 mouse kidneys, and the following primers were used: sense, 5′-tgctcctgacatgagtaacg-3′ and antisense, 5′- tgtaccctcgttctctctctc-3′ (nucleotides 179–1329, GenBank accession number M32490) (18). CCN1 cDNA was TA-cloned into pGEM-T Easy vector (Promega, Madison, WI) and transferred into pBacPAK vector (Clontech, Palo Alto, CA). The transfer vector along with BacPAK6 viral DNA (Clontech) was delivered into cells by liposome-mediated transfection to obtain a recombinant virus. SF9 cells grown to 10^6 cells/ml in SFM-II medium were infected with the recombinant virus and incubated for 48 h at 28°C. The conditioned media centrifuged at 5,000 g for 5 min at 4°C were adjusted to 50 mM sodium phosphate buffer (pH 6.0) containing 2 mM EDTA and 1 mM PMSF and applied to a Hitrap SP column (Bio-Rad, Hercules, CA). Protein concentration was determined by the modified Bradford method with a protein assay kit (Bio-Rad, Hercules, CA).

**Cell culture.** Human umbilical vein endothelial cells (HUVEC; Clonetics, Walkersville, MD) were maintained in the basal medium with growth supplements (EGM-2; Clonetics) containing 2% FCS (Cansaer, International, Ontario, Canada). Cell cultures between passages 5 and 7 were used for each experiment. Mesangial cells established from glomeruli of 6-wk-old Sprague-Dawley rats were cultured in DMEM (Invitrogen) containing 10% FCS and antibiotics (32) and used between passages 7 and 10. Human mesangial cells (Clonetics) were maintained in the basal medium with growth supplements (CC-3147; Clonetics). Cell cultures between passages 5 and 8 were used for each experiment. Conditionally immortalized mouse podocytes (a kind gift from Dr. Peter Mundel) were cultured with RPMI 1640 medium (Nihonseiyaku, Tokyo, Japan) containing 10% FCS and antibiotics on dishes coated with type I collagen (Koken, Tokyo, Japan) as described elsewhere (24, 32). The cells proliferate when cultured at 33°C with 10 U/ml IFN-γ (Life Technologies, Gaithersburg, MD), whereas they halt growing and begin to differentiate when cultured at 37°C without IFN-γ. For CCN1 stimulation experiments, cells were differentiated for 2 wk and cultured with RPMI 1640/1% FCS for 24 h, until stimulation with recombinant CCN1 for 24 h on collagen I-coated dishes (Iwaki Glass, Chiba, Japan). Cells were used between passages 15 and 20. Conditionally immortalized human podocytes were cultured as described previously (33). These cells proliferate when cultured at 33°C, whereas they halt growing when cultured at 37°C. Cells were cultured

**Fig. 1.** Immunohistochemical analyses of cysteine-rich protein 61 (Cyr61; CCN1) expression in normal adult human kidney. CCN1 expression was detected at podocytes exclusively within the glomeruli (A and F, black arrows). CCN1-positive cells (F, black arrows) were also positive for WT1 (F, brown arrows). Weak CCN1 expression was also observed in some of arterioles (A, white arrowhead). Parietal epithelial cells, mesangial cells, and endothelial cells were negative for CCN1 in the glomeruli (A, E, and F). CCN1 expression was localized to the distal and proximal tubules (B), the thick ascending limb of Henle’s loop (C, white arrows), and collecting ducts (C, black arrowheads). There was no detectable CCN1 expression within endothelial cells or smooth muscle cell of arteries (D). Cultured mouse and human podocytes significantly expressed a higher level of CCN1 compared with cultured rat and human mesangial cells, respectively (G). Confocal fluorescence microscopy revealed both cytoplasmic and nuclear expression of CCN1 (H and J, green) in human cultured podocytes (I, counterphase of H; J, merged image of H and I). Magnification: x400 (A, B, D, H–J); x200 (C); x1,000 (E and F).
with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (Sigma-Aldrich) and an insulin-transferrin-sodium selenite medium supplement (Sigma-Aldrich) on tissue culture dish (Corning, Corning, NY). Cells were differentiated at 37°C for 2 wk without passage and were subcultured on 96-well plates (146520, Nunc, Roskilde, Denmark). Cells were used between passages 15 and 18.

For CCN1 expression analysis, cells grown at 33°C were used.

**Northern and Western blot analyses.** Northern blot analysis was performed as described elsewhere (32). In brief, after RNA extraction by TRIzol Reagent (Invitrogen), total RNA (20 μg in each lane) was electrophoresed on 1.0% agarose gels and transferred to nylon membranes (GeneScreen Plus, NEN, Boston, MA). The cDNA fragments of mouse synaptopodin (nucleotides 2165–2766, GenBank accession number XM_619543), mouse podocalyxin (nucleotides 1184–1601, GenBank accession number AF290209), mouse podoplanin (nucleotides 420–809, GenBank accession number BC026551), and mouse α-actinin-4 (nucleotides 2425–2809, GenBank accession number NM_021895) were generated by RT-PCR and used as probes. The membranes were hybridized with [32P]dCTP-labeled probes, and the filters were incubated with secondary antibodies (Santa Cruz Biotechnology) and a chemiluminescence kit (ECL plus, Amersham). The blots were exposed to a BAS-III imaging plate. The amount of RNA loaded in each lane was normalized for 28S ribosomal RNA.

Western blot analysis was performed as described elsewhere (16, 33). In brief, cells were lysed on ice in solution containing 20 mM Tris-HCl (pH 7.5), 12 mM glycerophosphate, 0.1 M EDTA, 1 mM pyrophosphate, 5 mM NaF, 5 mg/ml aprotinin, 2 mM dithiothreitol, 1 mM PMSF, 1% Triton X-100, and 1 mM sodium orthovanadate (Sigma-Aldrich). The lysate was centrifuged at 15,000 g on 96-well plates (146520, Nunc, Roskilde, Denmark). Cells were used between passages 15 and 18.

**RESULTS**

**CCN1 expression in normal adult human kidney.** The immunohistochemical staining pattern of CCN1 in normal adult kidneys was essentially consistent in the 12 samples analyzed (Fig. 1, summarized in Table 3). Within the glomeruli, podocytes were invariably positive for CCN1 staining, whereas parietal epithelial cells, mesangial cells, and endothelial cells were virtually negative for CCN1 (Fig. 1A). Podocytes were identified morphologically as cells located at the outer aspect of the glomerular basement membrane (GBM) and immunohistochemically as WT1-positive cells (Fig. 1F). CCN1 expression in podocytes was detected mainly in the cytoplasm and also in the nucleus (Fig. 1, A and E). In tubular epithelial cells, the expression pattern was similar to that of podocytes but expressed mainly in the nucleus and cytoplasm.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Normal</th>
<th>IgAN</th>
<th>MCNS</th>
<th>FSGS</th>
<th>MN</th>
<th>DN</th>
</tr>
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<tbody>
<tr>
<td>Glomerulus</td>
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<tr>
<td>Mesangial cells</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>++</td>
<td>++</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>Collecting ducts</td>
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<td>+</td>
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<td>Interstitial cells</td>
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</table>

CCN1, cysteine-rich protein 61.
CCN1 staining was positive in the thick ascending limb of Henle’s loop, distal tubules, proximal tubules, and collecting ducts (Fig. 1, B and C). CCN1 expression in the tubular cells was prominent in the cytoplasm, and to a lesser extent in the nucleus (Fig. 1, B and C). Weak expression of CCN1 protein was also observed in some of afferent and efferent arterioles (Fig. 1A), but the protein was not expressed in larger vessels (Fig. 1D). Negative controls with nonimmune goat or rabbit serum gave no staining in all analyzed normal and diseased kidneys (data not shown). Compatible with human kidney tissue, cultured podocytes expressed abundant CCN1 compared with mesangial cells (Fig. 1G). Confocal fluorescence microscopy revealed both cytoplasmic and nuclear expression of CCN1 in human cultured podocytes (Fig. 1, H–J).

**CCN1 expression in developing human kidney.** In the developing human kidneys, CCN1 showed a distinct localization pattern (Fig. 2, summarized in Table 4). In early glomerular stages, i.e., comma-shaped and S-shaped bodies, CCN1 was almost completely negative in the glomeruli (Fig. 2, A and B). From the late capillary loop stage, CCN1 expression was detected in the glomeruli, restricted to the developing podocytes (Fig. 2, B–D). CCN1 expression in fetal podocytes was detected mainly in the nucleus and also in the cytoplasm (Fig. 2D). Intensity of CCN1 expression in podocytes was stronger in glomeruli at the maturing stage (Fig. 2, B and C). Other glomerular cell types, including developing endothelial cells, mesangial cells, and parietal epithelial cells, were uniformly negative or faintly positive in early glomerular stages (A, white arrow). From the late capillary loop stage, CCN1 expression was detected in developing podocytes (B–D, black arrows). There was also CCN1 expression in developing thick ascending limb of Henle’s loop, distal tubules (C and E, white arrowheads), proximal tubules (C, black arrowheads), and collecting ducts (E, asterisks). CCN1 was also detected at endothelial cells in arteries and arterioles (F). Magnification: ×400 (A, C, and F); ×200 (B and E); ×1,000 (D).

<table>
<thead>
<tr>
<th>Site</th>
<th>CCN1 Expression</th>
</tr>
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<tbody>
<tr>
<td>Glomerulus</td>
<td></td>
</tr>
<tr>
<td>Comma-shaped body</td>
<td>–</td>
</tr>
<tr>
<td>S-shaped body</td>
<td>–</td>
</tr>
<tr>
<td>Capillary loop stage</td>
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</tr>
<tr>
<td>Maturing-stage glomeruli</td>
<td></td>
</tr>
<tr>
<td>Tubules</td>
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</tr>
<tr>
<td>Distal tubules</td>
<td>+ + +</td>
</tr>
<tr>
<td>Thick ascending limb of Henle</td>
<td>+ + +</td>
</tr>
<tr>
<td>Proximal tubules</td>
<td>+ +</td>
</tr>
<tr>
<td>Collecting ducts</td>
<td>+</td>
</tr>
<tr>
<td>Vasculature</td>
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<tr>
<td>Arterial endothelial cells</td>
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<tr>
<td>Smooth muscle cells</td>
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Fig. 2. Immunohistochemical analyses of CCN1 expression in fetal human kidney. CCN1 was negative or faintly positive in early glomerular stages (A, white arrow). From the late capillary loop stage, CCN1 expression was detected in developing podocytes (B–D, black arrows). There was also CCN1 expression in developing thick ascending limb of Henle’s loop, distal tubules (C and E, white arrowheads), proximal tubules (C, black arrowheads), and collecting ducts (E, asterisks). CCN1 was also detected at endothelial cells in arteries and arterioles (F). Magnification: ×400 (A, C, and F); ×200 (B and E); ×1,000 (D).
negative for CCN1 (Fig. 2D). Strong CCN1 expression was detected in the developing thick ascending limb of Henle’s loop, distal tubules, and proximal tubules (Fig. 2, C and E). Weak expression was present in developing collecting ducts (Fig. 2E). In the vasculature, CCN1 was detected at endothelial cells in arteries and arterioles (Fig. 2F).

**CCN1 expression in IgAN.** The CCN1 staining pattern was studied in 33 specimens with IgAN (Fig. 3, summarized in Tables 1–3). Cases included minimal lesions, those with mesangial hypercellularity and/or mesangial expansion, and those with severe focal necrosis and crescents (represented in Fig. 3, F, H, and J, respectively). In cases with IgAN, glomerular CCN1 expression was also confined to podocytes (Fig. 3, C, E, G, and I) as in normal glomeruli (Fig. 3A). WT1 staining in the nucleus as a podocyte marker in adjacent sections confirmed podocyte-specific expression of CCN1 (Fig. 3, A–D). In IgAN, however, the intensity of CCN1 staining in podocytes was decreased in various degrees compared with normal controls (Fig. 3M). Furthermore, some podocytes seemed to have lost CCN1 expression (Fig. 3, C and E). In contrast, WT1 staining intensity was not reduced in IgAN (Fig. 3M). Closer examination revealed that expression of CCN1 was markedly decreased in podocytes surrounding glomerular tufts with severe mesangial expansion (Fig. 3G) and in those adjacent to segmental sclerosis (Fig. 3I). Thus CCN1 staining intensity at podocytes was significantly reduced in cases with severe mesangial expansion (group II) compared with those with mild mesangial lesion (group I) (Fig. 3N, Table 2). The number of CCN1-positive cells per glomerulus was also significantly decreased in group II (Fig. 3N). Tubular CCN1 expression and its localization were well preserved in IgAN, even in tubular cells with advanced tubulointerstitial lesion (Fig. 3, K and L).

**CCN1 expression in other glomerular diseases.** Next, we evaluated cases with MCNS, FSGS, MN, and DN as glomerular tuft.

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**Fig. 3.** CCN1 expression in IgA nephropathy (IgAN). CCN1 staining (A, C, E, G, I, K, L), WT1 staining (B and D), and periodic acid-Schiff (PAS) staining (F, H, and J) of renal biopsy specimens from patients with IgAN (C–L) and normal controls (A and B) is shown. Compared with normal controls (A), CCN1 staining intensity at podocytes was decreased in IgAN (C, E, G, and I). Expression of CCN1 was downregulated especially in podocytes surrounding the glomerular tuft with mesangial expansion (G, black arrows), and in podocytes next to segmental sclerosis (I, black arrowheads). CCN1-negative podocytes were frequently observed (white arrows). CCN1 expression was well preserved in the tubular cells (K and L). Intensity of CCN1 expression in podocytes was significantly decreased in IgAN compared with normal controls, whereas intensity of Wilms’ tumor-1 (WT1) was not altered (M). Decrease in CCN1 intensity and the number of CCN1-positive cells was prominent in IgAN with severe mesangial expansion (group II) compared with those with only mild mesangial expansion (group I; N). ns, Not significantly different. Magnification: ×400 (A–J); ×200 (K and L). #P < 0.005 vs. normal control. *P < 0.005 vs. group I.
ular disease with various degrees of mesangial expansion (Fig. 4, summarized in Table 3). CCN1 expression was also confined to podocytes in these glomerulopathies (Fig. 4, C, E, G, and I). In cases with MCNS or FSGS, the intensity of CCN1 expression in podocytes was decreased in some glomeruli, but not with statistical significance (Fig. 4, C, E, and G). Podocyte CCN1 expression was significantly decreased in cases with MN (Fig. 4, G and K), and also in DN, which had a prominent decrease in cytoplasmic CCN1 (Fig. 4, I and K, Table 3). CCN1-positive cells per glomerular cross section were decreased in all of the glomerular diseases examined, which in part may be due to a loss of podocytes, indicated by decreased WT1-positive cells per glomerular cross section (Fig. 4L). However, the ratio of CCN1-positive cells to WT1-positive cells was significantly decreased in IgAN by 23% but was not altered in MCNS, FSGS, MN, and DN (Fig. 4L). CCN1-negative podocytes frequently seen in IgAN were not observed in these glomerular diseases. WT1 staining intensity was not altered in any of these diseases (Fig. 4, D, F, H, J, and K). Tubular CCN1 was preserved in MCNS, FSGS, MN, and DN (data not shown), as was the case with IgAN (Fig. 3, K and L).

Effects of CCN1 on mesangial cell adhesion and migration. To clarify the functional role of CCN1 expressed at podocytes, effects of recombinant CCN1 protein were examined in vitro. Because CCN1 expression at podocytes was decreased in glomeruli with mesangial expansion, we examined the effects of CCN1 on mesangial adhesion and migration. Although CCN1 significantly augmented endothelial cell attachment (Fig. 5A) as in previous reports (13, 14), CCN1 caused a marked inhibition of mesangial cell adhesion (Fig. 5A). Next, we examined the effect of CCN1 on mesangial migration. In the presence of PDGF-BB, CCN1 potently abolished PDGF-induced mesangial migration (Fig. 5B). These results show that CCN1 can act on mesangial cells as a potential inhibitor of adhesion and migration.

Effects of CCN1 on podocyte differentiation. To explore the role of CCN1 on podocytes, podocytes cultured under differ-
entiating condition for 2 wk were incubated with recombinant CCN1 for an additional 24 h. Synaptopodin, a marker widely used to identify differentiated phenotypes of podocytes, was upregulated dose dependently (Fig. 6, A and C). Other podocyte-specific genes including podocalyxin, podoplanin, and αH9251-actinin-4, were not significantly induced by addition of CCN1. Since differentiation of podocytes leads to a quiescent phenotype both in vitro and in vivo (5, 25, 34), we examined the effect of CCN1 on p27Kip1 (p27) expression, a cyclin-dependent kinase inhibitor (CKI) involved in the maintenance of G0/G1 phase arrest in podocytes (5). CCN1 upregulated p27 protein dose dependently (4.2-fold with 3 g/ml CCN1) (Fig. 6, B and C). Because the migratory phenotype of podocytes is reported to be involved in glomerulosclerosis (17, 30, 31), we finally examined the effect of CCN1 on podocyte migration. CCN1 significantly suppressed podocyte migration by 26% (Fig. 6D). Thus CCN1 may act as an autocrine/paracrine factor, promoting podocyte differentiation and maintaining its quiescent phenotype.

DISCUSSION

In the present study, we investigated CCN1 protein expression in normal fetal and adult human kidneys and those with various nephropathies. In normal glomeruli, CCN1 was predominantly expressed at podocytes from the capillary loop stage to mature glomeruli. CCN1 was also present in the thick ascending limb of Henle’s loop, distal and proximal tubules, and collecting ducts. Decrease in CCN1 expression at podocytes was observed in IgAN, DN, and MN. Especially in IgAN, CCN1-negative podocytes were frequently observed, and the intensity of CCN1 staining at podocytes was significantly reduced in cases with severe mesangial expansion. In vitro, CCN1 prominently inhibited adhesion and migration of mesangial cells. Furthermore, CCN1 induced p27 and synaptopodin expression, indicating that CCN1 may act as an autocrine factor to stimulate podocyte differentiation.

CCN1 at podocytes may exert its function in two ways: one in a secreted form, and the other as a factor inside the nucleus. Secreted CCN1, which may be quantified by CCN1 staining at the cytoplasm, can act on glomerular cells in a GBM-bound form, since CCN1 has a high affinity for heparan sulfate proteoglycans (37), a major component of the GBM (8). Furthermore, CCN1 interacts with a variety of integrins (3, 9, 14, 19, 28), e.g., acting through integrin αvβ3 on endothelial cells and through αvβ5 on fibroblasts (9, 14), both of which are expressed in mesangial cells and podocytes (10). It was recently reported that production of vascular endothelial growth factor A, a homodimeric glycoprotein of 48 kDa, from podocytes is required for mesangial cell survival in vivo (6), suggesting that secreted factors from podocytes can act on glomerular cells, counteracting against the flow from the capillary lumen to the urinary space. It is therefore likely that CCN1, an extracellular matrix-associated protein with a similar molecular weight, secreted from podocytes can either act on

Fig. 6. Effects of CCN1 on cultured podocytes. Recombinant CCN1 enhanced the expression of synaptopodin mRNA (A) and cyclin-dependent kinase inhibitor p27 protein (B) in a dose-dependent manner (C). Podocyte migration was significantly suppressed by addition of CCN1 at 3 μg/ml (D). *P < 0.05 vs. 0 μg/ml; #P < 0.05 vs. 1 μg/ml; n = 4.
themselves in an autocrine manner or act on mesangial cells as a GBM-bound form, through integrin complexes on these cells.

Podocytes are terminally differentiated, quiescent cells, possessing well-developed processes, and express podocyte-specific proteins such as WT1 and synaptopodin (27). In contrast, typical “dedifferentiated” podocytes proliferate and have decreased synaptopodin expression (17). Abnormal podocyte function, such as dedifferentiation, loss, or inflammatory change, is postulated to play a central role in nephron degeneration (17). Therefore it is important to understand the underlying mechanisms, but very few factors are known to be involved in the maintenance of podocytes at differentiated phenotypes (7, 12, 21, 36). In this study, we revealed that CCN1 upregulates synaptopodin expression in vitro and showed that CCN1 is induced in vivo at the capillary loop stage, when synaptopodin is first detected during kidney development. Synaptopodin is essential for podocyte foot process formation, by blocking the actin-branching activity of α-actinin-4 to develop unbranched actin bundles (1). Our observation that CCN1 upregulated synaptopodin, but not α-actinin-4, implies the role of CCN1 in podocyte foot process formation, which needs to be clarified in the future studies.

CKIs maintain G0/G1 cell cycle arrest in mature podocytes, and their downregulation is responsible for podocyte proliferation in pathological conditions (5, 25, 34). We showed in this study that CCN1 enhances the expression of CKI p27 in vitro and that CCN1 is upregulated in vivo at the capillary loop stage, when p27 is also induced in the developing kidney (5, 25). Decreased CCN1 expression at podocytes in IgAN may contribute to downregulation of podocyte p27, as reported previously in proliferative IgAN (29).

A decrease in the ratio of CCN1-positive cells to WT1-positive cells was prominent in IgAN, indicating the appearance of CCN1-negative podocytes in IgAN. In fact, CCN1-negative podocytes were frequently observed in IgAN. CCN1-negative podocytes may contribute to the pathological lesion seen in IgAN in two ways: first, a decrease in GBM-bound CCN1 may enhance mesangial migration, which leads to glomerular tuft occlusion and to glomerulosclerosis. Second, excessive podocyte migration can lead to podocyte bridging, and further on to glomerular crescent formation (17, 20, 23, 26, 30, 31). The functional significance of CCN1 downregulation in IgAN should await further clarification.

Downregulation of CCN1 at podocytes may be merely a consequence of podocyte loss or damage, but we assume it unlikely for the following reasons. First, we found that WT1 expression intensity was not altered despite a dramatic change in CCN1 expression in podocytes. Second, a reduction in CCN1 expression at podocytes was significant in IgAN, DN, and MN compared with MCNS and FSGS. Since MCNS and FSGS are supposed to have more pronounced podocyte damage compared with IgAN (15), it is unlikely that podocyte damage alone leads to CCN1 downregulation. Third, the findings that podocyte CCN1 expression is decreased in glomeruli with overt mesangial expansion, and that CCN1 is markedly downregulated at podocytes adjacent to mesangial expansion or segmental sclerosis, supports the possible mesangial-repellent activity of CCN1. Taken together, we hypothesize that decreased CCN1 expression in podocytes may, at least in part, play a role in disease progression in these glomerulopathies.

In conclusion, we show that CCN1, which acts as a potential mesangial-repellent factor and a podocyte differentiation factor, is predominantly expressed by podocytes in glomeruli of adult human kidneys and that CCN1 expression at podocytes is downregulated in various diseases associated with mesangial expansion. Although the functional relevance is still unclarified, these findings raise the possibility that impairment of CCN1 expression at podocytes may play a role in the progression of various human glomerulopathies.

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