CD44 Deficiency Increases Tubular Damage But Reduces Renal Fibrosis in Obstructive Nephropathy

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Abstract. CD44 is a glycoprotein involved in inflammation and cell-cell/cell-matrix interactions. CD44 is upregulated in the kidney upon injury; however, its role in the pathogenesis of renal damage and fibrosis remains largely unknown. The authors show that mice lacking CD44 developed more tubular damage, associated with decreased proliferation and increased apoptosis of tubular epithelial cells, but less renal fibrosis after unilateral ureteral obstruction. In addition, impaired influx of macrophages and decreased accumulation of myofibroblasts was observed in the obstructed kidney of CD44−/− mice compared with CD44+/+ mice. Hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) exert reciprocal functions in the progression of renal diseases and interact with CD44 in vitro. For the first time, the authors establish diminished HGF-signaling, via its high affinity receptor c-Met, in the absence of CD44 in vivo. In parallel, the signaling of TGF-β1 reflected by the relative phosphorylation and nuclear translocation of Smad-2 and Smad-3 was reduced in the obstructed kidney of CD44−/− mice. In conclusion, CD44 exerts protective effects on tubuli but contributes to renal fibrogenesis at least in part through enhancement of HGF and TGF-β1 signaling pathway in obstructive nephropathy.

Tubulointerstitial injury is a common finding in the chronically diseased kidney and is the main predictor for the progression to end-stage renal disease. Progression of renal diseases is characterized by tubular damage, macrophage infiltration, accumulation of myofibroblasts, and renal fibrosis. One of the molecules that may orchestrate this cascade is CD44. Under normal conditions, CD44 is hardly expressed in the kidney except for passenger leukocytes (1,2). However, in inflammatory renal diseases, CD44 expression is markedly enhanced, particularly in crescents and injured tubuli as documented in human diseases and in several animal models (1,3–5). Altogether, these observations suggest a central but still unknown role for CD44 in renal injury.

CD44 family glycoproteins are encoded by a single gene consisting of 19 exons. By alternative splicing, different isoforms can be generated (6,7). These isoforms have been implicated in many important physiologic and pathologic processes, such as cell-cell and cell-matrix interaction, lymphocyte extravasation, wound healing/scarring, cell migration, lymphocyte activation, and binding/presentation of growth factors (8–11).

Hyaluronic acid (HA) and osteopontin are the major ligands of CD44 (9,12). HA is a glycosaminoglycan of the extracellular matrix, which markedly accumulates in the kidney cortex upon injury and may undergo degradation into low–molecular weight products (3,13) that exert proinflammatory effects (14,15). Interestingly, HA fragments accumulate in the absence of CD44 at the site of injury (16), suggesting a role for CD44 in the clearance of HA. Osteopontin, the second major ligand of CD44, promotes accumulation of macrophages, decreases renal cell apoptosis and participates in the regeneration of tubular epithelial cells (TEC) upon renal injury (17,18).

The CD44-variant containing variable-exon 3 (CD44v3) is capable of binding growth factors at its attached heparan sulfate-chain and presents these factors to their high-affinity receptors (19,20). Due to binding to heparan sulfate, a local gradient is created that facilitates cross-linking of growth factor receptors (10,21). One of the heparan sulfate-binding growth factors that exerts potent renoprotective actions is hepatocyte growth factor (HGF) (22–25). In a B cell line it was shown that CD44v3 binds HGF and presents it to its high-affinity receptor c-Met (11).

CD44 is also implicated in the activation and signaling of the profibrotic agent, transforming growth factor-β1 (TGF-β1). After binding to CD44, the matrix metalloproteinase-9 (MMP-9) is able to cleave pro–TGF-β1 into its active form (26). Furthermore, upon binding with HA, CD44 interacts with TGF-β receptor I, thereby enhancing TGF-β1 signaling (27).

These results suggest an important, yet unknown role for CD44 in renal injury. Our results reveal that CD44 disruption leads to increased tubular injury but decreased renal fibrosis during obstructive nephropathy.

Materials and Methods

Mice and Experimental Protocol

Mice, CD44 knockout on C57Bl/6 background (CD44−/−) (28) and C57Bl/6 wild-type (CD44+/+) origin were bred in our animal...
facility. Right kidney unilateral ureteral obstruction (UUO) or sham surgery was performed under general anesthesia (0.07 ml/10 g mouse of FFM mixture, containing: 1.25 mg/ml midazolam [Roche, Mijdrecht, The Netherlands], 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanisone [Janssen Pharmaceutica, Beerse, Belgium]) on 6- to 8-wk-old male mice. The right ureter was ligated with 6-0 silk and all mice received postoperative analgesia (0.15 mg/kg buprenorfine, subcutaneously; Shering-Plough, Brussels, Belgium). Sham-operated mice underwent the same procedure without the ligation of one ureter. To determine the quantitative and qualitative changes in CD44 expression and tubular damage in obstructive nephropathy, we performed experiments using real-time PCR and immunohistochemistry.

Table 1. Sequences of primer-pairs

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<th>Antisense Primer</th>
<th>Size (bp)</th>
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<td>5’-CCACACCTTCTCTACACTTgAC-3’</td>
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Figure 1. De novo CD44-expression after unilateral ureteral obstruction (UUO). Quantitative real-time PCR for CD44 presented as x-fold increase of CD44 transcripts, corrected for the number of TATA-box binding protein (TBP) transcripts, of the obstructed kidney versus the contralateral kidney. Sham-operated CD44+/−/− (hatched bars) and UUO CD44+/−/− (white bars), presented as mean ± SEM, n = 6 (A). Immunostaining for pan-CD44 on CD44+/−/− kidneys, 1 d (B), 3 d (C), 7 d (D), and 14 d (E) after UUO. Magnification, ×100; representative for n = 6.

Figure 2. Tubular damage after UUO. (A) Semiquantitative scoring of tubular injury revealed more damage in CD44+/−/− compared with CD44+/−/− and representative picture of tubular lesions at day 3 after UUO (periodic acid Schiff [PAS-D] staining; magnification, ×200). (B) Tubular epithelial cells (TEC) proliferation as assessed by the number of 5-bromo-2-deoxyuridine (BrdU)–positive nuclei per 1000 TEC showing more proliferation of TEC in CD44+/−/− compared with CD44+/−/− and representative immunostaining for BrdU 3 d after UUO (magnification, ×200). (C) More apoptotic TEC in CD44+/−/− compared with CD44+/−/− were counted, and representative immunostaining of anti-active caspase 3 (magnification, ×400). White bars represent CD44+/−/−, and black bars represent CD44+/−/−. Mean ± SEM, n = 6.
mark proliferating cells, 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co, St. Louis, MO) was injected intraperitoneally (50 mg/kg body wt) 1 h before sacrifice. Mice (n = 6 per group) were sacrificed 1, 3, 7, and 14 d. All experimental procedures were approved by the Animal Care and Use Committee of the University of Amsterdam, the Netherlands.

**Antibodies**

Rat IgG2b anti-CD44 was obtained from concentrated supernatant of the hybridoma IM 7.8.1 (ATCC, Livermore, CA). Goat anti-osteopontin was purchased from R&D Systems (Abingdon, UK), the biotinylated HA binding protein from Calbiochem (Darmstadt, Germany), anti-β-actin and anti-BrdU antibodies from Sigma, anti-active caspase 3 from Cell Signaling Technology (Beverly, MA), anti-F4/80 antibodies from Serotec (Oxford, UK), anti-c-Met (B2) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho c-Met (pY-pYpY1230/1234/1235) from Biosource International (Nivelles, Belgium), anti-α-smooth muscle actin (SMA) and all HRP-labeled secondary antibodies from DAKO (Glostrup, Denmark), anti–TIMP-1 and anti–TIMP-2 from Oncogene (Cambridge, UK), and anti-Smad-2/3 antibodies from BD Pharmingen (San Diego, CA). The anti-phospho-Smad-2 and –3 antibodies used for Western blotting were a kind gift of P. ten Dijke (NKI, Amsterdam, the Netherlands). The anti-phospho-Smad-2/3 antibody used for immunohistochemistry was purchased from Santa Cruz Biotechnology.

**Histology and Immunohistochemistry**

Renal tissues were fixed in 10% formalin for 12 h and embedded in paraffin in a routine fashion. Four micrometer sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS-D), or Sirius Red. For detection of CD44, osteopontin, macrophages, and apoptosis, antigen retrieval was performed by microwave treatment. To detect BrdU, DNA was denatured in 2 N HCl, and antigen retrieval was performed by 0.4% pepsin (Sigma). Immunostainings were performed in accordance with standard procedures. The slides were counterstained with methyl green (Sigma). Anti-phospho c-Met and anti-phospho Smad-2 and –3 were stained using frozen sections.

**Histopathological Scoring**

All histopathological scorings were made in the cortex and performed in a blinded fashion. Tubular injury was assessed by grading tubular dilatation, epithelial simplification and brush border loss in ten randomly chosen, non-overlapping fields (×200 magnification). Lesions were graded on a scale from 0 to 4: 0 = normal; 1 = mild, involvement of less than 25% of the cortex; 2 = moderate, involvement of 25 to 50% of the cortex; 3 = severe, involvement of 50 to 75% of the cortex; 4 = extensive damage involving more than 75% of the cortex. To evaluate the number of proliferating TEC, BrdU-positive TEC were counted for a total of 1000 TEC. The number of active caspase-3–positive apoptotic tubular cells, macrophages, and TEC positive for nuclear p-Smad-2/3 were counted in ten non-overlapping fields.

Osteopontin was expressed as the percentage of positive tubuli. An area of 10 mm² was analyzed for HA and Sirius Red using a digital image analysis program (Image pro-plus; Mediacybernetics, Germany). Results are expressed as a percentage of the analyzed cortex.

*Figure 3.* Macrophages infiltration after UUO. (A) Immunostaining for macrophages revealed impaired influx of macrophages into CD44⁺/⁻ compared with CD44⁺/+ kidneys 3 d after UUO (magnification, ×100). (B) Data are presented as number of macrophages/mm² cortex. White bars represent CD44⁺/⁺, and black bars represent CD44⁻/⁻. Mean ± SEM, n = 6.
Real-Time Quantitative RT-PCR and Conventional RT-PCR

Total RNA was isolated from frozen kidney cortex or microdissected tubuli sections (performed with a PALM laser-microbeam system; PALM GmbH, Bernried, Germany) using Trizol reagent (Life Technologies, Breda, The Netherlands). cDNA was synthesized using anchored 5'(dT)14-d(A/G/C)-d(A/G/C/T)-3' primers. To exclude genomic-DNA amplification, RNA samples were analyzed without RT-procedure. Real-time RT-PCR was performed on a LightCycler system (Roche Diagnostics, Almere, the Netherlands) using FastStart DNA Master SYBR Green I reagent (Roche). Specific primers (synthesized by Sigma-Genosys, Cambridgeshire, UK) for CD44-pan, CD44v3, collagen IV, and house-keeping gene TATA-box binding protein (TBP) were designed and are listed in Table 1. To adjust for variable input, values were corrected for TBP mRNA. Values are expressed as x-fold upregulation (obstructed kidney versus contralateral kidney).

Total Collagen Assay

Hydroxyproline concentrations in hydrolyzed (6 M HCL, 110°C, 12 h) accurately weighed frozen kidney samples were chemically measured according to the method of Kivirikko et al. (29). Total collagen was assumed to contain 12.7% hydroxyproline by weight, and final results were expressed as µg of collagen/mg of kidney weight.

Gelatin Zymographic Analysis

Frozen tissue was sonicated in extraction buffer (10 mM NaCacodilate, 1 M NaCl, 0.1% Triton, 1 µM ZnCl2, and 0.1 mg/ml NaN3). Equal quantities of protein were loaded onto a 10% polyacrylamide gel containing 1% gelatin (Bloom 225, Sigma) next to a protein marker. To induce MMP activity, gels were incubated overnight in a buffer containing 50 mM Tris, 5 mM CaCl2, and 1% Triton, pH 7.5. To visualize MMP activity gels were stained with Coomassie Brilliant Blue and subsequently destained.

HGF and TGF-β1 ELISA

Kidney cortex was sonicated in PBS containing 1% Triton, 1 mM EDTA, and 1% protease inhibitor cocktail (P8340, Sigma). Kidney tissue HGF levels were assayed by two-site ELISA using a mouse anti-HGF antibody (R&D Systems) and a goat anti-HGF (R&D Systems) in accordance with standard procedures. Activated TGF-β1 was determined using a Quantikine TGF-β1 ELISA kit in accordance with the protocol of the manufacturer (R&D systems). Activation of latent TGF-β1 was done by incubation kidney lysates with an equal volume of 2.5 N acetic acid/10 M urea.

Immunoblotting

Kidney cortex lysates were prepared as described for the ELISA with addition of 1 mM sodium orthovanadate (Sigma). Samples (20-µg proteins) were separated by SDS-PAGE and transferred onto activated PVDF membranes (Millipore, Etten Leur, The Netherlands). Membranes were blocked with either 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% Tween (TBS-T) (α-SMA, c-Met, Smad-2/3, phospho Smad-3, β-actin) or 5% bovine serum albumin (Sigma) in TBS-T (phospho c-Met and phospho Smad-2). The blots were probed with primary antibody followed by incubation with HRP-conjugated secondary antibody. HRP activity was visualized.

Figure 4. Hyaluronic acid (HA) accumulation after UUO. (A) Representative microphotographs of HA staining (magnification, ×200) revealed accumulation of HA in CD44+/+ mice (left panel) and CD44+-/- (right panel) mice 7 d after UUO. (B) Quantification by digital image analysis revealed less HA accumulation in CD44+/+ (white bars) than in CD44+-/- (black bars) kidneys. Mean ± SEM, n = 6.
with ECL-reagent (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Densitometric quantification analysis was conducted on directly scanned images using National Institutes of Health Image 1.62 for Macintosh software.

**Statistical Analyses**

All data were analyzed by comparison with unpaired t test, except for tubular injury, which was analyzed by using a nonparametric Mann-Whitney U test.

**Results**

**Expression of CD44 Is Induced by UUO**

As assessed by real-time RT-PCR, a strong upregulation of CD44 mRNA was observed in obstructed kidneys that peaks 7 d after UUO (Figure 1A). To localize CD44 expression, immunostainings were performed. One day after obstruction, CD44 protein was hardly detectable, except for a few passenger leukocytes (Figure 1B). Three days after UUO, CD44 expression was increased on tubuli and capillary endothelial cells (Figure 1C). At 7 d (Figure 1D) and 14 d (Figure 1E) after UUO, the expression of CD44 was even more pronounced.

**Increased Tubular Injury in CD44<sup>−/−</sup> Obstructed Kidneys**

To study the physiologic role of de novo expression of CD44 in UUO, we compared renal injury in CD44<sup>−/−</sup> and CD44<sup>+/+</sup> mice. Tubular damage was significantly more severe in CD44<sup>−/−</sup> compared with CD44<sup>+/+</sup> obstructed kidneys at all time points (Figure 2A). Increased tubular damage in CD44<sup>−/−</sup> kidneys was associated with decreased proliferation (Figure 2B) and increased apoptosis of CD44<sup>−/−</sup> compared with CD44<sup>+/+</sup> TEC (Figure 2C).

**Decreased Macrophage Influx in CD44<sup>−/−</sup> Obstructed Kidney**

At all time points after UUO, the influx of macrophages was significantly impaired in the CD44<sup>−/−</sup> compared with CD44<sup>+/+</sup> mice (Figure 3, A and B).

**Hyaluronic Acid and Osteopontin Expression in CD44<sup>−/−</sup> and CD44<sup>+/+</sup> Obstructed Kidneys**

Because HA and osteopontin are the principal ligands of CD44 and promote inflammation, we analyzed HA and osteopontin expression by immunohistochemistry. Interstitial HA-positive areas expanded in the obstructed kidneys of both genotypes (Figure 4A), reaching up to a tenfold increase relative to the contralateral kidneys by day 7 after UUO (Figure 4B). Interestingly, the increase in HA was significantly higher in the CD44<sup>−/−</sup> mice compared with the CD44<sup>+/+</sup> mice. In contrast, osteopontin expression was comparable in CD44<sup>−/−</sup> and CD44<sup>+/+</sup> obstructed kidneys (Figure 5, A and B).

![Figure 5. Osteopontin expression after UUO. (A) Immunostaining for osteopontin revealed comparable expression of osteopontin in renal cortex after UUO in CD44<sup>+/+</sup> and CD44<sup>−/−</sup> mice (magnification, ×100). (B) The number of positive tubuli were counted and expressed as percentage of the total. White bars represent CD44<sup>+/+</sup>, and black bars represent CD44<sup>−/−</sup>. Mean ± SEM, n = 6.](image-url)
Attenuation of Renal Fibrosis in CD44\(^{-/-}\) Obstructed Kidney

As expected, total kidney collagen increased in time in CD44\(^{+/+}\) mice in response to UUO. In sharp contrast, the increase in collagen deposition was attenuated in CD44\(^{-/-}\) mice (Figure 6A). These data were confirmed by Sirius Red staining (Figure 6, B and C).

Myofibroblasts play an important role in interstitial fibrosis; therefore, we followed the accumulation of \(\alpha\)-SMA\(^+\) cells. Accumulation of myofibroblasts was delayed in CD44\(^{-/-}\) mice compared with CD44\(^{+/+}\) mice as quantified by digital image analysis (Figure 7A). This was confirmed by Western blot analysis, showing less \(\alpha\)-SMA in renal homogenates of CD44\(^{-/-}\) \textit{versus} CD44\(^{+/+}\) obstructed kidneys at day 1 and day 3 (Figure 7B).

To determine whether the absence of fibrosis in CD44\(^{-/-}\) obstructed kidneys was caused by differences in synthesis or degradation of collagen, quantitative real-time RT-PCR was...
performed for collagen type IV, which transcripts were comparable in CD44+/+ and CD44−/− mice (Figure 8A). We further assessed MMP activity in renal homogenates by zymography. A marked but similar induction of MMP-2 and MMP-9 activity was observed in CD44+/+ and CD44−/− obstructed kidneys (Figure 8B). To further assess the MMP’s capacity to degrade collagens, we determined the level of tissue inhibitors of MMP (TIMP) by immunoblotting. TIMP-1, the inhibitor with the highest affinity for MMP-9, was less present at days 7 and 14 in CD44−/− compared with CD44+/+ obstructed kidney (Figure 8C). The level of TIMP-2, with the highest affinity for MMP-2, was comparable in both groups (Figure 8C).

CD44 Facilitates HGF-Signaling In Vivo

Because signaling by HGF is facilitated by the expression of the v3 isoform of CD44 (CD44v3) (11), we determined mRNA levels of CD44v3 in the CD44+/+ and CD44−/− mice by quantitative real-time PCR (Figure 9A). Upon obstruction, mRNA levels of CD44v3 increased, starting at day 1 to culminate after 3 d, indicating its potential role early after obstruction. To get insight in the cellular localization of CD44v3, tubuli were microdissected and conventional PCR for CD44v3 mRNA performed. As shown in Figure 9B, CD44v3 mRNA was clearly present in tubuli 3 d after obstruction.

The HGF level of the obstructed CD44+/+ kidneys decreased after 7 d and was nearly undetectable at day 14. In obstructed CD44−/− kidneys, a clear increase in HGF was observed after 3 d followed by a rapid decrease (Figure 10A).

To study whether CD44 expression could enhance HGF signaling via its high-affinity receptor, c-Met, expression and phosphorylation of the receptor were determined. c-Met expression increased as obstruction continued (data not shown). Initially (data not shown) and 1 d after obstruction, c-Met expression is more pronounced in the CD44−/− kidney compared with the CD44+/+ kidney (Figure 10B). After 3 d, no difference in expression of c-Met was observed between CD44+/+ and CD44−/− obstructed kidneys, but the capacity of
HGF to activate c-Met was less efficient in CD44+/H11001/H11001 than in CD44+/H11002/H11002 mice (Figure 10, B and C). After 7 d of obstruction, phosphorylation of c-Met was hardly detectable in both groups (data not shown). To obtain insight into the site of c-Met phosphorylation, immunostainings for phospho-c-Met were performed that showed diffuse positive tubuli in CD44+/H11001/H11001 obstructed kidney but only a few positive tubuli in CD44+/H11002/H11002 obstructed kidneys (Figure 10D).

CD44 Contributes to TGF-β1 Signaling In Vivo
Despite the difference in TGF-β1 levels in CD44+/+ and CD44−/− obstructed kidneys (Figure 11, A and B) and the higher expression of Smad-2 and Smad-3 (the major signaling molecules of TGF-β1) in CD44−/− kidneys, comparable phosphorylation of Smad-2 and Smad-3 was observed in CD44+/+ and CD44−/− obstructed kidneys (Figure 11C), suggesting an impaired TGF-β1 signaling pathway in CD44−/− mice. In addition, nuclear translocation of p-Smad-2/3, essential for TGF-β1 signaling, was impaired in the absence of CD44 (Figure 11D). Both observations suggest a crucial role for CD44 in TGF-β1 signaling.

Discussion
The decline of renal function in a variety of pathologic states closely correlates with the degree of tubulointerstitial damage. A cascade of events takes place during the progression of tubulointerstitial lesions, including release of cytokines/chemokines and growth factors, expression of adhesion molecules, inflammatory infiltrate, renal epithelial cell damage, accumulation of myofibroblasts, and finally fibrosis. In this study, we show that CD44 plays a crucial role in this cascade.

First, we show by quantitative real-time PCR that CD44 mRNA peaked at day 7 after UUO and by immunohistochemistry that CD44 is predominantly expressed by damaged tubuli and inflammatory cells. This is in agreement with previous studies reporting the expression of CD44 in different models of kidney diseases (2–4) and in human nephropathies (1,5).

Second, we demonstrate that CD44 expression decreases tubular injury as a consequence of increased tubular proliferation and decreased tubular apoptosis. In vitro, CD44 has been implicated in cell proliferation (30) and apoptosis (31). CD44 expression may promote the maintenance of tubular cell viability in response to renal injury, because cell-cell and cell-matrix interactions might be facilitated by CD44 (4). Moreover, ligand-receptor interaction of osteopontin with CD44 induces proliferation and decreases apoptosis of TEC (17,18). The survival signal induced by CD44-osteopontin interaction involves activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (32), which is also used by HGF, a well-known renoprotective molecule (22,33). In vitro studies re-
revealed that CD44v3 binds HGF and presents it to its high-affinity receptor c-Met (11). In this study, we observed for the first time a role for CD44 in HGF signaling in vivo because phosphorylation of c-Met was attenuated in obstructed CD44−/− kidneys. Altogether facilitation of HGF signaling by CD44 is very likely to contribute to the preservation of tubuli in our model.

Third, we observed that the two major ligands of CD44, HA, and osteopontin are highly expressed in obstructed kidneys. Interactions of CD44 with HA and osteopontin play an important role in renal inflammation (3,34,35). Interaction of CD44 with HA is a crucial step in the rolling of leukocytes along endothelial cells (36,37). Lack of CD44 was associated with increased HA accumulation in obstructed kidneys. This is in agreement with the study of Teder et al. (16), in which HA accumulated in a model of pulmonary fibrosis as a consequence of CD44 deficiency, suggesting a crucial role for CD44 in the homeostasis of HA. Osteopontin binds to macrophages and mediates their adhesion and migration (18,38). Absence of functional interaction between CD44 and both HA and osteopontin results in less infiltration of macrophages in the CD44−/− compared with CD44+/+ obstructed kidneys. Although macrophages are often considered to induce damage to the tubulointerstitial compartment (39), the influx of macrophages is not correlated to the severity of tubular damage in our model, which indicates that macrophage influx is not the main determinant of the tubular response leading to tubular cell loss. Macrophage clearance capacity of apoptotic bodies is critical in resolution of inflammation. If apoptotic clearance capacity is exceeded, apoptotic cells may progress to secondary necrosis, resulting in the release of harmful cellular contents and in damage to the surrounding tissue. Lack of CD44 results in decreased clearance capacity of apoptotic bodies (16) and may therefore contribute to increased tubular damage.

Fourth, our study reveals that renal fibrosis was clearly attenuated, despite the extensive tubular damage observed in CD44−/− mice after UUO. This was associated with less myofibroblasts accumulation compared with CD44+/+ mice. Although the differences in accumulation of myofibroblasts are only evident early in obstruction (days 1 and 3), the earlier myofibroblast recruitment may contribute to the development of fibrosis in CD44+/+ obstructed kidneys as the obstruction continues. Because accumulation of extracellular matrix may be caused by either increased synthesis, decreased degradation, or both, the levels of collagen type IV mRNA were assessed by quantitative PCR, which mRNA was induced at the same levels in CD44−/− and CD44+/+ obstructed kidneys. Since MMP play a central role in the degradation of matrix proteins, we analyzed MMP activity by zymography. No differences in activity could be detected between CD44+/+ and CD44−/− mice that could explain the striking difference in collagen accumulation. The differences in collagen accumulation between CD44−/− and CD44+/+ mice may be related to altered production of other collagens, such as collagen type III, differential expression of other MMP, or altered expression of tissue inhibitors of MMP (TIMP) (40,41). Accordingly, Western blot analyses revealed enhanced expression of TIMP-1, but not TIMP-2, in the CD44−/− compared with CD44+/+ obstructed kidneys. Although TIMP-1 deficiency has not been shown to attenuate renal fibrosis (42), increased TIMP-1 ex-
pression in the presence of CD44 is one of the possible genes that may contribute to the development of fibrosis. This does not rule out other factors involved in fibrogenesis.

TGF-β1 is probably the most important pro-fibrotic agent during progression of renal disease. Indeed, TGF-β1 induces myofibroblastic transition (43–45), promotes collagen type IV production (46), decreases MMP expression, and increases expression of TIMP (47). Unexpectedly, regarding the absence of renal fibrosis in CD44−/−/− mice, higher levels of TGF-β1 and activated TGF-β1 were observed in obstructed CD44−/− kidneys compared with CD44+/−/− kidneys. TGF-β1 is secreted in a latent form and needs to be converted into an active form to exert its biologic activity. In vitro, TGF-β1 is activated by a variety of mechanisms, but in vivo mechanisms are still not fully understood. A novel mechanism has been suggested that involves direct interaction of CD44 and MMP-9 to activate TGF-β1 in vivo (48). In a model of pulmonary fibrosis, the lack of CD44 was found to lead to decreased levels of active TGF-β1 (16). Our findings in the obstructed kidney are at variance because we found higher levels of active TGF-β1 in CD44−/− obstructed kidney compared with CD44+/−/− (circa 60% in CD44−/− versus circa 35% in CD44+/−/− of total TGF-β1). Thrombospondin-1 is also considered to be a major activator of TGF-β1 in vivo (49), yet thrombospondin-1 expression was comparable in both types of mice (data not shown). Although higher levels of TGF-β1 and Smad-2 and Smad-3 (two major signaling proteins of TGF-β1) were found in CD44−/− renal homogenates compared with CD44+/−/−, relatively less phosphorylation and nuclear translocation (essential for TGF-β1 signaling) of Smad-2 and Smad-3 was measured in CD44−/− compared with CD44+/−/− obstructed kidneys. An essential role for CD44 in TGF-β1 signaling in vivo is suggested by the fact that, in the absence of CD44, more Smad-2 and Smad-3 expression is required to obtain identical levels of phosphorylated Smad-2 and Smad-3 and that, despite equal levels of phosphorylated Smad-2 and Smad-3, less nuclear translocation was observed in CD44−/− mice. Accordingly, Bourguignon et al. (27) recently showed that HA promotes signaling interaction between CD44 and TGF-β1 receptor in metastatic mammary carcinoma.

In conclusion, de novo expression of CD44 in injured kidneys promotes macrophage recruitment. In addition, expres-
Figure 11. Transforming growth factor–β1 (TGF-β1) levels and Smad-2 and Smad-3 activation after UUO. Total TGF-β1 (A) and activated TGF-β1 (B) levels were quantified by ELISA and corrected for quantity of protein, CD44+/+ (white bars), and CD44+/− (black bars); data are presented as mean ± SEM, n = 6. (C) TGF-β1 signaling was determined by Western blotting by assessment of the total levels of Smad-2 and Smad-3 and their phosphorylation; β-actin was used as loading control. Blots were analyzed by densitometric analysis, the ratio of phosphorylated Smad-2 and Smad-3 versus Smad-2 and Smad-3 is depicted in the graphs. Data are presented as mean ± SEM, n = 6. (D) Representative immunostainings for phospho-Smad-2/3 of CD44+/+ and CD44+/− renal cortex 7 d after obstruction (magnification, ×500); the percentage of TEC with positive nuclear staining for phospho Smad-2 and Smad-3 are depicted in the graph.
sion of CD44 protects TEC at least in part by enhancing survival signals through its interaction with osteopontin and HGF. Moreover, expression of CD44 in the injured kidney contributes to the development of fibrosis at least in part through enhanced TGF-β1 signaling. Altogether, our data suggest that CD44 contributes to the delicate balance between HGF and TGF-β1 in the progression of renal disease (50).

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References


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