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Opposite role of CD44-standard and CD44-variant-3 in tubular injury and development of renal fibrosis during chronic obstructive nephropathy

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Chronic kidney diseases (CKDs) are characterized by tubular atrophy and interstitial fibrosis. We previously showed that in obstructive nephropathy *de novo* CD44 renal expression contributes to renal fibrosis but attenuates tubular damage/apoptosis. As CD44-standard (CD44s) has been linked to TGF-β1-mediated actions and CD44-variant-3 (CD44v3) favors HGF-c-Met binding, we compared the functional properties of these CD44 isoforms in the progression of obstructive nephropathy, using specific CD44-variant knockout/knockin mice. The presence of CD44v3 diminished tubular damage during obstructive nephropathy, decreased apoptosis, and increased proliferation of tubular epithelial cells, and prevented renal fibrosis development. In contrast, expression of CD44s led to increased tubular damage and tubular epithelial cell apoptosis, and more renal fibrosis. A relative increase in renal β-catenin expression, HGF production, and HGF/c-Met signaling, together with a relative inhibition of TGF-β1 downstream signaling and TGF-β type I receptor expression, was found in CD44v3 mice compared with CD44s littermates. In line with this, Wnt3a/HGF treatment of tubular cells resulted in higher β-catenin/p-AKT levels in CD44v3$^+$ tubular epithelial cells, whereas TGF-β1 induced a mild collagen I upregulation in CD44v3$^+$ mouse embryonic fibroblasts as compared with CD44s$^+$ cells. Thus, CD44s and CD44v3 exert opposite roles in the progression of obstructive nephropathy, with CD44v3–v10 being the protective isoform that delays evolution of the renal pathology.

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Chronic kidney disease (CKD) is a common condition in which there is a loss of renal function over time, regardless of etiology. Progression of renal diseases is characterized by tubular damage and atrophy, renal leukocyte recruitment, rarefaction of the peritubular capillary network, myofibroblast accumulation, and eventually exceeded extracellular matrix (ECM) deposition that result in scar tissue formation and loss of renal function.[1] The reciprocal imbalance between protective factors such as hepatocyte growth factor (HGF) and deleterious factors, such as transforming growth factor-β1 (TGF-β1) is closely involved in the progression of tissue fibrosis.[2] We formerly showed that CD44 contributes to the signaling activation of both HGF and TGF-β1 in injured kidneys,[3] and previous reports indicated its involvement in wound healing,[4] fibrosis,[5] and inflammation.[6,7]

CD44 family members are widely expressed class I transmembrane glycoproteins. They mediate cell responses to the cellular microenvironment, functioning as specialized 'platforms' for growth factors, chemokines, matrix metalloproteases (MMPs), and components of the ECM,[8,9] including hyaluronan (HA) and osteopontin.[10,11] CD44 glycoproteins are encoded by a single gene consisting of 20 exons: exons 1–5 and 16–18 are constants, whereas exons 6–15 and 19–20 are variably expressed and inserted by alternative splicing.[12] Direct splicing from constant exon 5 to constant exon 16 generates the shortest and most common isoform CD44-standard (CD44s).[9] Bourguignon *et al.*[13] showed that, upon HA binding, CD44 can interact with TGFβ receptor type I (TGFβR-I) in metastatic breast tumor cells. Recent reports highlighted the importance of CD44s in TGF-β1-mediated epithelial-to-mesenchymal transition,[14,15] and our earlier study showed that CD44s expression by tubular epithelial cells (TECs) sensibilizes cells to TGFβ-1 profibrotic effects.[16] Another isoform of CD44 that has been extensively studied is CD44-variant-3 (CD44v3/CD44v3–v10), which contains variable exons 3–10 and has heparan sulfate (HS) chains attached to its extracellular domain. Owing to the HS chains, CD44v3 is capable of binding growth factors

containing an HS-binding site, such as the renoprotective HGF, and facilitates the cross-linking with their receptors.[8,9] Moreover, CD44 acts as a co-receptor for the HGF receptor c-Met, and isoforms containing variant exon v6 are strictly required for c-Met activation by HGF in carcinoma cells.[17]

Under normal conditions, CD44 is hardly expressed in the kidney except for passenger leukocytes.[18,19] However, in inflammatory renal diseases, CD44 expression is markedly enhanced, particularly in glomerular crescents and injured tubules, as documented in human diseases and in several animal models.[3,18–21] Furthermore, the degree of CD44 expression on tubules closely correlates with inflammation in renal transplantation,[22] and the degree of tubular damage and interstitial fibrosis in IgA nephropathy,[21] and renal transplantation.[23]

As CD44s has been linked to TGF-β1-mediated actions and CD44v3 is known to facilitate HGF-c-Met binding, we hypothesized that expressing either CD44s or CD44v3 by using specific knockout/knockin (KO/KI) mice would aggravate CKD progression or exert beneficial effects during chronic obstructive nephropathy, respectively.

RESULTS

CD44 upregulation after unilateral ureteral obstruction (UUO)

The evolution of renal damage after ureteric ligation was compared in $CD44^{+/+}$ wild type (WT) C57Bl/6 mice, specific KO/KI C57Bl/6 mice expressing solely CD44s (CD44s mice) or CD44v3 (CD44v3 mice). Both CD44 KO/KI mouse strains bred normally and presented no macroscopic abnormalities and have been previously used.[24,25] In physiological situation, CD44s and CD44v3 mice showed normal kidney histology and function and no expression of CD44, except for passenger leukocytes, as in WT mice (data not shown).

In all mouse strains, CD44 was upregulated in renal tissues after unilateral ureteral obstruction (UUO) (Figure 1a); CD44 was predominantly expressed at the basolateral membranes of TECs, as well as by interstitial cells (Figure 1b). Renal CD44 expression in CD44s mice was increased to a lesser extent as compared with WT and CD44v3 mice (Figure 1a). Quantitative real-time PCR (Q-PCR) analysis revealed specific expression of CD44s and CD44v3 mRNAs, respectively, in CD44s and CD44v3 mice 3 days after obstruction, when CD44 protein starts to be upregulated (Figure 1c and d). As CD44 gene transcription is at least in part activated by Wnt/β-catenin signaling,[26,27] which is activated in TECs during obstructive nephropathy,[28] we next determined β-catenin abundance in UUO kidneys to elucidate the differences in CD44 expression among the mouse strains. In line with CD44 expression, we found more cytoplasmic and nuclear β-catenin in TECs of WT and CD44v3 kidneys than in CD44s kidneys at days 3 and 7 of UUO (Figure 1e–g).

Differential impact of CD44s and CD44v3 on tubular damage and proliferation/apoptosis after UUO

The importance of CD44s and CD44v3 in the development of chronic renal injury was first studied by assessing tubular injury after ureter ligation (Figure 2a). At day 3 of UUO, tubular damage in CD44v3 mice was less compared with WT and CD44s animals (Figure 2b); at day 7, renal injury was milder in WT and CD44v3 mice as compared with CD44s animals; finally, at day 14 the maximum level of renal damage was reached, and therefore, no differences were observed among the strains.

We next questioned whether the dissimilarities in tubular damage among the mouse strains could be explained by differences in TEC proliferation/apoptosis rate. In response to the renal injury, tubular cells started proliferating at similar levels in all mouse strains (day 3); however, at a later stage of UUO, proliferation drastically decreased, with exception of the TECs of CD44v3 mice (Figure 3a and b). Tubular cellular apoptosis progressively increased during UUO, although the expression of solely CD44s exacerbates this process (Figure 3c and d).

Together with tubular damage, we analyzed macrophage renal infiltration, which is strongly associated with the progression of renal injury and fibrosis.[1,29] Macrophage influx into the obstructed kidneys occurred similarly in all groups (Supplementary Figure S1A online). Next, we studied the interstitial expression of osteopontin and HA, two components of the ECM and major CD44 ligands that accumulate upon injury and promote inflammation and leukocyte recruitment.[30,31] We found that both osteopontin and HA markedly increased from day 3 UUO (Supplementary Figure S1B and C online). Osteopontin accumulation was comparable among the strains (Supplementary Figure S1B online), whereas HA expression was significantly more elevated in the KO/KI mice at day 3 and in CD44s mice at day 7 as compared with WT mice (Supplementary Figure S1C online).

Reduced fibrosis in CD44v3 mice

Obstructive nephropathy eventually leads to renal fibrosis caused by myofibroblasts, which are marked by *de novo* expression of α-SMA in rodents.[32] Myofibroblast accumulation was comparable among the strains after 3 and 14 days of obstruction; at day 7, CD44v3 kidneys displayed significantly more myofibroblasts (Figure 4a and b). In line with previous reports,[33,34] CD44 was expressed by activated fibroblasts, as shown in CD44v3-obstructed kidneys (Figure 4c).

The augmentation of collagen deposition was assessed by hydroxyproline assay, picrosirius red, and collagen type I stainings (Figure 4d–f). Despite more myofibroblasts, fibrosis formation in CD44v3 kidneys was mild and less as compared with that in WT and CD44s-obstructed kidneys, which displayed a significant increase in collagen (Figure 4d, day 14 vs. sham: $P = 0.04$ for WT, $P = 0.007$ for CD44s).

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