Lack of hyaluronidases exacerbates renal post-ischemic injury, inflammation, and fibrosis

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Renal ischemia–reperfusion injury (IRI) is a pathological process that may lead to acute renal failure and chronic dysfunction in renal allografts. During IRI, hyaluronan (HA) accumulates in the kidney, but suppression of HA accumulation during IRI protects the kidney from ischemic insults. Here we tested whether *Hyal1* −/− and *Hyal2* −/− mice display exacerbated renal damage following unilateral IRI due to a higher HA accumulation in the post-ischemic kidney compared with that in the kidney of wild-type mice. Two days after IRI in male mice there was accumulation of HA and CD44 in the kidney, marked tubular damage, infiltration, and increase creatininemia in wild-type mice. Knockout mice exhibited higher amounts of HA and higher creatininemia. Seven days after injury, wild-type mice had a significant decrease in renal damage, but knockout mice still displayed exacerbated inflammation. HA and CD44 together with α-smooth muscle actin and collagen types I and III expression were increased in knockout compared with wild-type mice 30 days after IRI. Thus, both HA-degrading enzymes seem to be protective against IRI most likely by reducing HA accumulation in the post-ischemic kidney and decreasing the inflammatory processes. Deficiency in either HYAL1 or HYAL2 leads to enhanced HA accumulation in the post-ischemic kidney and consequently worsened inflammatory response, increased tubular damage, and fibrosis.

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Acute kidney injury remains associated with high morbidity and mortality rates in humans and can lead to end-stage renal disease. One of the major causes of acute kidney injury is ischemia–reperfusion (IR) injury (IRI), a pathological process that often occurs in renal allografts[1] and after major vascular surgery.[2] IRI results from a combination of events including cellular hypoxia, tubular and endothelial injuries, and inflammatory processes. IRI may be followed by a repair process that can restore normal morphology and function in case of moderate damage,[3] but when incomplete leads to permanent damage, progressive fibrosis, and chronic kidney disease (CKD).[4]

Despite intensive research, none of the attempts at reducing early post-ischemic renal damage in experimental animals has turned into an effective treatment of IRI in humans.[5] Therefore, novel avenues of treatment need to be identified. Among the factors involved in inflammation and regeneration processes of the post-ischemic kidney are hyaluronan (HA) and its main receptor CD44. HA is a ubiquitous glycosaminoglycan of the extracellular matrix with a fast turnover.[6] It is found in most parts of the body and composed of repeating units of the disaccharide [-D-glucuronic acid-β1,3-*N*-acetyl-D-glucosamine-β1,4]$_n$.[7] In the healthy kidney, HA is abundant in the interstitium of the inner medulla but almost absent in the cortex, with intermediate levels in the outer medulla.[8,9] During pathophysiological conditions such as diabetic nephropathy,[10] allograft rejection,[11] and ischemia reperfusion,[12] HA accumulates in the cortex. Declèves *et al.*[13] have previously demonstrated that IR in rats induces HA accumulation in both cortex and outer medulla, thereby modifying the physicochemical environment of interstitial cells. The dynamics of HA deposition in IR suggest that it might initially attract inflammatory and regenerative cells and later participate in the persistence of tissue inflammation, possibly through an interaction with its main receptor, CD44, which is expressed on basolateral membranes of damaged tubules, as well as on inflammatory cells.[13]

Regulation of HA levels is required to maintain homeostasis and is achieved by coordinative expression of its metabolic enzymes. HYAL1 and HYAL2 are the major hyaluronidases in somatic tissues, whereas HA synthesis is achieved by three HA synthases.[7] Declèves *et al.*[14] have demonstrated that HA

accumulation in the post-ischemic kidney is related to temporal changes in the level of expression of its metabolic enzymes: *Hyal1* and *Hyal2* mRNA expression are repressed during the first 24 h post-IR, whereas *Has1* is upregulated, thereby promoting the accumulation of HA. *Has2* mRNA expression is markedly increased 7 and 14 days after IR, whereas *Has1* mRNA expression decreases at this time point. Moreover, we have shown that suppression of HA accumulation in the post-ischemic kidney through 4-methylumbelliferone treatment is protective against IRI.[15]

A pathogenic role of HA has also been suggested in CKD. For instance, expression of the HA-CD44 pair is highly increased in chronic cyclosporine A–induced nephropathy,[16] experimental crescentic glomerulonephritis,[17] and obstructive nephropathy.[18] Therefore, blocking the interaction between HA and CD44 may prevent the development of fibrosis in CKD.[18]

On the basis of the above observations, we hypothesized that deficiency in either HYAL1 or HYAL2 could cause a higher accumulation of HA in the post-ischemic kidney of *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice (knockout (KO) mice). In turn, that could lead to an exacerbated inflammatory response and more severe renal damages compared with the wild-type (WT) mice littermates.

RESULTS

HYAL1- and HYAL2-deficient mice display much higher HA accumulation in association with damaged tubules in the post-ischemic kidney

As shown in Figure 1a and b, IR induced a marked HA accumulation in the cortex and outer medulla of all mice 2 days after surgery, but *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice showed even higher concentrations of HA in comparison to their respective WT littermates (HYAL1: 181 ± 30 vs. 80 ± 18 ng/mg; HYAL2: 153 ± 21 vs. 39 ± 6 ng/mg). Baseline levels of cortex/outer medulla HA were slightly higher in both types of KO mice versus WT mice. The levels of HA in the inner medulla did not change after IR (data not shown). Post-IR HA accumulated predominantly in the interstitium and was associated with damaged tubules (Figure 1f and h).

Seven days after IR, HA levels had returned to baseline in WT mice but remained strikingly elevated in *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice (i.e., as high as on Day 2): HYAL1: 206 ± 32 vs. 11 ± 4 ng/mg; HYAL2: 125 ± 26 vs. 17 ± 9 ng/mg (Figure 1a and b). One week post-IR, HA remained associated with damaged tubules; the latter were much more prominent in KO mice than in WT mice (Figure 1i–k).

Renal HA content remained elevated in both WT mice 30 days post-IR but was significantly higher in *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice (HYAL1: 64 ± 19 vs. 20 ± 5 ng/mg; HYAL2: 44 ± 7 vs. 21 ± 4 ng/mg; Figure 1l–n).

Lower HYAL expression and higher HAS1 expression drive HA accumulation in KO mouse post-ischemic kidney

The expressions of *HYAL1-2* and *HAS1-2* mRNAs were measured at baseline and after IR using quantitative real-time reverse-transcriptase–PCR (Tables 1 and 2). At baseline, *Hyal1*$^{-/-}$ mice had a lower *Hyal2* mRNA expression in comparison to the WT mice, whereas *Hyal2*$^{-/-}$ mice had a higher level of *Hyal1* mRNA expression than the WT littermates. Regarding *HAS1-2* mRNA expression, there was no difference between KO and WT mice.

Two, 7, and 30 days after IR, *Hyal1* and *Hyal2* mRNA expressions decreased in WT mice. Similarly, *Hyal2* mRNA expression also decreased in *Hyal1*$^{-/-}$ mice (even more than in WT mice), but *Hyal1* mRNA expression remained higher in *Hyal2*$^{-/-}$ mice than in their littermates. In other words, low or absent HYAL2 expression accompanies HA accumulation during IRI in all three genotypes.

Regarding HA synthases, *Has1* mRNA expression increased between 3.5- and 35-fold at day 2 and 7 after IR in each genotype. The highest levels were found in KO mice at day 7. The levels of *Has1* mRNA tended to return to baseline at day 30. *Has2* mRNA expression showed brief and mild increases at day 2 or 7 in all genotypes without significant differences between WT and KO mice.

CD44 expression is also increased in the post-ischemic kidney of HYAL1- and HYAL2-deficient mice

The main HA receptor, CD44, was barely detectable in the kidney of control mice (Figure 2c–e). Two days after IR, its expression increased significantly and was located mainly on interstitial cells around the necrotic tubules (Figure 2a and b, f–h). No difference was observed between WT and KO mice at this time point. However, 7 days after surgery, CD44 had returned to basal levels in WT mice but remained extremely high (as high as on day 2) in *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice (HYAL1: 21.0 ± 2.9 vs. 0.9 ± 0.4% of scanned area; HYAL2: 20.6 ± 4.3 vs. 1.8 ± 0.7%; Figure 2a and b, i–k). CD44 remained elevated in both WT mice 30 days post-IR but was significantly higher in *Hyal1*$^{-/-}$ and *Hyal2*$^{-/-}$ mice (Figure 2a and b, l–n). The pattern of CD44 expression closely mirrored that of HA accumulation.

HYAL1 or HYAL2 deficiency increases renal injury after IRI

Two days after surgery, IR induced a large increase in plasma creatinine concentration in WT mice; this increase was slightly higher in *Hyal1*$^{-/-}$ mice (70 ± 5 vs. 58 ± 5 μmol/l; Figure 3a) and in *Hyal2*$^{-/-}$ mice (73 ± 11 vs. 50 ± 7 μmol/l; Figure 3b). Seven and 30 days after IR, creatininemia values returned to baseline, although they remained slightly higher in *Hyal1*$^{-/-}$ mice in comparison to their WT littermates at Day 7 (Figure 3a and b).

Renal lesions were evaluated using periodic acid–Schiff staining. Acute tubular injury, including tubular cell detachment, cell debris in tubular lumens, and tubular dilation, was detected to the same extent in WT and KO mice 2 days after IR (Figure 3c–j) but, although these lesions were clearly improved in WT mice, they remained at a high level in both types of KO mice at day 7 (similar to day 2) and day 30 (Figure 3k–p). At that time, the percentage of damaged tubules was significantly higher in *Hyal1*$^{-/-}$ mice in comparison to *Hyal1*$^{+/+}$ mice (Figure 3c) as well as in *Hyal2*$^{-/-}$ mice in