Synthesis and fragmentation of hyaluronan in renal ischaemia

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Abstract

Background. The turnover of hyaluronan (HA), especially the production of low-molecular-weight fragments of HA, was examined in a model of unilateral renal ischaemia–reperfusion (IR) in rats.

Methods. HA was extracted from the outer and inner stripe of the outer medulla (OSOM and ISOM) at different times following IR. Its fragmentation was measured using membrane filtration and size-exclusion chromatography. Quantitative reverse transcription–polymerase chain reaction, zymography and immunohistochemistry were used to assess the expression and localization of various forms of HA synthase (HAS) and hyaluronidase (HYAL). Macrophage infiltration was evaluated using immunohistochemistry.

Results. HA accumulated at Day 1 mostly as high-molecular-weight (HMW) species with an elution profile similar to a reference 2500 kDa HA and at Day 14 mostly as medium- to low-size fragments. Within 1 day, HAS1 messenger RNA was up-regulated >50- and 35-fold in OSOM and ISOM, respectively. Thereafter, HAS1 tended to normalize, while HAS2 increased steadily. Both synthetic enzymes were localized around tubules and in the interstitium. Conversely, HYAL1, HYAL2 and global hyaluronidase activity were repressed during the first 24 h. The patterns were identical in the OSOM and ISOM despite markedly different amounts of HA at baseline. There was no obvious correlation between HA deposits and macrophage infiltration.

Conclusions. In the post-ischaemic kidney, HA starts to accumulate at Day 1 mostly as HMW species. Later on, a large proportion becomes degraded into smaller fragments. This pattern is explained by coordinated changes in the expression of HA synthases and hyaluronidases, especially an early induction of HAS1. The current data open the door to timed pharmacological interventions blocking the production of HA fragments.

Keywords: extracellular matrix; hyaluronan synthase; hyaluronidase; inflammation; renal outer medulla

Introduction

Hyaluronan (HA), a linear glycosaminoglycan of the extracellular matrix with a native mass of >1000 kDa, plays various biological roles including space filling, filtering, maintenance of water homeostasis and modulation of inflammation and tumourigenesis [1]. It is usually assumed that in order to perform pro-inflammatory activities, HA must be partially degraded into low-molecular-weight (LMW) forms [2, 3]. In vitro, LMW-HA exhibits distinct functions compared with high-molecular-weight (HMW) HA. For instance, HA fragments either produced in vitro or collected from the serum of individuals with acute lung injury stimulate innate immunity through toll-like receptor-4 (TLR4) and -2 (TLR2) [4, 5] while HMW-HA inhibits pulmonary vascular leakiness [6]. However, up to now, the demonstration of the existence of LMW HA species in vivo has been limited.

The kidney is an organ where HA accretion has been observed under various conditions, including ischaemia–reperfusion (IR) injury [7–9]. In rats, if the ischaemic challenge is ≤30 min, HA does not accumulate except on a diabetic background [10]. We previously reported that besides the cortex, HA amasses in the rat outer and inner stripes of the outer medulla (OSOM and ISOM) after a 60-min clamp of the renal artery followed by reperfusion [11]. The outer medulla is a critical ischaemic and inflammatory tissue when renal blood flow is compromised [12]. The most severe tubular injuries and largest leucocyte infiltrations are found in this zone. However, it is unknown whether and how HA breaks up during renal IR.

The aim of the present study was therefore to determine if and how HA accumulates and gets degraded following severe IR injury in the rat kidney. We focussed on OSOM, which is devoid of HA at baseline, and ISOM, which contains fairly large amounts of this polysaccharide. The expression pattern of the main HA synthases (HAS1, HAS2 and HAS3) and somatic hyaluronidases (HYAL1 and HYAL2) as well as the presence of different populations of HA fragments within the renal tissue were measured up to Day 14 after IR.
Materials and methods

Animals and renal IR model

The study conformed to the American Society of Physiology’s Guiding Principles in the Care and Use of Animals and the protocols were approved by the local Ethical Commission on Animal Welfare. Experiments were performed on male Wistar rats weighing 220–260 g purchased from Harlan Nederland (Horst, The Netherlands) and maintained in our animal facility. Animals were anesthetized with 60 mg kg⁻¹ body weight sodium pentobarbital intraperitoneally, placed on a heated table and underwent left renal artery occlusion for 60 min as described previously [11]. Right nephrectomy was performed as the clamp on the left artery was removed. Rats were returned to cages for up to 12, 24 (Day 1), 48 (Day 2), 168 (Day 7) and 336 h (Day 14) before euthanasia and analysis of the remaining kidney. To separate OSOM, ISOM and the inner medulla, the kidney was rapidly removed, decapsulated and cut along the corticopapillary axis. The tissue was then carefully dissected on ice under a stereomicroscope. The boundary between OSOM and ISOM was identified as the transition between a very pale and thin area (OSOM, also easily distinguished from cortex) and the adjacent dark red zone corresponding to ISOM. The dissected tissue was immediately frozen and stored at −80°C for RNA and HA extraction or prepared for zymography (fresh samples) or immunohistochemistry (paraffin embedding). Plasma creatinine concentration was determined using the Jaffé method. In control groups, rats were not submitted to any surgical preparation.

Separation and detection of LMW and HMW HA polymers in renal tissue

Snap-frozen samples were lyophilized before digestion with Pronase E (Sigma) at 1 mg/mL in ammonium/formic acid buffer, pH 7–8, at 55°C for 24 h followed by immersion in boiling water during 10 min. Samples were centrifuged (1000 g, 20 min) and the supernatants were lyophilized and stored at −70°C. The samples were analysed using two different methods performed in a blinded fashion in two different laboratories. Firstly, the amount of LMW HA was assessed using a size-exclusion chromatography. Here, the lyophilized samples were dissolved in the elution buffer (0.5 M Na-acetate, pH 5.8, containing 0.1% CHAPS) and analysed on a 0.5 × 20 cm column of Sephacryl S-1000 (Amersham Biosciences, Uppsala, Sweden) eluted at 0.07 mL/min. Fractions of 0.22 mL were collected, diluted in 1% bovine serum albumin in phosphate-buffered saline and assayed for HA using a sandwich-type ELISA-like assay based on a biotinylated probe containing aggrecan G1 domain and link protein, as described previously [14]. The supernatants of the Pronase digestion were filtered during centrifugation at 2500 g for 30 min in Centrisart I columns (Sartorius Technologies SV, Belgium) with a nominal 100-kDa cut-off. The HMW (retained) and LMW (filtered) HA fractions were collected separately. In a second step, HA concentration in these samples was measured using a commercial competitive pseudo-enzyme-linked immunosorbent assay (HA-ELISA from Echelon Biosciences, Inc., Salt Lake City, UT). The second method was a size-exclusion chromatography. Here, the lyophilized samples were dissolved in the elution buffer (0.5 M Na-acetate, pH 5.8, containing 0.1% CHAPS) and analysed on a 0.5 × 20 cm column of Sephacryl S-1000 (Amersham Biosciences, Uppsala, Sweden) eluted at 0.07 mL/min. Fractions of 0.22 mL were collected, diluted in 1% bovine serum albumin in phosphate-buffered saline and assayed for HA using a sandwich type ELISA-like assay based on a biotinylated probe containing aggrecan G1 domain and link protein, as described previously [14]. For calibration of this method, samples of standard HA of 150, 500 and 1000 kDa (Hyalose, Oklahoma City, OK) were eluted and assayed like the standard curves and the proportions of LMW and HMW species calculated from the control group. The latter were determined as the transition between a very pale and thin area and a very dark area along the corticopapillary axis. The tissue was then carefully dissected on ice under a stereomicroscope. The boundary between OSOM and ISOM was identified as the transition between a very pale and thin area (OSOM, also easily distinguished from cortex) and the adjacent dark red zone corresponding to ISOM. The dissected tissue was immediately frozen and stored at −80°C for RNA and HA extraction or prepared for zymography (fresh samples) or immunohistochemistry (paraffin embedding). Plasma creatinine concentration was determined using the Jaffé method. In control groups, rats were not submitted to any surgical preparation.

Real-time reverse transcription–polymerase chain reaction

Total RNA was extracted from 30 mg of renal tissue (RNaseasy miniKit; Qiagen, Stanford, CA) and reverse transcribed using random hexamers and SuperScript II MMLV-reverse transcriptase (Invitrogen, Cergy Pontoise, France). Real-time polymerase chain reaction (PCR) reactions were performed on ABI PRISM 7000 Sequence Detection System using TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression Probes (Applied Biosystems Applera France, Courtabœuf, France) for the HAS1 and HYALs genes (HAS1 assay ID: Rn00597231_m1; HAS2 assay ID: Rn00565774_m1; HAS3 assay ID: Rn00597204_m1; HYAL1 assay ID: Rn02133715_s1; HYAL2 assay ID: Rn00597038_m1 and 18S assay ID: HS99999901_s1). Analysis of relative target gene expression was performed using the comparative Ct method [15]. Results were normalized with the data obtained from 18S rRNA internal control. The amount of target gene messenger RNA (mRNA), normalized to an endogenous control and relative to a calibrator, is given by 2ΔΔCt. The change in gene expression after IR was calculated as the ratio between the average 2ΔΔCt value corresponding to IR rats and the average 2ΔΔCt value corresponding to control rats.

Detection of hyaluronidase activity by zymography

Samples were homogenized in 0.25 M sucrose in the presence of protease inhibitors (Complete, Roche) and 50 % of proteins from each sample were applied to 10% sodium dodecyl sulphate-polyacrylamide gels containing 1.7 mg/mL rooster comb HA (Sigma, St Louis, MO). After electrophoretic run on ice, gels were rinsed with 3% Triton X-100 for 120 min at room temperature, washed and incubated with 0.1 M formate buffer (pH 3.7) containing 0.1 M NaCl for 16 h at 37°C. After washing, gels were treated with 0.1 mg/mL Pronase (Sigma) in 20 mM Tris-HCl buffer (pH 8.0) for 2 h at 39°C. To visualize HA digestion, gels were stained with 0.005% Stains-All (3,3′-dioethyl-9-methyl-4,5,4′,5′-dibenzothia-carbocyanine; Sigma) in 50% formamide/water. Gels were kept at room temperature in the dark during several days. After destaining in water, hyaluronidase activity was visualized by pink bands on gels and photographed with a digital camera on a transilluminator.

Detection of HASs and macrophages by immunohistochemistry and HA by a HA-binding protein

Immunostaining on paraffin-embedded rat kidney was performed as described previously [11]. Primary antibodies included HAS1 and HAS2 goat anti-rat polycolonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 1:40) as well as ED1 mouse anti-rat monoclonal antibodies (Serotec, Oxford, UK; 1:75). For HA detection, kidney sections were incubated for 1 h with a biotinylated HA-binding protein (HABP, Calbiochem, San Diego, CA) and the presence of HABP was revealed by the method of Tyramin-Signal Amplification (TSA; Perkin Elmer, Boston, MA). Counterstaining was performed with hemalum and Luxol fast blue.

Calculations and statistics

Results are presented as means ± SEM. The level for statistical significance was defined as P < 0.05. A non-parametric Kruskal–Wallis one-way analysis of variance on ranks was applied for multiple intergroup comparisons followed by the Dunn’s test to identify significant differences from the control group.

Results

General observations

Within 12 h of renal IR, creatininaemia increased from 31 ± 3 to 227 ± 10 μM (Figure 1, P < 0.05), signalling a severe acute renal failure. Creatininaemia was maximal at Day 2 post-IR (345 ± 49 μM, P < 0.05 versus control) and returned near baseline levels at Days 7 and 14.

Separation and detection of LMW and HMW HA polymers in OSOM and ISOM

Total HA content in OSOM (Figure 2) under control conditions averaged 28.6 ± 10.3 ng/mg of dry tissue weight. This corresponds to the sum of 21.0 ± 8.6 ng/mg for HMW HA molecules (white box in Figure 2a) and 7.6 ± 2.2 ng/mg for LMW HA molecules (black box in Figure 2a). The latter were defined as HA molecules capable of filtering through a Centrisart™ filter with a 100-kDa cut-off. Therefore, the proportion of LMW HA molecules in OSOM at the basal level is 26 versus 74% HMW HA.
In ISOM (Figure 3), the proportion of LMW HA was similar (34%) although the total HA content was >10-fold richer (370 ± 45 ng/mg, corresponding to the sum of 243 ± 28 ng/mg for HMW HA molecules [white box in Figure 3a] and 127 ± 27 ng/mg for LMW HA (black box in Figure 3a)).

After 24 h of IR injury (Figures 2a and 3a), total HA content in OSOM reached 305 ± 51 ng/mg, corresponding to 241 ± 49 ng/mg (79%) of HMW HA and 64 ± 8 ng/mg (21%) of LMW HA. Fourteen days after IR, the total HA content had increased 40-fold compared to the control condition, reaching 1200 ± 241 ng/mg. The absolute value of LMW HA was 607 ± 154 ng/mg, representing a proportion of 50% of total HA. Thus, the increase in LMW HA between Day 2 and Day 14 was proportionally larger than the increase in HMW HA.

In ISOM at 24 h after IR, the total HA content reached 774 ± 318 ng/mg and was made of 722 ± 294 ng/mg (93%) of HMW HA and 52 ± 30 ng/mg (<7%) of LMW HA. Therefore, the relative proportion of LMW HA in ISOM decreased significantly from a baseline value of 34.3 ± 4.5 to 6.7 ± 2.4% (P < 0.05) at 24 h. This inhibition of LMW HA production in ISOM was then reversed at Day 14 when LMW HA content increased to 46% of total HA, which corresponds to an absolute value of 695 ± 193 ng/mg.

To illustrate the degradation of HA occurring between Days 1 and 14, exclusion chromatography was used to assess the molecular weight (MW) profile of accumulating HA molecules more accurately (Figures 2b and c, and 3b and c). The elution pattern of extracted HA was comparable in OSOM and ISOM: the mean size of HA molecules accrued at Day 1 was very similar to a 2500-kDa reference HA, while those accumulated at Day 14 had an elution profile closer to those of 500-kDa and 150-kDa reference HA standards. The relative amounts of HA in combined fractions #16 to 21, containing most of the LMW HA molecules, were 13% at Day 1 versus 36% at Day 14 in OSOM (Figure 2b) and 15% at Day 1 versus 44% at Day 14 in ISOM (Figure 3b). These proportions are comparable to those found with the filter elution method. One limitation of the chromatography results,
however, was the fact that the absolute amounts of HA measured in this system (after integration of all fractions) were much lower than those obtained with the filter elution system. This may be due to the different types of assays, the buffers or other factors that we were not able to identify. Thus, no firm conclusion can be drawn regarding the exact MW of the HA molecules extracted from the pre- and post-ischaemic kidney. Globally, however, the similar patterns of HMW and LMW HA detection based on blinded measurements using two very different methods confirm that there is a delayed process of HA fragmentation in the post-ischaemic kidney, both in OSOM and ISOM.

Expression of HASs and HYALs in control and post-ischaemic OSOM

Figure 4 illustrates the temporal evolution of gene expression of HAS1, HAS2, HYAL1 and HYAL2, as well as zymographic hyaluronidase activity, in OSOM. HAS3 mRNA was not detected. At baseline, HAS1 and HAS2 were both expressed at very low levels in OSOM (Figure 4A). This is consistent with the observation that this zone is almost free of HA. After IR, HAS1 expression increased very rapidly within 12 h, reaching levels 50-fold higher than baseline. This peak was largely attenuated 12 h later. Thereafter, HAS1 mRNA levels remained elevated and relatively stable until the end of the observation period (14 days post-IR). On the other hand, HAS2 expression showed a slowly increasing trend that became statistically significant at Day 2. Apart from Day 1, the relative levels of expression of HAS1 and HAS2 were similar throughout the following 2 weeks. Regarding HYALs (Figure 4B), the expression of HYAL1 and HYAL2 decreased significantly at 12 and 24 h post-IR before returning to baseline values between Days 2 and 7. At all time periods before and after ischaemia, the absolute amounts of HYAL2 mRNA surpassed those of HYAL1. To confirm that the transient decrease in HYAL1 and HYAL2 mRNAs had functional consequences, hyaluronidase activity was measured in renal tissue samples using zymography (Figure 4C). Under control conditions, an enzymatic complex formed by double bands ~74 kDa was detected. Between 12 and 48 h post-IR, however, hyaluronidase activity was localized in a single band and globally reduced. The double band re-appeared 7 and 14 days after IR. In summary, these unexpected biphasic changes in HASs and HYALs in post-IR OSOM are consistent with the swift generation of HMW HA (high HAS1 and low HYALs) followed by a continuous production of LMW species (high HAS2 and high HYALs).

Localization of HASs and HA in control and post-ischaemic OSOM

In control rats (Figure 5a and b), staining for HAS1 and HAS2 was faint. HAS2 could be detected at the basal pole of some tubular cells or in scattered interstitial cells; small amounts of HA were also detected in these areas (Figure 5c). One day after IR (Figure 5d and e), a significant increase in HAS1 and a lesser one for HAS2, as well as deposition of HA (Figure 5f), were observed in the same areas. Seven (data not shown) and 14 days post-IR (Figure 5g–h and j–k), the tubular staining of HAS1 appeared less pronounced, whereas that of HAS2 markedly increased in remodelling areas, where HA also concentrated (Figure 5i and l). Throughout this time course, the overlay between HAS2 and HA expression was obvious. A few interstitial cells surrounded by a coat of HA could be clearly identified (e.g. in Figure 5f and l); the same pattern was sometimes observed for HAS2 (e.g. in Figure 5h).
Expression of HASs, HYALs and HA in control and post-ischaemic ISOM

These observations, illustrated in Figure 6 (mRNA levels and zymography) and Figure 7 (immunohistochemistry), should be put in perspective with a much higher baseline amount of HA in ISOM (Figure 7c) than in OSOM (Figure 5c). In ISOM, the baseline levels of HAS1 mRNA and immunostaining were low (Figures 6A and 7a), whereas HAS2 mRNA and protein were more readily detected (Figures 6A and 7b). One day post-IR, the patterns of HAS2 and HA distribution (Figure 7d–f) were not very different from baseline while HAS1 staining appeared stronger. At the mRNA level, however, there was a rapid and intense increase in HAS1 expression measured at 12 h, exactly as in OSOM but even more short-lived since HAS1 mRNA had returned to baseline levels by Day 2 in ISOM (Figure 6a). The increase in HAS2 mRNA followed the same pattern in both zones until Day 7 but during the second week, HAS2 mRNA levels continued to rise in ISOM. Histological examination at Day 14 (Figure 7g–l) showed a severely distorted tissue with regenerating and necrotic tubules, heterogeneous HA deposition (Figure 7i and l) and markedly increased HAS2 immunostaining (Figure 7h and k). Again, the pattern of HAS2 expression mirrored that of HA (e.g. compare Figure 7h and i). The same early decreases in the mRNA levels of HYAL1 and HYAL2 and in their zymographic activity were observed in ISOM (Figure 6B and C) as compared to OSOM (Figure 4B and C). The strikingly parallel down-regulation of HYAL1 and HYAL2 persisted throughout Day 2 in ISOM. The mRNA amounts of these proteins recovered their pre-IR values at Days 7 and 14.

Localization and quantification of macrophages in control and post-ischaemic kidney

ED-1 antibody was used to identify and quantify monocytes/macrophages (hereby collectively called macrophages). Figure 8 illustrates the time course of
Macrophage infiltration in the interstitium of OSOM and ISOM. Macrophages appeared at Day 1; their number peaked at Day 7 and they remained abundant at Day 14 (Figure 8A). Overall, they were slightly more abundant in OSOM than ISOM. Thus, the number of macrophages in each part of the outer medulla did not parallel the amounts of either total HA or LMW HA. The same conclusion can be drawn from comparing the pattern of macrophage infiltration (Figure 8B), which was patchy in both OSOM and ISOM, and the deposition of HA, which was much more localized in OSOM (see Figure 5) than ISOM (see Figure 7) throughout the time course of our observations. There was no strict temporal or local correlation between HA deposition and macrophage infiltration.

Fig. 5. Immunolocalization of HAS1 and HAS2 and distribution of HA labelling in OSOM on consecutive serial sections of control rats (a–c) and at Day 1 (d–f) and Day 14 (g–l) post-IR. HAS1 and HAS2 immunoreactivity was low in control kidney (a and b) and was mainly detected on interstitial cells (arrowheads) and on the basal pole of tubules (arrow), in correlation with HA labelling (c). At Day 1 post-IR, HAS1 staining (d), localized around necrotic tubules (NT), was more pronounced than that of HAS2 (e) and was again closely associated with HA labelling (f). At Day 14, HAS1 immunoreactivity appeared weaker (g and j), while HAS2 staining was highly increased (h and k), in association with basement tubular membranes (arrows) and in the interstitium (arrowheads) of remodelling areas. HA accumulation (i and l) was closely associated with HAS2 staining. PT, proximal tubule. Magnifications: a, b, c, g, h and l: ×260; d, e and f: ×290 and j, k and l: ×120.
Discussion

In the current study, we demonstrate for the first time the time course of HA accumulation and degradation in the post-ischaemic kidney. There is a fast but transient increase in HMW HA deposition followed by a delayed accumulation of lower size HA. This phenomenon can be explained by remarkably coordinated up- and down-regulation of HA synthases and hyaluronidases.

The HA synthetic machinery was in fact activated in two steps: first HAS1 alone and then a combination of HAS1 and HAS2. To our knowledge, such a phenomenon was never reported in vivo. Göransson et al. [8] could not detect HAS1 mRNA in pre- or post-ischaemic rat kidneys but they did not explore HAS expression earlier than 72 h after IR. Wu et al. [9] described comparable overexpression of all three HASs 1–9 days after a bilateral IR injury in C57BL/6 mice while the mRNA was extracted from the whole kidney. Induction of various HASs has been found in many other types of tissue injury [2], from skin stripping [14] to ischaemic stroke in man [13]. Of note, an early activation of HAS1 was also observed in rat cardiomyocytes 24 h after abdominal aorta ligation [16] while in vitro, HAS1 gene transcription can be induced selectively and rapidly, e.g., by tumour growth factor-β in synoviocytes [17]. It is possible that isolated HAS1 induction, especially if coupled with a repression of hyaluronidases, results in a particular arrangement of HA molecules in the matrix with effects on resident cells or infiltrating leucocytes. At this stage, HAS1 may be tentatively considered as an ‘early induction gene’ in renal IR and possibly other types of ischaemia and its selective inhibition could be tested.

We have also described the intrarenal distribution of HAS1 and HAS2 immunoreactivity in the outer medulla. At baseline and during IR, HASs were localized at the
basal side of the tubules and in some interstitial cells. Their expression coincided nicely with HA, confirming that they are the main drivers of HA accumulation. At Day 14, while tubular lesions were not completely healed, there was a marked overlay between HAS2 expression and HA deposits. HAS2 may be involved in the maintenance of inflammatory pockets in the post-ischaemic kidney as well as in other tissues [2].

The first wave of HA deposition was clearly of HMW. At 24 h post-IR, the proportion of LMW HA was at its lowest (21% in ISOM and 7% in OSOM) and the mean size of the HA molecules detected in the chromatographic method approximated 2500 kDa, although we cannot exclude the presence of higher or much lower MW species. Accretion of HMW HA may create anti-inflammatory conditions [18], stimulate regeneration [19] or

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**Fig. 7.** Immunolocalization of HAS1 and HAS2 and distribution of HA labelling in ISOM on consecutive serial sections of control rats (a–c) and at Day 1 (d–f) and Day 14 (g–l) post-IR. Under control conditions, HAS2 immunoreactivity (b) and HA labelling (c) were more detectable than in OSOM. At Day 1 post-IR, HAS1 staining appeared more pronounced and was associated with necrotic tubules (NT) (d). At Day 14, the tissue was severely distorted with the presence of both regenerating (RT) and necrotic tubules (NT), heterogeneous HA deposition (i and l) and markedly increased HAS2 immunostaining (h and k). The pattern of HAS2 expression was quite similar to that of HA. At that time, HAS1 immunoreactivity appeared less pronounced (g and j). Magnifications: a, b, c, d, e, f, g, h and i: ×260 and j, k and l: ×100.
affect trafficking leucocytes, e.g. by preventing them from attaching to cable-like HA structures around the cells [20]. Similarly, intravenous administration of HMW HA to mice has been shown to prevent, e.g. lipopolysaccharide-induced pulmonary vascular leakiness [6] or diabetic kidney inflammation [21].

Fragments of HA started to appear later in OSOM and ISOM. At Day 14, LMW HA had increased 80-fold and 6-fold versus baseline in OSOM and ISOM, respectively, and it made up half of all HA molecules. Although HA fragments are often invoked as a major pro-inflammatory factor [2, 3], there are only a few examples of successful isolation of LMW HA species in inflammatory tissues in vivo, e.g. in the brain [13], aortic lesions or plaques [22, 23], cervix [24], prostate cancer [25] and lung [26–29]. In the post-IR kidney, we show that the creation of HA fragments results from a remarkably coordinated up-regulation of HAS1-2 and temporary down-regulation of HYAL1-2. Similar events were not detected previously in other tissues. In lung IR, for instance, LMW HA fragments accumulate already during the first day after injury when HAS1 and HAS2 are simultaneously induced [29]. Post-ischaemic events likely imply different mechanisms in different tissues. Strikingly, Li et al. [30], using a transgenic mouse approach, have recently demonstrated the critical role of HAS2 in myoﬁbroblasts during the development of bleomycin-induced lung ﬁbrosis. Mesenchymal HAS2 overexpression could be similarly involved in post-ischaemic or post-transplant renal ﬁbrosis.

Our histochemical results suggest that renal HASs are stromal enzymes which may respond to various inflammatory factors. On the other hand, HYALs may well reside in tubular cells so their biphasic changes would reﬂect successive tubular necrosis and regeneration in the outer medulla (this issue is under further examination). The rise in tissue HA fragments may also be explained by the presence of reactive oxygen species or peroxynitrite in addition to hyaluronidases or by a lack of efﬁcient clearance mechanisms for these fragments.

At the time of the increase in biochemically deﬁned HA fragmentation (Days 7 and 14), the outer medulla displayed active remodelling including HA deposits. Those

Fig. 8. Macrophage infiltration. (A) Quantitative analysis of ED1-positive staining (representing macrophages) in OSOM and ISOM. Values are means ± SEM; n = 6 in each group. Statistical analysis: Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn’s test; *P < 0.05. (B) Representative photomicrographs of ED1-positive cells (dark brown staining) in kidney sections of control rat (a, OSOM) as well as at Day 7 (b, OSOM and c, ISOM) and Day 14 (d, OSOM and e, ISOM). Magniﬁcations: ×250.
were patchy in OSOM and more extensive in ISOM. Since the methods used to measure LMW HA were based on the same probe (HABP) as the histochemical method, one may surmise that HA fragments are mostly localized to those remodelling areas and thus may play an active role in the maintenance of inflammation or the development of fibrosis. This is compatible with current thinking [2, 3]. However, we could not demonstrate any temporal or local correlation between HA deposition and macrophage infiltration (the latter peaked at Day 7). The situation is undoubtedly complex. To define the exact role of HA fragments in the midst of large inflammatory, HA accumulation, which includes HMW HA, will require specific measurements or preventive measures. It will be crucial to explore, e.g. the role of endogenous LMW HA in the presence of different levels of HMW HA for the activation of TLR2 and TLR4, both of which seem clearly involved in the pathogenesis of renal IR injury while they cannot be stimulated by HMW HA [9, 31, 32].

In summary, we have used a rat model of severe renal IR injury exhibiting large HA deposits to demonstrate the sequential activation of two different forms of HASs, HAS1 (which may be considered as an early induction gene) and HAS2. In parallel, there was a transient down-regulation of the main hyaluronidases, HYAL1 and HYAL2. This resulted in two successive HA environments: the first one, lasting 24–48 h, was rich in HMW molecules (at least 2500 kDa) and the second one was enriched in lower size HA species (up to 500 kDa). During the second phase, HA accumulated within residual inflammatory pockets but did not correlate with macrophage infiltration. In fact, the consequence of these varying HA climates on the fate of renal tissue, especially in the development of long-term fibrosis, needs further exploration. The rat model of severe renal ischaemia to a single kidney that was used in this study mimics the situation of most renal allografts and the prolonged renal hypoperfusion observed in shock. Whether delayed HA fragmentation occurs in other post-ischaemic or inflammatory situations should be explored as well.

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Conflict of interest statement. None declared.

References
Methods.

Brain tissue.

Promote endothelial repair in ischemic limb, heart and tubular cell proliferation. Administration of CD133+ cells to vehicle- or CD133- derived CD133+ cells unexpectedly exacerbate ischemic

Conclusions.

To FVB/N mice post-I/R also augmented kidney injury.

Plasma tumor necrosis factor-\(\alpha\) exacerbates tubular necrosis and apoptosis, increased

Results.

Biochemistry and kidney histology.

Blood were injected intravenously at the time of reperfusion of progenitor cells facilitates recovery from AKI. Human umbilical cord-derived CD133+ progenitor cells promote endothelial repair in ischemic limb, heart and brain tissue.

Methods. We examined the effects of human CD133+ progenitor cells in bilateral ischemia–reperfusion (I/R) kidney injury in non-obese diabetic severe combined immunodeficient mice. CD133+ cells from human cord blood were injected intravenously at the time of reperfusion and the extent of injury was determined by plasma biochemistry and kidney histology.

Results. In mice with I/R, fluorescently labeled CD133+ cells were detected in blood 2 min after injection but decreased rapidly thereafter with no evidence of homing to the kidneys. In mice subjected to I/R, CD133+ cells significantly increased plasma urea and Cr at 24 h compared to vehicle- or CD133− cell-treated mice. CD133+ cells exacerbated tubular necrosis and apoptosis, increased plasma tumor necrosis factor-\(\alpha\) and increased kidney neutrophil infiltration. In contrast, CD133− cells did not affect tubular cell proliferation. Administration of CD133− cells to FVB/N mice post-I/R also augmented kidney injury.

Conclusions. These data indicate that human cord blood-derived CD133+ cells unexpectedly exacerbate ischemic

AKI in mice, possibly through soluble factors. Our study highlights the importance of caution in cell-based therapies for human AKI.

Keywords: acute kidney injury; apoptosis; inflammation; ischemia–reperfusion; stem cells

Introduction

Acute kidney injury (AKI) affects 7–18% of all hospitalized patients and >60% of patients in the critical care setting [1, 2]. Despite advances in our understanding of AKI, mortality still approaches 50% and therapeutic options remain supportive [2]. The major cause of human AKI is ischemic injury, typically resulting from shock/hypovolemia [3]. While the regeneration of tubular epithelium is fundamental to post-AKI recuperation, restoration of endothelial function is a major determinant of recovery. Animal models of ischemic AKI are associated with a 30–50% reduction in vascular density in the kidney, which can persist and impart a risk of future chronic kidney disease [4]. Endothelial permeability in the peritubular capillaries is increased along with the expression of cellular adhesion molecules, resulting in increased leukocyte–endothelial interactions, tissue infiltration and inflammation [5]. Vascular rarefaction and inflammation ultimately prolong hypoperfusion and

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Human cord blood CD133+ cells exacerbate ischemic acute kidney injury in mice

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Abstract

Background. Acute kidney injury (AKI) in humans has few therapeutic options. In experimental models, administration of progenitor cells facilitates recovery from AKI. Human umbilical cord-derived CD133+ progenitor cells promote endothelial repair in ischemic limb, heart and brain tissue.

Methods. We examined the effects of human CD133+ progenitor cells in bilateral ischemia–reperfusion (I/R) kidney injury in non-obese diabetic severe combined immunodeficient mice. CD133+ cells from human cord blood were injected intravenously at the time of reperfusion and the extent of injury was determined by plasma biochemistry and kidney histology.

Results. In mice with I/R, fluorescently labeled CD133+ cells were detected in blood 2 min after injection but decreased rapidly thereafter with no evidence of homing to the kidneys. In mice subjected to I/R, CD133+ cells significantly increased plasma urea and Cr at 24 h compared to vehicle- or CD133− cell-treated mice. CD133+ cells exacerbated tubular necrosis and apoptosis, increased plasma tumor necrosis factor-\(\alpha\) and increased kidney neutrophil infiltration. In contrast, CD133− cells did not affect tubular cell proliferation. Administration of CD133− cells to FVB/N mice post-I/R also augmented kidney injury.

Conclusions. These data indicate that human cord blood-derived CD133+ cells unexpectedly exacerbate ischemic

AKI in mice, possibly through soluble factors. Our study highlights the importance of caution in cell-based therapies for human AKI.

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Acute kidney injury (AKI) affects 7–18% of all hospitalized patients and >60% of patients in the critical care setting [1, 2]. Despite advances in our understanding of AKI, mortality still approaches 50% and therapeutic options remain supportive [2]. The major cause of human AKI is ischemic injury, typically resulting from shock/hypovolemia [3]. While the regeneration of tubular epithelium is fundamental to post-AKI recuperation, restoration of endothelial function is a major determinant of recovery. Animal models of ischemic AKI are associated with a 30–50% reduction in vascular density in the kidney, which can persist and impart a risk of future chronic kidney disease [4]. Endothelial permeability in the peritubular capillaries is increased along with the expression of cellular adhesion molecules, resulting in increased leukocyte–endothelial interactions, tissue infiltration and inflammation [5]. Vascular rarefaction and inflammation ultimately prolong hypoperfusion and