Deficiency in mouse hyaluronidase 2: a new mechanism of chronic thrombotic microangiopathy

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ABSTRACT

Hyaluronan is a major component of the extracellular matrix and glyocalyx. Its main somatic degrading enzymes are hyaluronidases 1 and 2, neither of which is active in the bloodstream. We generated hyaluronidase 2-deficient mice. These animals suffer from chronic, mild anemia and thrombocytopenia, in parallel with a 10-fold increase in plasma hyaluronan concentration. In this study we explored the mechanism of these hematologic anomalies. The decreased erythrocyte and platelet counts were attributed to peripheral consumption. The erythrocyte half-life was reduced from 25 to 8 days without signs of premature aging. Hyaluronidase 2-deficient platelets were functional. Major intrinsic defects in erythrocyte membrane or stability, as well as detrimental effects of high hyaluronan levels on erythrocytes, were ruled out in vitro. Normal erythrocytes transfused into hyaluronidase 2-deficient mice were quickly destroyed but neither splenectomy nor anti-C5 administration prevented chronic hemolysis. Schistocytes were present in blood smears from hyaluronidase 2-deficient mice at a level of 1% to 6%, while virtually absent in control mice. Hyaluronidase 2-deficient mice had increased markers of endothelial damage and microvascular fibrin deposition, without renal failure, accumulation of ultra-large multimers of von Willebrand factor, deficiency of ADAMTS13, or hypertension. There was no sign of structural damage in hepatic or splenic sinusoids, or in any other microvessels. We conclude that hyaluronidase 2 deficiency induces chronic thrombotic microangiopathy with hemolytic anemia in mice. The link between this uncommon condition and hyaluronidase 2 remains to be explored in humans.

Introduction

Hyaluronan, a major constituent of the extracellular matrix of vertebrates, belongs to the glycosaminoglycan family. Depending on its molecular size, it acts as a scaffold and a signaling molecule between the matrix and the cells.1 It can also be found in the plasma at concentrations of about 10-100 ng/mL in humans2 and 400-600 ng/mL in C57Bl/6 mice.3 Hyaluronan is catabolized by somatic hyaluronidases, principally hyaluronidase 1 (HYAL1) and hyaluronidase 2 (HYAL2), neither of which is active in the bloodstream at physiological pH. The role of each hyaluronidase in hyaluronan turnover is poorly understood. HYAL2 is known to be a ubiquitous glycosylphosphatidylinositol-anchored membrane protein.4 HYAL2-deficient (Hyal2−/−) mice were generated in our laboratory through a conditional Cre-lox system.5 These Hyal2−/− mice display mild skeletal and hematologic abnormalities, which include thrombocytopenia and chronic compensated hemolytic anemia with markedly elevated plasma hyaluronan levels.

The main purpose of the current study was to characterize the cause of this chronic anemia accompanied by thrombocytopenia and determine how it relates to HYAL2 deficiency. In particular, we investigated whether the cause of hemolysis was intra- or extra-corpuscular by performing a complete hematologic analysis and measuring the intrinsic properties of red blood cells (RBC) (membrane deformability, signs of senescence) as well as survival in Hyal2−/− and control mice.

Methods

Animals

Controls (Hyal2+/+ or Hyal2−/− mice; animals with these genotypes had indistinguishable phenotypes) and knockout (Hyal2−/−) outbred 129P1.CD1 Hyal2−/− mice were used. Males from the same litters were selected except where otherwise noted. For bone marrow and transfusion experiments, donor and recipient inbred mice, backcrossed on a C57Bl/6 background, were used. Blood was collected via retro-orbital, tail vein, or cardiac puncture. All experiments were approved by the local animal ethics committee at the University of Namur, Belgium.

Hematologic analyses

The hematologic analyses were run on an ADVIA 120 (Siemens). Eosin-5′-maleimide was assayed using a BDIS FACS Canto II® cytometer (BD Biosciences) and immature reticulocyte and platelet fractions were assessed using an XE-2100 instrument (Sysmex).

Red blood cell survival, senescence and deformability

In order to measure RBC survival, mice were given an intravenous injection of 3 mg sulfo-NHS-LC-biotin (Pierce) and RBC collected at various time points were incubated with Alexa 488-streptavidin (Life


Results

Hyal2<sup>-</sup> mice display chronic macrocytic anemia and thrombocytopenia

Congenital HYAL2 deficiency in mice is accompanied by thrombocytopenia and chronic, mild anemia with high reticulocyte counts and elevated plasma lactate dehydrogenase levels, suggesting chronic hemolytic anemia.<sup>4</sup> Erythrocytic and thrombocytic indices were analyzed in Hyal2<sup>-/</sup>- and control (Hyal2<sup>+/</sup> and Hyal2<sup>-/-</sup>) outbred young adult mice (8 weeks old) (Table 1). Whereas the main parameters of Hyal2<sup>+/</sup>- and Hyal2<sup>-/-</sup> mice did not differ significantly, Hyal2<sup>-/-</sup> mice had a mild (10%) but significant decrease in both circulating hemoglobin levels and hematocrit, as well as a 5-fold increase in reticulocyte count, suggestive of chronic regenerative anemia. In addition, both reticulocyte and immature platelet fractions were elevated in Hyal2<sup>-/-</sup> mice. This indicates a strong stimulation of erythropoiesis and thrombopoiesis, probably due to peripheral consumption. Similar hematologic abnormalities were found when comparing control C57Bl/6 inbred versus Hyal2<sup>-/-</sup> mice (Online Supplementary Table S1). These observations were thus independent of the mouse’s genetic background.

Hyal2<sup>-/-</sup>-deficient mice also displayed macrocytosis (Table 1). The mean corpuscular volume and mean corpuscular hemoglobin content of RBC were significantly higher in Hyal2<sup>-/-</sup> mice than in control animals, even after in vitro maturation of reticulocytes into RBC, suggesting macrocytosis was not due to the high proportion of large reticulocytes. Nor was it due to vitamin B12 deficiency or folic acid deficiency (Table 1). In addition, histograms of RBC volume and hemoglobin concentration revealed anisocytosis. Both macrocytosis and anisocytosis may result from enhanced erythropoiesis.

The absence of a significant difference in mean corpuscular hemoglobin concentration and the results of eosin-5'-maleimide binding tests (Table 1) ruled out hereditary spherocytosis.

The half-life of Hyal2<sup>-/-</sup> red blood cells is dramatically reduced without signs of intrinsic membrane anomalies, premature aging, or high C3 deposition

To confirm peripheral destruction, the lifespan of control and Hyal2<sup>-/-</sup> RBC was measured following sulfo-NHS-LC-biotin injections. This system labels RBC proteins simply and efficiently without altering their biological activity.<sup>4</sup> The half-life of biotinylated RBC was only 8 days for Hyal2<sup>-/-</sup> RBC (Figure 1A) compared with 25 days for control RBC (P<0.001).

The accelerated turnover of Hyal2<sup>-/-</sup> RBC suggests premature aging. We, therefore, measured the main markers of RBC aging, i.e. phosphatidylserine exposure and auto-IgG binding. Phosphatidylserine externalization did not differ significantly between Hyal2<sup>-/-</sup> and control RBC (1.1±0.1% versus 0.9±0.1%, respectively; n=8, P=NS). The amount of IgG bound to RBC was significantly lower in Hyal2<sup>-/-</sup> RBC than in normal RBC (1.5±0.2% versus 3.6±0.4%, respectively; n=6, P<0.001).

Besides IgG, C3 deposition on the RBC surface may also trigger auto-immune hemolytic anemia. However, C3 exposure on RBC was measured using cytometry and found to be too low (<1% cells were positive for C3) to cause significant hemolysis in either HYAL2-deficient or
control RBC, even though the proportion of C3-positive cells was higher among Hyal2-/- cells than control cells (0.81±0.15% versus 0.03±0.01%, respectively; n=14; P<0.0001).

To further test for intrinsic membrane defects, RBC deformability was measured using ectactometry (Figure 1B). There was no significant difference between the curves of elongation index measured over a range of shear stresses in control and Hyal2-/- mice. In particular, the elongation index at 7.8 Pa (the murine mean physiological shear stress)^10 was identical in both genotypes (0.41±0.01 versus 0.41±0.01 in control and Hyal2-/- mice, respectively; n=10; P=NS), indicating no difference in RBC surface area under physiological conditions.

With regards to platelets, electron microscopy revealed that Hyal2-/- platelets did not differ morphologically either before or after ADP- or thrombin-induced aggregation (Online Supplementary Figure S1A-F). Furthermore, the amount of platelet microparticles did not differ between genotypes, ruling out premature aging of Hyal2-/- platelets (Online Supplementary Figure S1G).

Taken together, these data point to an accelerated clearance of platelets and RBC without any sign of premature aging or eryptosis.

Hyal2-/- red blood cells include acanthocytes and schistocytes

Close observations of blood smears (Figure 1C-F) revealed abnormalities in Hyal2-/- RBC, including global poikilocytosis and shape irregularities (acanthocytosis). Moreover, various amounts of fragmented cells (schistocytes) were visible. These schistocytes were quantified precisely on blood smears. As shown in Figure 1G, schistocytes were significantly (P<0.001) more frequent in Hyal2-/- than in control blood smears. Figure 1H shows a strong correlation (Pearson r = 0.862, P<0.001) between the proportions of schistocytes (0% to 6%) and reticulocytes (1% to 10%), pointing to a common process underlying both abnormalities.

The presence of schistocytes indicates mechanical destruction of Hyal2-/- RBC. Bone marrow histology did not reveal any abnormality and hemoglobin electrophoresis excluded a hemoglobinopathy (data not shown).

To clarify the relative contribution of intrinsic defects versus peripheral consumption of RBC, a more radical experiment, i.e. bone marrow transplantation, was performed.

**Bone marrow transplantation does not reproduce all Hyal2-/- blood abnormalities**

Fourteen C57Bl/6 normal recipient mice were irradiated. Seven received bone marrow cells from a Hyal2-/- inbred donor mouse while the others received bone marrow cells from a control inbred mouse. Twelve weeks later (i.e. the time necessary for bone marrow reconstitution), the engraftment of the donor cells was verified by genotyping circulating leukocytes: those of all recipients of Hyal2-/- cells were indeed homozygous for the null allele (data not shown).

Concurrent blood counts (Table 2) showed significant but small (7 to 8%) differences in hemoglobin concentration between recipients of control and Hyal2-/- bone marrow without significant difference in reticulocyte counts. Platelet levels remained 35% lower in recipients of Hyal2-/- bone marrow. In summary, these results cannot exclude a minor intrinsic abnormality in Hyal2-/- RBC and platelets but they are insufficient to explain the chronic hemolytic anemia in Hyal2-/- mice. The role of extrinsic factors was, therefore, further explored using RBC transfusions.

The Hyal2-/- environment is responsible for a shortened red blood cell life span

In vivo labeled RBC from one control mouse and one Hyal2-/- inbred donor mouse were infused into recipient mice of both genotypes and the percentage of labeled RBC was monitored over a 1-month period (Figure 2). In control recipient mice, the survival of Hyal2-/- RBC was completely normal whereas in Hyal2-/- recipient mice the half-life of both normal and Hyal2-/- RBC was dramatically reduced to the level of that of endogenous outbred Hyal2-/- RBC. These observations strongly suggest that the origin of hemolysis in Hyal2-/- mice is almost exclusively extracorporeal and could be explained by mechanical destruction, a finding in agreement with the presence of schistocytes in Hyal2-/- blood smears.

Hyal2-/- mice displayed marked splenomegaly (Online Supplementary Table S2). It was, therefore, important to exclude splenomegaly as a cause of RBC and platelet destruction before analyzing various causes of mechanical stress. RBC survival was measured immediately before and 1 month after splenectomy in Hyal2-/- mice. This

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**Table 1. Corpuscular indices in outbred mice.**

<table>
<thead>
<tr>
<th></th>
<th>Hyal2+/+ (n=15)</th>
<th>Hyal2+/+ (n=24)</th>
<th>P</th>
<th>Hyal2-/- (n=49)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6 cells/L)</td>
<td>9.79 ± 0.20</td>
<td>9.66 ± 0.10</td>
<td>NS</td>
<td>7.75 ± 0.13</td>
<td>***</td>
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<tr>
<td>Hb (g/dL)</td>
<td>14.14 ± 0.59</td>
<td>13.67 ± 0.16</td>
<td>NS</td>
<td>12.51 ± 0.23</td>
<td>**</td>
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<tr>
<td>HCT (%)</td>
<td>46.03 ± 1.30</td>
<td>45.17 ± 0.47</td>
<td>NS</td>
<td>39.47 ± 0.68</td>
<td>**</td>
</tr>
<tr>
<td>Ret (10^12 cells/L)</td>
<td>327 ± 24</td>
<td>240 ± 14</td>
<td>NS</td>
<td>922 ± 46</td>
<td>***</td>
</tr>
<tr>
<td>Ret (%)</td>
<td>3.60 ± 0.24</td>
<td>2.39 ± 0.11</td>
<td>NS</td>
<td>12.12 ± 0.67</td>
<td>***</td>
</tr>
<tr>
<td>IRF (%) (n=9)</td>
<td>44.97 ± 1.60</td>
<td></td>
<td></td>
<td>56.86 ± 2.08</td>
<td>***</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>45.87 ± 0.56</td>
<td>44.30 ± 0.34</td>
<td>NS</td>
<td>51.16 ± 0.43</td>
<td>**</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.42 ± 0.19</td>
<td>14.14 ± 0.15</td>
<td>**</td>
<td>16.17 ± 0.12</td>
<td>***</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.69 ± 0.44</td>
<td>31.71 ± 0.20</td>
<td>NS</td>
<td>31.66 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>CH (pg)</td>
<td>14.94 ± 0.25</td>
<td>13.77 ± 0.11</td>
<td>*</td>
<td>17.58 ± 0.12</td>
<td>**</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>13.61 ± 0.31</td>
<td>12.65 ± 0.27</td>
<td>NS</td>
<td>14.72 ± 0.32</td>
<td>***</td>
</tr>
<tr>
<td>HDW (g/dL)</td>
<td>2.25 ± 0.07</td>
<td>1.95 ± 0.03</td>
<td>NS</td>
<td>2.73 ± 0.05</td>
<td>***</td>
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<tr>
<td>PLT (10^6 cells/L)</td>
<td>1140 ± 79</td>
<td>1252 ± 45</td>
<td>NS</td>
<td>402 ± 26</td>
<td>***</td>
</tr>
<tr>
<td>IPF (%) (n=9)</td>
<td>0.58 ± 0.04</td>
<td>0.55 ± 0.04</td>
<td>NS</td>
<td>2.33 ± 0.61</td>
<td>***</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>7.06 ± 0.13</td>
<td>6.06 ± 0.18</td>
<td>*</td>
<td>7.71 ± 0.15</td>
<td>***</td>
</tr>
<tr>
<td>Vitamin 12 (nmol/L)</td>
<td>23.0 ± 3.7</td>
<td>33.1 ± 2.3</td>
<td>*</td>
<td>100.0 ± 11.9</td>
<td>***</td>
</tr>
<tr>
<td>Serum folates (nmol/L)</td>
<td>52.7 ± 5.5</td>
<td>98.0 ± 11.9</td>
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</table>

_RBC: red blood cell count; Hb: hemoglobin; HCT: hematocrit; Ret, reticulocytes; IRF: immature reticulocyte fraction; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration (calculated); CHCM: corpuscular hemoglobin concentration mean; CH: corpuscular hemoglobin content; RDW: red cell volume distribution width; HDW: hemoglobin concentration distribution width; PLT platelet count; IPF: immature platelet fraction; MPV: mean platelet volume; P for ANOVA 1 (using the Kruskal-Wallis test) vs <0.001 for all parameters except for MCHC (P=NS) and CHCM (P=NS) values of P for Hyal2-/- vs Hyal2+/+ and P for Hyal2-/- vs Hyal2+/+-calculated based on Dunn’s multiple comparison test, are indicated. The Mann Whitney test was used for IRF and MPV comparisons as well as for MCV and RDW after reticulocyte maturation: NS: non significant; *P<0.05; **P<0.01; ***P<0.001._
removal of the spleen had no influence on RBC half-life (Online Supplementary Figure S2).

In addition, anti-C5 antibodies were administered for 3 weeks in order to rule out complement-dependent hemolysis. Anti-C5 treatment has been shown to suppress plasma hemolytic activity by at least 60% in C57Bl/6 mice. In our hands, plasma hemolytic activity was reduced to 18.5±3.7% of baseline after 3 weeks of treatment in our outbred mice (n=9; P<0.0001). However, after a total of six biweekly injections of anti-C5 antibodies, the hematologic differences between control and Hyal2−/− mice persisted (Online Supplementary Table S3). We conclude that neither hemolysis nor thrombocytopenia of Hyal2−/− mice is C5-dependent.

**High hyaluronan concentrations do not increase phosphatidylserine exposure in vitro or blood viscosity in vivo**

We hypothesized that the elevated plasma hyaluronan levels in Hyal2−/− mice could damage RBC either through direct toxicity or through increased blood viscosity and mechanical stress. To test this hypothesis, RBC were first incubated with hyaluronan-rich plasma (either Hyal2−/− plasma or normal plasma supplemented with exogenous high molecular mass hyaluronan). These incubations did not induce signs of premature aging, such as increased phosphatidylserine exposure, in any type of RBC (Figure 3A). However, Hyal2−/− RBC were more sensitive than normal RBC to plasma incubation in general.
Second, whole blood viscosity was measured in blood samples from control and \textit{Hyal2}\textsuperscript{−/−} mice at different physiological shear rates ranging from 11 to 450 s\textsuperscript{−1} (Figure 3B). No significant difference was detected between genotypes. Similarly, plasma viscosity did not differ (1.02±0.03 versus 1.03±0.01 Pa.s in control and \textit{Hyal2}\textsuperscript{−/−} mice, respectively; n=3, P=NS). These results rule out a toxic effect of hyaluronan on RBC, either directly or through blood viscosity.

\textbf{Hyal2}\textsuperscript{−/−} mice are normotensive

Malignant hypertension is another possible cause of mechanical RBC fragmentation.\textsuperscript{21} Arterial blood pressure was thus measured using a validated noninvasive method in trained mice. There was no difference in mean blood pressure between genotypes (76.5±2.0 mmHg versus 72.0±1.5 mmHg in control and \textit{Hyal2}\textsuperscript{−/−} mice, respectively; n=7, P=NS).

\textbf{Hyal2}\textsuperscript{−/−} mice have significant endothelial cell injuries with signs of thrombosis but normal renal function and no deficiency in ADAMTS13

Hemolytic anemia of extrinsic origin can also be a sign of microangiopathy. We, therefore, measured markers of endothelial injury, i.e. vascular adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin. As shown in Figure 4A-C, all three markers were significantly higher in \textit{Hyal2}\textsuperscript{−/−} mice than in control mice. This endothelial impairment was not accompanied by changes in serum creatinine or urine albumin/creatinine ratio at 6 and 11 months of age (Online Supplementary Table S4). In other words \textit{Hyal2}\textsuperscript{−/−} mice showed no sign of renal dysfunction, even at an advanced age. Moreover, careful screening of microvesSEL histology in various tissues, particularly in liver and spleen, revealed no obvious signs of endothelial damage (data not shown) or glycoscalyx scarcity (Online Supplementary Figure S3). In contrast, the endothelial glycoscalyx tended to be thicker in \textit{Hyal2}\textsuperscript{+/-} than in control mice, while vascular fibrin deposition, a sign of thrombotic events, was found in numerous microvessels of \textit{Hyal2}\textsuperscript{−/−} mice but not in control mice (Figure 4D-G).

In humans, thrombotic microangiopathy is often associated with a deficiency in the vWF-cleaving protease, ADAMTS13. However, the activity of ADAMTS13 in \textit{Hyal2}\textsuperscript{−/−} mice was only minimally affected and there was no sign of hemolysis. Similarly, and perhaps even more convincingly, no significant difference was detected between control and \textit{Hyal2}\textsuperscript{−/−} mice (Figure 4I), further supporting a normal activity of ADAMTS13.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|}
\hline
 & Control marrow & \textit{Hyal2}\textsuperscript{−/−} marrow & \textit{P} \\
\hline
WBC (10\textsuperscript{9} cells/L) & 14.19±0.62 & 13.86±0.60 & NS \\
RBC (10\textsuperscript{10} cells/L) & 9.87±0.19 & 9.13±0.19 & * \\
Hemoglobin (g/dL) & 13.42±0.19 & 12.49±0.24 & ** \\
Hematocrit (%) & 45.13±0.61 & 42.55±0.99 & NS \\
Reticulocytes (%) & 3.21±0.10 & 3.49±0.09 & NS \\
Platelets (10\textsuperscript{10} cells/L) & 1246±37 & 919±46 & ** \\
\hline
\end{tabular}
\caption{Effect of bone marrow reconstitution on blood parameters of irradiated \textit{Hyal2}\textsuperscript{−/−} mice.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{RBC transfusion. In vivo survival of sulfosuccinimidyl-6-lysyl succinimidyl biotin labeled RBC transfused into wild-type and \textit{Hyal2}\textsuperscript{−/−} recipient inbred mice. The donors were one female control inbred mouse (control RBC represented by plain symbols, \(\bullet\) and ▲) and one female \textit{Hyal2}\textsuperscript{−/−} inbred mouse (\textit{Hyal2}\textsuperscript{−/−} RBC represented by empty symbols, ○ and △). The recipients were three female inbred wild-type (circles, \(\bullet\) and ○) and three female inbred \textit{Hyal2}\textsuperscript{−/−} (triangles, ▲ and △) mice. Transfusion was performed via the tail vein. The percentage of circulating donor biotin-labeled erythrocytes (donor biotin+ RBC) was calculated as the ratio of labeled RBC to all RBC. Approximately 7% of the RBC were labeled immediately after the transfusion. Each value represents the mean ± SEM of three recipient mice in each group. \textit{Hyal2}\textsuperscript{−/−} recipient mice differed significantly from control recipient mice (two-way ANOVA, \(P<0.001\)) but there was no difference in survival between control and \textit{Hyal2}\textsuperscript{−/−} donor RBC infused into mice of the same genotype.}
\end{figure}
wild-type RBC transfused into Hyal2− mice disappeared as quickly as Hyal2− RBC.

RBC turn over very quickly in Hyal2− mice; their half-life is a mere 8 days instead of 25 days in normal mice. Several experiments were performed to demonstrate that their rapid removal is not related to an overactive reticuloendothelial system or spleen. Indeed, despite splenomegaly (probably a sign of extramedullary erythropoiesis in Hyal2− mice), splenectomy had no effect on the fast RBC turnover. In addition, no obvious platelet dysfunction or markers of platelet and RBC senescence were found.

Several elements, including the presence of schistocytes, point to a mostly intravascular mechanical destruction of RBC and platelets in Hyal2− mice. The cause of this destruction could be the elevated plasma levels of hyaluronan. For instance, hyaluronan has recently been found to interact with the RBC surface leading to a reduction in erythrocyte deformability and aggregability. However, potential changes in erythrocyte rheological properties in the presence of hyaluronan do not necessarily imply direct involvement of hyaluronan itself was not toxic to the RBC. High concentrations of hyaluronan could also increase blood viscosity. For this reason, we measured plasma and blood viscosity at different shear rates, including those present in murine microvessels under physiological conditions but there was no significant difference in viscosity between control and Hyal2− mice. Thus, hyaluronan does not seem to be directly involved in RBC destruction.

A typical or atypical hemolytic uremic syndrome can also be excluded because (i) the hemolytic process in Hyal2− mice is chronic, (ii) renal function remains normal even at an advanced age, and (iii), more importantly, while activated C5 is required for complement-dependent destruction of RBC, repeated administrations of anti-C5 antibody (BB5.1, the murine equivalent of eculizumab), though effectively repressing plasma hemolytic activity, did not prevent erythrocyte elimination. Eculizumab is very efficient for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. BB5.1 is similarly effective in several complement-dependent murine disease models such as collagen-induced arthritis and cardiac allograft rejection. We can, therefore, reliably exclude C5 activation as a key step in HYAL2 deficiency-induced hemolysis. A few forms of chronic atypical hemolytic uremic syndrome, such as defects in membrane cofactor protein, may present with preserved renal function.

The remaining possibility is a complement-independent form of MAHA. In humans, these forms, essentially characterized by thrombocytopenia and the presence of schistocytes in addition to anemia, are described as thrombotic microangiopathies, thrombotic microangiopathic anemias (TMA), or simply MAHA. They represent a spectrum of disorders that usually present as hemolytic uremic syndrome or thrombotic thrombocytopenic purpura. They are mostly acute conditions linked to bacterial toxin exposure, pregnancy, organ transplantation, malignancy, malignant hypertension, or vasculitis. Most TMA are linked to ADAMTS13 deficiency or endothelial damage leading to partial occlusion of small vessels, mechanical trauma to RBC, and platelet consumption. In Hyal2− mice, plasma levels of three typical endothelial cell injury markers, i.e. vascular adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin, were all significantly elevated but, unlike most human forms of MAHA, HYAL2-deficient MAHA was not accompanied by abnormalities in regulatory elements of the complement cascade, deficiency in vWF-cleaving protease, or any degree of organ dysfunction. RBC fragmentation (1% to 6%) and thrombocytopenia (to one third of normal levels) were mild. There was no visible purpura. Thrombotic events were identified through fibrin deposition in microvessels without visible tissue damage.

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**Figure 3.** In vitro incubations with hyaluronan and blood viscosity. (A) Phosphatidylserine exposure (annexin V labeling) on RBC was measured using FACS after 24-h incubation of control (gray bars, ) and Hyal2− (white bars, ) RBC. 1: No incubation. 2: Incubation in control hyaluronan-poor plasma. 3: Incubation in Hyal2− hyaluronan-rich plasma. 4: Incubation in control plasma + 7.5 μg/mL high molecular mass (3.9×10^6 Da) hyaluronan. The differences between genotypes were statistically significant for all samples except those which underwent no incubation [two-way repeated measure ANOVA (**P<0.01**) followed by Bonferroni tests (P<0.01-0.001) for each condition]. (B) Blood viscosity was measured in five control (gray bars, ) and six Hyal2− (white bars, ) mice at four predefined shear rates ranging from 11 s^−1 to 450 s^−1 using a rotation viscosimeter. The differences between genotypes were not statistically significant (two-way ANOVA, P<0.06).
Microangiopathy due to HYAL2 deficiency

Figure 4. Endothelial and ADAMTS13 phenotypes. (A-C) Serum levels of (A) vascular cell adhesion marker-1 (VCAM-1), (B), intercellular adhesion molecule-1 (ICAM-1), and (C) P-selectin were measured using enzyme-linked immunosorbent assays in control (closed circles, ●) and Hyal2-/-(open circles, □) mice. Each point represents one mouse. Horizontal lines represent means ± SEM. ***P<0.001 (Mann-Whitney tests). (D-G) Representative images of small vessels with fibrin staining (in brown). Renal vessels are shown in glomeruli (D-E) and medulla (F-G), in both control (D & F) and Hyal2-/-(E & G) mice. The bars indicate 50 μm. Positive staining was found exclusively in Hyal2-/-(open circles, □) mice, calculated according to the assay manufacturer’s instructions. Each point represents one mouse. Horizontal lines represent means ± SEM. *P<0.05 (Mann-Whitney test). (H) Percentage of plasma activity of ADAMTS13 in control (closed circles, ●) vs. Hyal2-/-(open circles, □) mice, calculated according to the assay manufacturer’s instructions. Each point represents one mouse. Horizontal lines represent means ± SEM. *P<0.05 (Mann-Whitney test). (I) Representative analysis of vWF multimers using 2% SDS agarose gels and western blots in four wild-type inbred mice (1-4) and four Hyal2-/inbred mice (5-8). NP indicates the vWF multimeric pattern of normal human plasma. There was no difference between genotypes. Similar results were obtained for outbred mice.
Since we did not find evidence of any of the spontaneous causes of TMA, such as autoimmune disorders, chronic vasculitis, hypertension, or the Kasabach-Merritt phenomenon, i.e., the presence of hemangiosarcoma, hemangiendothelioma or tufted angioma,\textsuperscript{12,13} we assume that HYAL2 deficiency represents a novel form of chronic MAHA. The link between HYAL2 deficiency, high plasma levels of hyaluronan, and endothelial damage remains to be elucidated. Although the final mechanism for intravascular hemolysis or TMA in Hyal2\textsuperscript{-/-} mice has not been uncovered so far, we have demonstrated that a yet undetermined environmental factor present in Hyal2\textsuperscript{-/-} mice (and perhaps in so far unexplained human cases of TMA) induces fibrin deposition, peripheral platelet consumption and RBC fragmentation (schistocytes). This observation represents the general mechanism of TMA in humans. Other causes of intravascular hemolysis or TMA, such as lack of ADAMTS13, were clearly excluded. Our next objective will be to identify the specific Hyal2\textsuperscript{-/-} environmental particularity at the origin of this novel form of TMA. Thereby, we hope to reach a better understanding of the mechanism creating intravascular hemolysis in these mice and possibly in some forms of similar diseases in humans.

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