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 glycocalyx. 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 A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motifs, member 13 (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 matrices of verterbrates, 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 humans[2] and 400-600 ng/mL in C57Bl/6 mice.[3] Hyaluronan is catabolized by somatic hyaluronidases, principally hyaluronidase 1 (HYAL1) and hyaluronidase 2 (HYAL2),[4] 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.[5] HYAL2-deficient (*Hyal2*$^{-/-}$) mice were generated in our laboratory through a conditional Cre-lox system.[6] 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) a 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*$^{tm1.1BFla}$ mice[6] 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).[7]

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

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Technologies) and analyzed using flow cytometry (FACS Calibur, BD Biosciences).[8]

RBC senescence was assessed by measuring the exposure of phosphatidylserine,[9] auto-immunoglobulin G (IgG),[10] and C3 on RBC after *in vitro* incubation with fluorescein isothiocyanate-(FITC)-annexin V (BD Biosciences), FITC-rabbit anti-mouse IgG (Dako), or rat monoclonal anti-mouse C3 antibody (Abcam) followed by Alexa-488-anti-rat antibody (Life Technologies), respectively.

For the deformability studies, a pre-determined volume of blood (23 to 30 μL for hematocrit values of 33% to 44%, respectively) was added to 5 mL of isotonic polyvinylpyrrolidone solution. One milliter of this suspension was submitted to 12 different shear stress values at 37°C using a LORCA ektacytometer (Mechatronics Instruments). A decrease in elongation index indicates a loss of RBC deformability.[11,12]

Transplantation of bone marrow

Mice were irradiated twice (2×6 Gy at a 3-h interval). One hour later, they were given an intravenous injection of either control or *Hyal2*$^{-/-}$ bone marrow (5x10^6 cells). The mice were kept under sterile conditions for 12 weeks to allow bone marrow reconstitution and blood analyses.[13]

Survival of transfused red blood cells

RBC from control or *Hya2*$^{-/-}$ donor mice were labeled by intravenous injection of sulfo-NHS-LC-biotin, collected 1 h later, washed, and re-suspended to achieve a hematocrit of 25%. Two hundred microliters of this suspension were injected into the tail vein of control and *Hya2*$^{-/-}$ recipient mice. The percentage of biotinylated erythrocytes remaining in the circulation at various time points was determined using FACS.[14]

Incubation of red blood cells

Buffy coat-free plasma was collected by centrifugation. Complement was inactivated (56°C for 30 min). RBC were incubated for 24 h in either *Hyal2*$^{-/-}$ plasma, control plasma, or the latter + 7.5 μg/mL high molecular mass (3.9×10^6 Da) hyaluronan, corresponding to plasma hyaluronan concentrations in *Hyal2*$^{-/-}$ mice. RBC were then incubated with FITC-annexin-V and analyzed using FACS.

Blood and plasma viscosities and blood pressure

The viscosity of blood and plasma was measured at 38°C under various shear rates using a rotation viscosimeter (LVDV-II+Pro C/P, Brookfield Engineering Laboratories).

Blood pressure was measured using the tail-cuff method (CODA, Kent Scientific, Torrington, CT, USA).[15,16]

Other measurements

Vascular adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin were measured using enzyme linked immunosorbent assays (R&D Systems), while the activity of A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motifs, member 13 (ADAMTS13) was determined using Lifecodes® Activity Assay (Immucor). von Willebrand factor (vWF) multimers were assessed in the laboratory of Claudine Caron, Lille, France. Fibrin was detected using polyclonal rabbit anti-human fibrinogen antibody (Dako).[17]

Statistical analyses

Unpaired and Mann-Whitney tests were used for single comparisons; two-way ANOVA and the Kruskal-Wallis test were applied for multiple comparisons. Correlations were assessed using Pearson r^2.

Results

Hyal2$^{-/-}$ 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.[6] Erythrocytic and thrombocytic indices were analyzed in *Hyal2*$^{-/-}$ and control (*Hyal2*$^{+/+}$ and *Hyal2*$^{+/-}$) outbred young adult mice (8 weeks old) (Table 1). Whereas the main parameters of *Hyal2*$^{+/+}$ and *Hyal2*$^{+/-}$ mice did not differ significantly, *Hyal2*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ mice (*Online Supplementary Table S1*). These observations were thus independent of the mouse's genetic background.

HYAL2-deficient mice also displayed macrocytosis (Table 1). The mean corpuscular volume and mean corpuscular hemoglobin content of RBC were significantly higher in *Hyal2*$^{-/-}$ 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$^{-/-}$ 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*$^{-/-}$ RBC was measured following sulfo-NHS-LC-biotin injections. This system labels RBC proteins simply and efficiently without altering their biological activity.[8] The half-life of biotinylated RBC was only 8 days for *Hyal2*$^{-/-}$ RBC (Figure 1A) compared with 25 days for control RBC (P<0.001).

The accelerated turnover of *Hyal2*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ 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 ektacytometry (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)[18] 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.

Table 1. Corpuscular indices in outbred mice.

	Hyal2$^{+/+}$ (n=15)	*Hyal2*$^{+/-}$ (n=24)	P^1	*Hyal2*$^{-/-}$ (n=49)	P^2
RBC (10^{12} cells/L)	9.79 ± 0,20	9.66 ± 0.10	NS	7.75 ± 0.13	***
Hb (g/dL)	14.14 ± 0.59	13.67 ± 0.16	NS	12.51 ± 0.23	**
HCT (%)	46.03 ± 1.30	43.17 ± 0.47	NS	39.47 ± 0.68	**
Ret (10^9 cells/L)	327 ± 24	240 ± 14	NS	922 ± 46	***
Ret (%)	3.60 ± 0.24	2.39 ± 0.11	NS	12.12 ± 0.67	***
IRF (%) (n=9)		44.97 ± 1.60		56.86 ± 2.08	***
MCV (fL)	45.87 ± 0.56	44.50 ± 0.34	NS	51.16 ± 0.43	***
MCH (pg)	15.42 ± 0.13	14.14 ± 0.15	**	16.17 ± 0.12	***
MCHC (g/dL)	32.69 ± 0.44	31.71 ± 0.20	NS	31.66 ± 0.20	NS
CHCM (g/dL)	32.04 ± 0.71	30.90 ± 0.11	NS	30.97 ± 0.21	NS
CH (pg)	14.94 ± 0.25	13.77 ± 0.11	*	15.78 ± 0.12	***
RDW (%)	13.61 ± 0.31	12.65 ± 0.27	NS	14.72 ± 0.32	***
HDW (g/dl)	2.25 ± 0.07	1.95 ± 0.03	NS	2.73 ± 0.05	***
PLT (10^9 cells/L)	1140 ± 79	1252 ± 45	NS	402 ± 26	***
IPF (%) (n=9)		0.58 ± 0.04		6.33 ± 0.61	***
MPV (fL)	7.06 ± 0.13	6.06 ± 0.18	*	7.71 ± 0.15	***
Vitamin 12 (nmol/L)		23,0 ± 3,7		33,1 ± 2,3	*
Serum folates (nmol/L)		52,7 ± 5,5		98,0 ± 11,9	**
After *in vitro* reticulocyte maturation:					
MCV (fL) (n=9)		40.70 ± 0.44		47.70 ± 0.63	***
RDW (%) (n=9)		14.88 ± 0.29		15.07 ± 0.66	NS

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) was <0.001 for all parameters except for MCHC (P=NS) and CHCM (P=NS). Values of P^1 for Hyal2$^{+/+}$ vs. Hyal2$^{+/-}$ *and* P^2 *for* Hyal2$^{+/-}$ vs. Hyal2$^{-/-}$*, calculated based on Dunn's multiple comparison test, are indicated. the Mann Whitney test was used for IRF and IPF comparisons as well as for MCV and RDW after reticulocyte maturation. NS: non significant; *P<0.05; **P<0.01; ***P<0.001.*

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^2 = 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 extracorpuscular 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

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.[19] Anti-C5 treatment has been shown to suppress plasma hemolytic activity by at least 60% in C57Bl/6 mice.[20] 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.

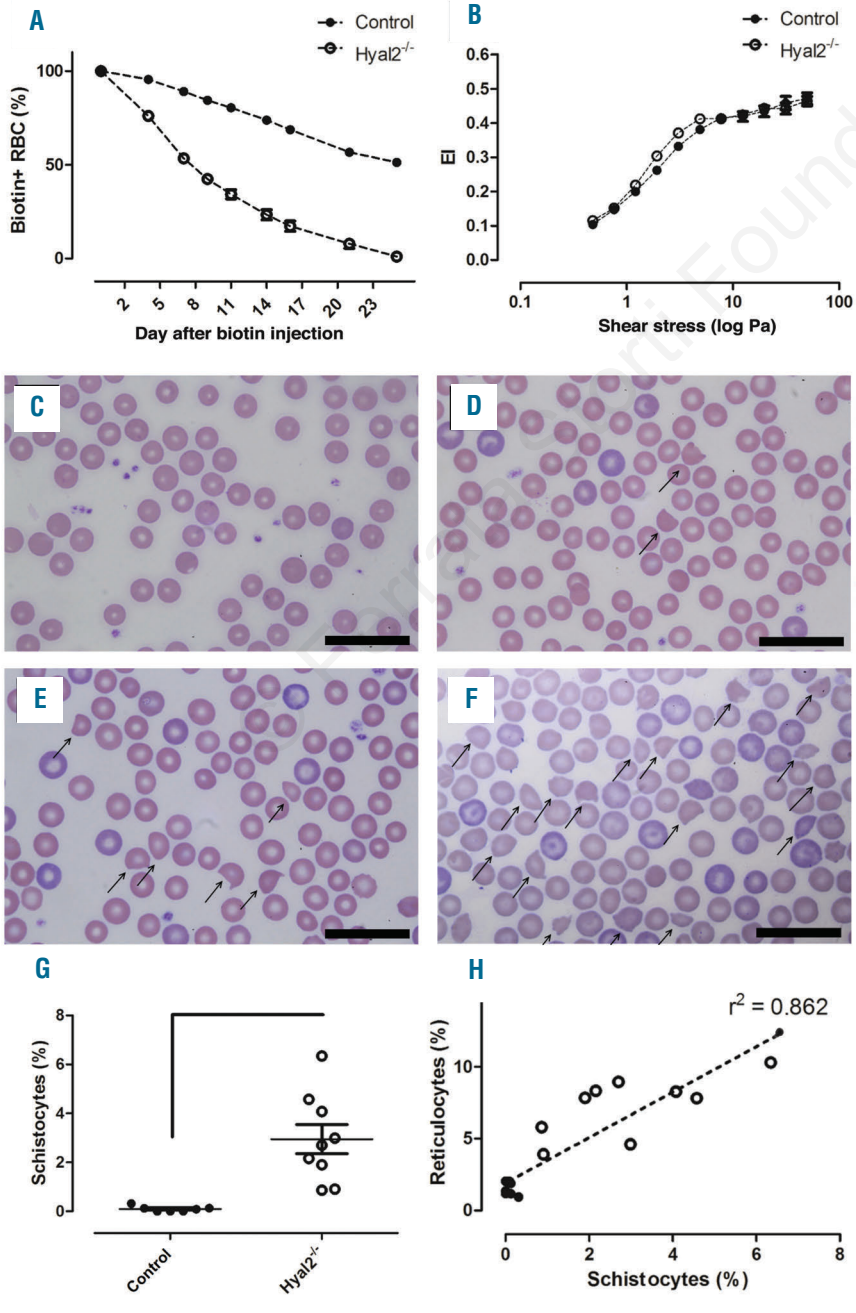

Figure 1. Erythrocyte phenotype. (A) Endogenous RBC clearance. Control (closed circle, ●) and *Hyal2*$^{-/-}$ (open circles, ❍) outbred mice were injected with sulfo-NHS-LC-biotin. Then, 5 μL of blood were taken at the indicated time points. The percentage of biotinylated erythrocytes (biotin+ RBC) was calculated as the ratio of positive cells to all RBC. Each value represents the mean ± SEM of six mice in each group. *Hyal2*$^{-/-}$ mice differed significantly from control mice (two-way ANOVA, P<0.001). Half-lives of control and *Hyal2*$^{-/-}$ RBC were estimated to be 25 days and 8 days, respectively. (B) Deformability curve (ektacytometry). Elongation index values *vs.* shear stress (Pa) are shown for RBC from wild-type (closed circle, ●) and *Hyal2*$^{-/-}$ (open circles, ❍) inbred mice. Each value represents the mean ± SEM of ten measurements on five mice in each group. Two-way ANOVA showed no significant difference between genotypes (P=0.18). Similar results were found for outbred mice. (C-F) Typical blood smears of control (C) and *Hyal2*$^{-/-}$ (D-F) outbred mice. The bar indicates 20 μm. Arrows point to schistocytes. (G) Percentage of schistocytes counted on blood smears in each genotype based on at least 500 RBC. Individual values of *control* (closed circle, ●, n=7) and *Hyal2*$^{-/-}$ (open circles, ❍, n=9) mice, and means ± SEM are shown. ***, P<0.001 (Mann-Whitney test). (H) Correlation (Pearson r^2) between the proportions of schistocytes (%) and reticulocytes (%) counted on blood smears of seven control (closed circle, ●) and nine *Hyal2*$^{-/-}$ (open circles, ❍) mice. P<0.001.

Second, whole blood viscosity was measured in blood samples from control and *Hyal2*$^{-/-}$ mice at different physiological shear rates ranging from 11 to 450 s^{-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 *Hyal2*$^{-/-}$ mice, respectively; n=3, *P*=NS). These results rule out a toxic effect of hyaluronan on RBC, either directly or through blood viscosity.

Hyal2$^{-/-}$ mice are normotensive

Malignant hypertension is another possible cause of mechanical RBC fragmentation.[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 *Hyal2*$^{-/-}$ mice, respectively; n=7, *P*=NS).

Hyal2$^{-/-}$ 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 *Hyal2*$^{-/-}$ 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 *Hyal2*$^{-/-}$ 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 glycocalyx scarcity (*Online Supplementary Figure S3*). In contrast, the endothelial glycocalyx tended to be thicker in *Hyal2*$^{-/-}$ than in control mice, while vascular fibrin deposition, a sign of thrombotic events, was found in numerous microvessels of *Hyal2*$^{-/-}$ 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 was not decreased in *Hyal2*$^{-/-}$ mice and was even higher than in control mice (Figure 4H). In addition, no ultra-large multimers of vWF were detected in either genotype (Figure 4I), further supporting a normal activity of ADAMTS13.

Discussion

Hyal2$^{-/-}$ mice were developed in our laboratory to study the exact function of HYAL2 in hyaluronan catabolism. Surprisingly, *Hyal2*$^{-/-}$ mice were found to suffer from mild thrombocytopenia and compensated hemolytic anemia with increased plasma lactate dehydrogenase levels.[6] Investigating these hematologic abnormalities, the current study revealed chronic thrombotic microangiopathic hemolytic anemia (MAHA) with fragmented RBC (i.e. schistocytes) in *Hyal2*$^{-/-}$ mice. HYAL2 deficiency seems to be a unique cause of chronic MAHA.

A thorough analysis of RBC parameters using ADVIA 120 equipment showed that the absence of HYAL2 leads to macrocytosis (without folate or vitamin B12 deficiency) and anisocytosis without spherocytosis (the latter was also ruled out by normal eosine-5-maleimide binding data). Blood smears confirmed the presence of poikilocytosis and showed numerous damaged RBC with a few schistocytes. RBC deformability was normal. Thus, poikilocytosis and macrocytosis likely result from accelerated erythropoiesis secondary to increased peripheral consumption. Bone marrow transplantation of either control or knockout stem cells into irradiated wild-type mice confirmed that the causes of erythrocyte and platelet abnormalities must be mostly environmental since erythrocyte and platelet production arising from *Hyal2*$^{-/-}$ precursors was only minimally affected and there was no sign of hemolysis. Similarly, and perhaps even more convincingly,

Table 2. Effect of bone marrow reconstitution on blood parameters of irradiated *Hyal2*$^{+/+}$ mice.

	Control marrow (n=7)	*Hyal2*$^{-/-}$ marrow (n=6)	*P*
WBC (10^9 cells/L)	14.19 ± 0.62	13.86 ± 0.60	NS
RBC (10^{12} 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 (10^9 cells/L)	1246 ± 37	919 ± 46	**

Bone marrow engraftment was confirmed using whole blood polymerase chain reaction. Blood analysis was performed 12 weeks after irradiation when blood parameters were stable. WBC: *white blood cell count;* RBC: *red blood cell count; One mouse receiving* Hyal2$^{-/-}$ *bone marrow was excluded and euthanized due to rectal prolapse with visible bleeding. Each value represents the mean ± SEM.* P *values for the differences between control and* Hyal2$^{-/-}$ *bone marrow were calculated using the Mann Whitney test. NS, non significant;* *P<*0.05*, **P<*0.01*.

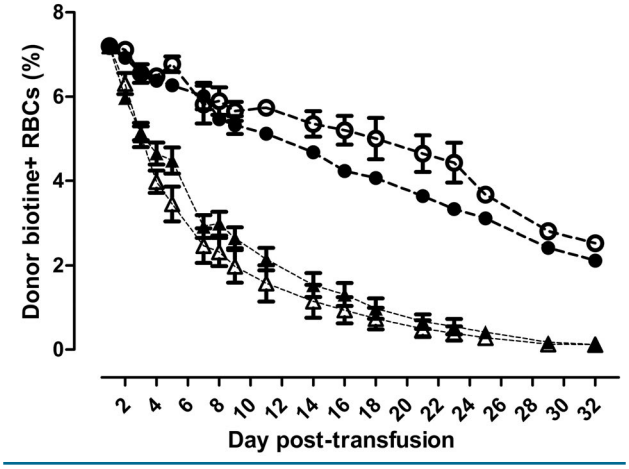

Figure 2. RBC transfusion. *In vivo* survival of sulfo-NHS-LC-biotin labeled RBC transfused into wild-type and *Hyal2*$^{-/-}$ recipient inbred mice. The donors were one female control inbred mouse (control RBC represented by plain symbols, ● and ▲) and one female *Hyal2*$^{-/-}$ inbred mouse (*Hyal2*$^{-/-}$ RBC represented by empty symbols, ❍ and △). The recipients were three female inbred wild-type (circles, ● and ❍) and three female inbred *Hyal2*$^{-/-}$ (triangles, ▲ and △) mice. Transfusion was performed *via* the tail vein. The percentage of circulating donor biotinylated 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. *Hyal2*$^{-/-}$ recipient mice differed significantly from control recipient mice (two-way ANOVA, *P*<0.001) but there was no difference in survival between control and *Hyal2*$^{-/-}$ donor RBC infused into mice of the same genotype.

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 reticulo-endothelial 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.[22] However, potential changes in erythrocyte rheological properties in the presence of hyaluronan do not necessarily imply hemolysis. In addition, we found that *in vitro* incubation of RBC with *Hyal2*$^{-/-}$ plasma or with high concentrations 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[18] 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 even 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.[23] BB5.1 is similarly effective in several complement-dependent murine disease models such as collagen-induced arthritis[20] and cardiac allograft rejection.[22] 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.[24]

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.[25-29] 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.[30,31] 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.

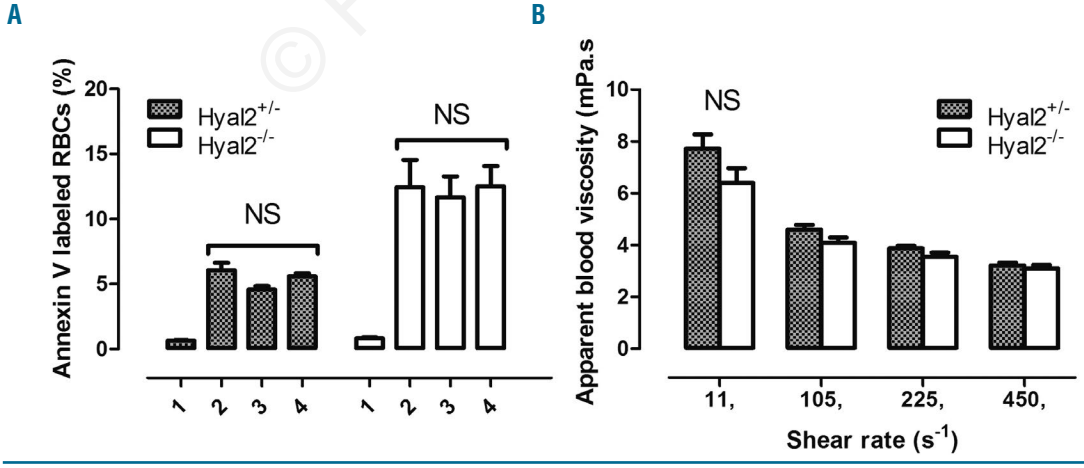

Figure 3. *In vitro* incubations with hyaluronan and blood viscosity. (A) Phosphatidylserine exposure (annexin V labeling) on RBC was measured using FACS after 24-h incubation of control (gray bars, ▩) and *Hyal2*$^{-/-}$ (white bars, ▭) RBC. 1: No incubation. 2: Incubation in control hyaluronan-poor 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).

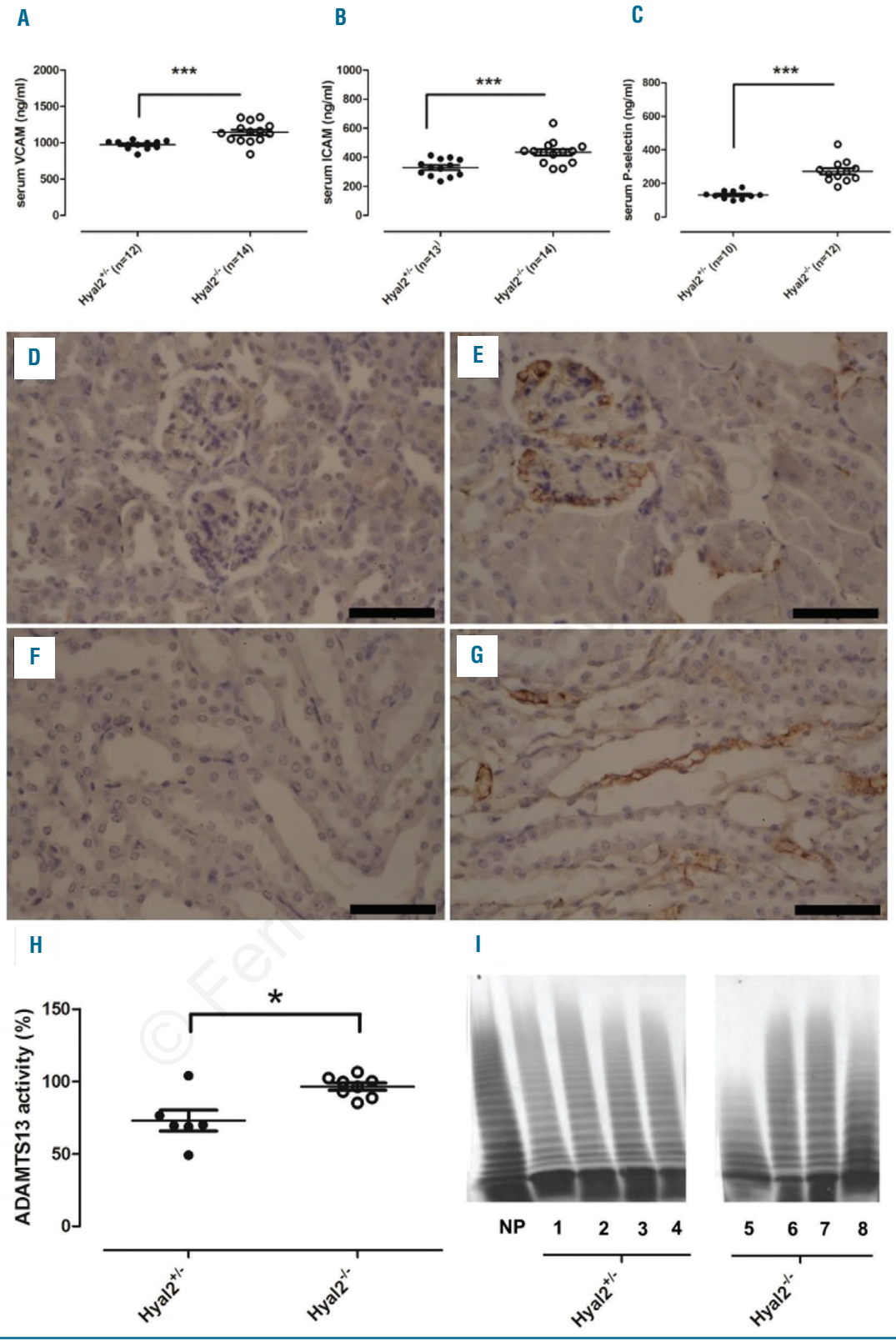

Figure 4. Endothelial and ADAMTS13 phenotypes. (A-C) Serum levels of (A) vascular cell adhesion marker-1 (VCAM-1), (B), intercellular adhesion molecule-1 (ICAM-1), and (C) P-selectin were measured using enzyme-linked immunosorbent assays in control (closed circles, ●) and *Hyal2*$^{-/-}$ (open circles, ○) 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*$^{-/-}$ mice. (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, hemangioendothelioma or tufted angioma,[32,33] 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*$^{-/-}$ mice has not been uncovered so far, we have demonstrated that a yet undetermined environmental factor present in *Hyal2*$^{-/-}$ 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*$^{-/-}$ 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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Authorship and Disclosures

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