SUPPLEMENTARY APPENDIX

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

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 doi:10.3324/haematol.2015.123828
The online version of this article has a Supplementary Appendix.
Manuscript received on January 29, 2015. Manuscript accepted on April 27, 2015.
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SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

.***Platelet phenotype****.* Platelet-rich fractions were prepared as described[34] using ACD (acid, citrate, dextrose) and apyrase (0,5 U/ml) as anticoagulants. ADP (10 µM) and thrombin (1 U/ml) were used to produce platelet partial and total activation, respectively. Platelet morphology was examined using electronic microscopy (JEOL and Philips Tecnai 10) observation as described elsewhere.[34] In addition, the amount of platelet microparticles was measured using a BD Accuri C6 flow cytometer as described elsewhere.[34]The following reagents were used: Megamix Beads (Biocytex), PE-rat anti mouse CD41 (BD Pharmingen, 558040), and FITC-Annexin V (BD Biosciences).

Splenectomy. *Control* and *Hyal2*$^{-/-}$ mice were anesthetized intraperitonally using a mixture of medetomidin (Medetor® 1mg/ml, Virbac Animal Health Belgium, Wavre, Belgium; 0.25 mg/kg) and ketamine HCl (Ceva®, Brussel, Belgium; 40 mg/kg). The mice were placed on a heating table to maintain body temperature during all the procedure. After asepsis, a small incision was made in the left flank region, and the spleen was gently removed**.** One month after splenectomy, mice were injected with sulfo-NHS-LC-biotin to measure RBC survival.

Anti-C5 administration. Mice received biweekly intraperitonal injections of 750 µg anti-mouse C5 monoclonal antibodies (BB5.1) or control isotype-matched antibodies (both a kind gift of Dr Paul Tamburini, Alexion Pharmaceuticals, Cheshire, CT, USA) for 3 weeks. Blood samples were then collected to evaluate blood parameters and to confirm that treatment with anti-C5 led to decreased complement activity, using a hemolytic assay as described.[20]

Glycocalyx staining in myocardial microvessels. Endothelial glycocalyx was fixed and stained with Alcian blue 8GX as described elsewhere.[35] Briefly, anesthetized mice were cannulated retrogradely at the aorta/brachiocephalic trunk bifurcation and the vena cava was transected. Three successive solutions were perfused at a constant pressure of 33 ± 5 mmHg: first, calcium-free cardioplegic solution (BSA 0.1% for 3 min); second, phosphate buffered fixative (pH 7.4) containing 30 mM $MgCl_2$ for 2 min; third, the same solution + 0.05% Alcian Blue 8GX (Sigma) for 30 min. Left ventricular wall was then cut into 2-mm segments. Samples were fixed as usual for transmission microscopy which was performed using a Tecnai instrument.

Additional serum assays. Serum creatinine was measured using *Crea+* (Roche Diagnostics), urine creatinine using *Urine Mouse Creatinine Assay Kit* (Crystal Chem Inc., Downers Grove, IL, USA), and urine albumin using *Mouse Albumin Elisa Kit* (Aviva Systems Biology, San Diego, CA, USA).

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL TABLES

Supplemental Table 1. Corpuscular indices in inbred mice.

	Control *(N=5)*	***Hyal2$^{-/-}$*** *(N=5)*	***P***
RBC (10^6 cells/µl)	9.05 ± 0.23	7.23 ± 0.48	*
Hb (g/dl)	12.8 ± 0.38	11.4 ± 0.68	NS
HCT (%)	38.4 ± 1.06	34.3 ± 1.23	*
Ret (%)	3.75 ± 0.28	16.5 ± 2.87	**
MCV (fL)	42.4 ± 0.57	48.0 ± 1.73	*
MCH (pg)	14.2 ± 0.23	15.9 ± 0.26	*
MCHC (g/dl)	33.4 ± 0.40	33.2 ± 1.02	NS
CHCM (g/dl)	34.2 ± 0.56	34.6 ± 0.98	NS
CH (pg)	14.6 ± 0.13	16.5 ± 0.18	*
RDW (%)	13.8 ± 0.29	15.4 ± 0.93	NS
HDW (g/dl)	2.60 ± 0.07	3.40 ± 0.12	*

RBC, red blood cell count; *Hb,* hemoglobin; *HCT,* hematocrit; *Ret,* reticulocytes; *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. Comparisons using Mann Whitney test. *NS,* non significant; *, P <0.05; **, P <0.01.

Supplemental Table 2. Weight of various organs in male mice.

	Control (*N*=10)	***Hyal2*$^{-/-}$** (*N*=10)	***P***
Animal (g)	33.1 ± 1.8	33.8 ± 1	NS
Heart (g)	0.158 ± 0.007	0.182 ± 0.006	*
Liver (g)	1.560 ± 0.098	1.553 ± 0.046	NS
Spleen (g)	0.095 ± 0.007	0.274 ± 0.022	***
Left Kidney (g)	0.220 ± 0.012	0.215 ± 0.015	NS
Right Kidney (g)	0.224 ± 0.011	0.227 ± 0.012	NS

Each value represents the mean ± SEM. P values for the differences between control and Hyal2$^{-/-}$ mice were calculated using unpaired Student's t-tests. NS, non significant; *, $P<0.05$; ***, $P<0.001$. Female *Hyal2*$^{-/-}$ mice had similar alterations compared with male *Hyal2*$^{-/-}$ mice (data not shown).

Supplemental Table 3. Effect of anti-C5 antibodies.

	Before injections			**After injections**					
				Control mAb			**Anti-C5 mAb**		
	Control (*N*=6)	***Hyal2*$^{-/-}$** (*N*=14)	***P***	***Control*** (*N*=3)	***Hyal2*$^{-/-}$** (*N*=7)	***P***	***Control*** (*N*=3)	***Hyal2*$^{-/-}$** (*N*=6)	***P***
WBC (10^3/µl)	4.97 ± 0.90	6.54 ± 0.87	NS	6.47 ± 1.34	5.27 ± 0.57	NS	5.85 ± 0.76	5.99 ± 0.35	NS
RBC (10^6/µl)	9.57 ± 0.18	7.83 ± 0.25	**	9.72 ± 0.46	7.52 ± 0.52	*	10.02 ± 0.25	7.26 ± 0.32	*
PLT (10^3/µl)	1237 ± 57	416 ± 40	***	1174 ± 75	507 ± 90	*	1459 ± 193	426 ± 101	*
Ret (%)	2.57 ± 0.13	10.48 ± 0.69	***	2.61 ± 0.12	12.00 ± 1.58	*	2.72 ± 0.30	11.74 ± 0.76	*

Blood parameters before and after 6 biweekly ip injections of control (isotype-matched) or anti-C5 monoclonal antibodies (mAb) in *control* and *Hyal2*$^{-/-}$ mice. One mouse was excluded from the active group because its plasma hemolytic activity was not decreased by the anti-C5 mAb. *WBC*, white blood cell count; *RBC*, red blood cell count; *PLT*, platelet count; *Ret*, reticulocytes. Each value represents the mean ± SEM. *P* values for the differences between *control* and *Hyal2*$^{-/-}$ mice were calculated using Mann-Whitney test; *NS*, non significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Supplemental Table 4. Serum creatinine and urine albumin-to-creatinine ratio in outbred mice.

	6 months (*N*=8)			**11 months (*N*=6)**		
	Control	***Hyal2*$^{-/-}$**	***P***	***Control***	***Hyal2*$^{-/-}$**	***P***
Serum creatinine (mg/dl)	0.41 ± 0.02	0.39 ± 0.02	NS	0.41 ± 0.03	0.44 ± 0.03	NS
Urine alb/creat (g/mol)	3.80 ± 0.29	4.43 ± 0.78	NS	4.66 ± 0.36	5.99 ± 1.21	NS

Statistical comparisons using unpaired t test. NS, non significant.

SUPPLEMENTAL FIGURE LEGENDS

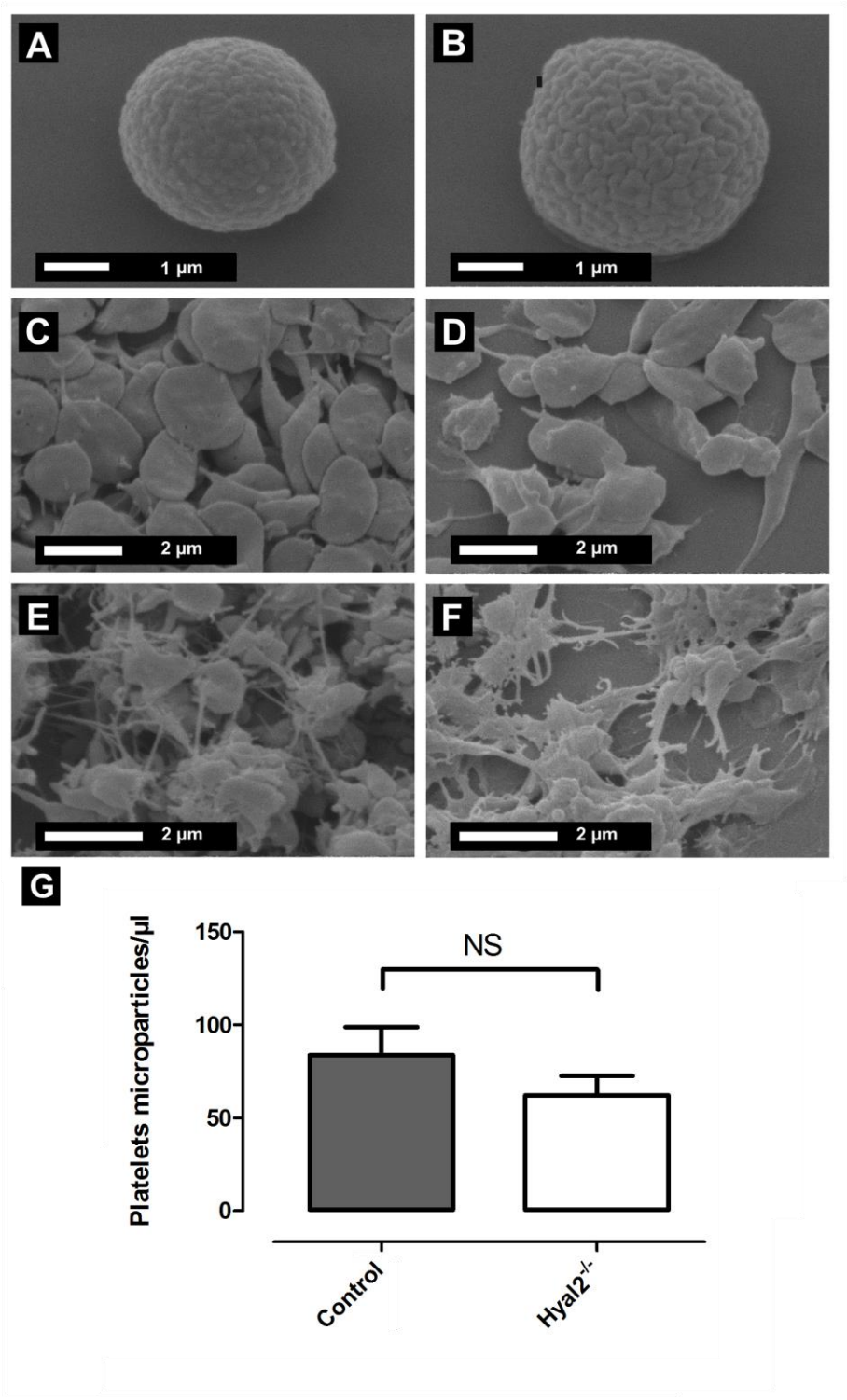
Supplemental Figure 1. Platelet Phenotype*.* (A-F) Platelet morphology observed using scanning electronic microscopy. (A-B) Morphological discoid appearance of washed platelets in *control* (A) and *Hyal2*$^{-/-}$ (B) mice. (C-F) Morphological changes of washed platelets during ADP (C-D) or thrombin (E-F) induced aggregation in *control* (C and E) and *Hyal2*$^{-/-}$ (D and F) mice. (G) Flow cytometric measurement of platelets microparticules in *control* (N=5) and *Hyal2*$^{-/-}$ (N=6) mice. Means ± SEM are shown. There is no difference (NS) between genotypes.

Supplemental Figure 2. Effect of splenectomy on RBC half-life*. Control* and *Hyal2*$^{-/-}$ outbred male mice were splenectomized. One month post-surgery, they were injected with sulfo-NHS-LC-biotin and 5 µl of blood were taken at the indicated time points. The percentage of biotinylated RBCs (biotin+ RBCs) was calculated as the ratio of positive cells to all RBCs in flow cytometry. The RBC survival of splenectomized *control* mice (▲) and *Hyal2*$^{-/-}$ mice (△) is very close to the survival of endogenous RBCs in the respective, non-operated animals (*control*, ●; *Hyal2*$^{-/-}$, ○). Each value represents the mean ± SEM of 3 mice in each group. There was no significant difference between RBC survival in splenectomized and non-splenectomized mice (two-way ANOVA).

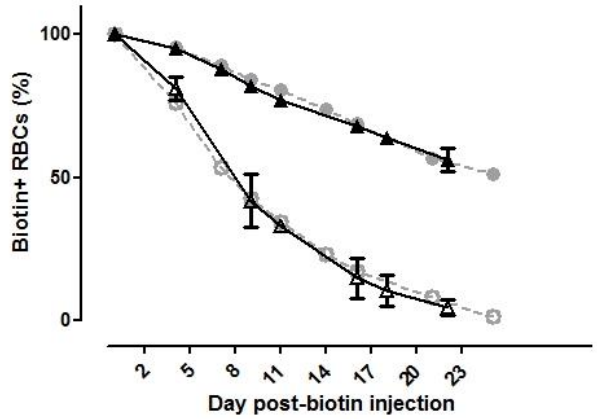
Supplemental Figure 3. Glycocalyx morphology in microvessels*.* Endothelial glycocalyx (stained in black using Alcian Blue 8GX) was observed using transmission electronic microscopy in myocardial microvessels. Glycocalyx was structurally intact in *Hyal2*$^{-/-}$ (B & D) compared with *control* (A & C) mice

SUPPLEMENTAL FIGURES

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3