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Survival protein anoctamin-6 controls multiple platelet responses including phospholipid scrambling and swelling

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The Scott syndrome is characterized as a mild bleeding disorder associated with a low prothrombin consumption in blood. Platelets from Scott patients show a defect in Ca^{2+}-induced phosphatidylserine (PS) exposure on the platelet surface and microparticle formation, but unchanged Ca^{2+} signaling and aggregation (1, 2). For long it has been recognized that the defective PS exposure in blood cells from Scott patients results from impaired phospholipid scrambling, a process that normally abolishes the asymmetric distribution of PS and phosphatidylethanolamine over the plasma membrane upon persistent elevation of cytosolic Ca^{2+}. Consequence of the defective PS exposure is a markedly impaired procoagulant activity of platelets, which agrees with the bleeding phenotype (3). Recently, in two unrelated Scott syndrome patients, dysfunctional mutations have been identified in the *ANO6* gene (alternatively named *TMEM16F*), which codes for the integral membrane protein anoctamin 6 (*ANO6*) (4, 5). The question can be raised if a gene defect in *ANO6* alone is sufficient for the altered blood cell properties in the Scott syndrome. In the present article, we used several molecular and functional approaches to unravel the apparently multiple and non-redundant functions of *ANO6* in platelets. We investigated the alterations in platelet properties of a Scott patient in comparison to platelets from healthy control subjects, and furthermore compared the platelet properties of several new strains of *Ano6*-deficient mice versus wild type mice.

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Methods

Blood was obtained from healthy volunteers and a patient with Scott syndrome, after full informed consent (Helsinki declaration), under protocols reviewed by the local ethics committees. The Scott patient has been genotyped as compound heterozygous for two different mutations in the *ANO6* gene (alias *TMEM16F*), with one splicing mutation (IVS6 + 1G→A) causing exon 6 skipping, and another mutation in exon 11 (c.1219insT) leading to a premature stop of translation (5). Mouse experiments were approved by the local animal care and use committees. Experiments were performed using genetically modified and corresponding wild-type animals from the same breeding, simultaneously at the same location. Platelets from these mice were extensively analyzed on molecular functions and compared with platelets from a patient with Scott syndrome.

Results

Platelet aggregation and PS exposure (procoagulant activity) in response to activation of the platelet via the main collagen receptor, glycoprotein VI (GPVI), can simultaneously be quantified in thrombi formed during whole-blood perfusion over a collagen surface. In flow studies with blood from a Scott patient, we established that platelet adhesion, aggregate formation and α-granule secretion (platelet surface expression of P-selectin) were similar to the platelet responses of healthy control subjects. In contrast, collagen-induced PS exposure was greatly reduced, but not completely abolished with the patient blood. Interestingly, the residual PS exposure appeared as patches on platelets with a normal, non-swollen shape. This contrasted to the large, ballooning morphology (diameter ~10 μm) of platelets from control subjects with high PS exposure (Figure 1A).

Earlier studies indicated that murine deficiency in *ANO6* affects mineral deposition in skeletal tissue and arterial thrombus formation in vivo (6, 7). By cross-breeding we could obtain viable and adult $ANO6^{-/-}$ offspring from mice of the $ANO6^{Avor}$, but not of the $ANO6^{AW}$ strain. We compared activation characteristics of platelets from mice of the $ANO6^{AW}$ an $ANO6^{AVOR}$ strains. Platelets from both types of heterozygous mice expressed normal levels of surface glycoproteins, and were unchanged in $\alpha_{IIb}\beta_3$ activation, P-selectin expression and PS exposure, when compared to corresponding wild-type platelets.

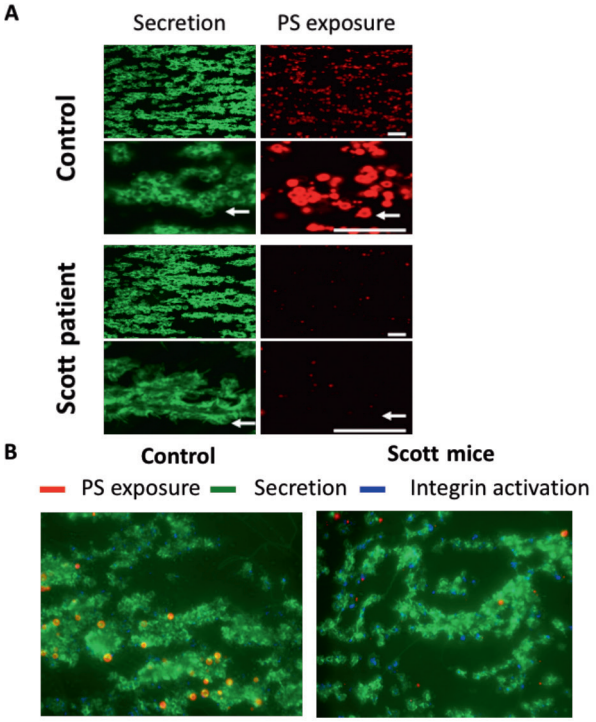

Figure 1. Normal thrombus formation with residual PS exposure and abolished ballooning in Scott syndrome and in the absence of *ANO6*.
A) Blood from healthy control subjects or a Scott patient was perfused over a collagen surface for 4 min at 1000 s^{-1}. Formed thrombi were stained with FITC-αCD62P (P-selectin) mAb and AF647-annexin A5 (PS exposure). B) Blood from corresponding *ANO6*$^{+/+}$ and *ANO6*$^{-/-}$ mice (*ANO6*^{Avor} strain) was perfused over collagen for 4 min at 1000 s^{-1}. Thrombi were stained with PE-JON/A mAb (integrin activation), FITC-αCD62P mAb (P-selectin) and AF647- annexin A5 (PS exposure). Shown are representative fluorescence images (bars 25 µm). Arrow indicates ballooning (control volunteers and wild type mice) and non-ballooning platelet with small patches of PS exposure (Scott patient and mice).

We then assessed thrombus formation on collagen using blood from surviving adult homozygous deficient *ANO6*$^{-/-}$ mice (*ANO6*^{Avor} strain). Thrombus formation as such was unchanged, with similar platelet adhesion, integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression, in comparison to thrombi from wild-type mice. However, while wild-type thrombi showed 1-2% of the surface as PS exposure, as before, the *ANO6*$^{Avor-/-}$ thrombi displayed a substantial 76.2 ± 2.6% (n = 4, $P<0.05$) reduction of this parameter. Notably, these platelets with residual PS exposure were smaller in size than the 8 µm-ballooning structures seen with wild-type platelets. Image quantification further learned that platelet ballooning was essentially abolished in *ANO6*$^{Avor-/-}$ blood samples (Figure 1B).

Discussion

In the present paper, we compared the activation properties of human Scott platelets and those of mouse platelets lacking the transmembrane protein anoctamin-6, previously linked to PS exposure, with their normal counterparts. We could obtain viable and adult *ANO6*$^{-/-}$ offspring from mice of the *ANO6*^{Avor}, but not of the *ANO6*^{AW} strain. Although this remains to be confirmed, the survival of the *ANO6*$^{Avor-/-}$ mice might be related to the expression of alternative Ano6 mRNA transcripts in key tissues.
A similar, combined defect in platelet PS exposure and ballooning was observed between *ANO6*-deficient platelets and blood from the Scott patient. The lack of balloon formation in *ANO6*-deficient platelets - like Scott platelets - suggests a defect in Ca^{2+}-dependent ion influx and swelling. This idea is confirmed by the observation that a hypertonic environment markedly delays the PS exposure and balloon formation. Together, these data indicate that the functional and morphological alterations found in platelets from the Scott patient are quite well phenocopied in platelets from *ANO6*-deficient mice.
The Scott syndrome has been described as a moderate bleeding disorder, with haemorrhagic episodes only after trauma or childbirth. The present data with *ANO6*$^{Avor-/-}$ mice - in spite of a reduced survival - point to limited hemostatic insufficiency, as mouse tail bleed times were increased. Our data point to a remarkable set of phenotypic changes linked to defective *ANO6* expression in both man and mouse, including low PS exposure, absence of swelling morphological changes and a moderate bleeding tendency.

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