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Abstract
Hemostasis is a tightly regulated physiological process to rapidly induce hemostatic plugs at sites of vascular injury. Inappropriate activation of this process may lead to thrombosis, i.e. pathological blood clot formation in uninjured vessels or on atherosclerotic lesions. ATP release through Pannexin1 (Panx1) membrane channels contributes to collagen-induced platelet aggregation in vitro.

Reference

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Selective inhibition of Panx1 channels decreases hemostasis and thrombosis in vivo

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A R T I C L E   I N F O

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A B S T R A C T

Background: Hemostasis is a tightly regulated physiological process to rapidly induce hemostatic plugs at sites of vascular injury. Inappropriate activation of this process may lead to thrombosis, i.e. pathological blood clot formation in uninjured vessels or on atherosclerotic lesions. ATP release through Pannexin1 (Panx1) membrane channels contributes to collagen-induced platelet aggregation in vitro.

Objective: To investigate the effects of genetic and pharmacological inhibition of Panx1 on hemostasis and thrombosis in vivo.

Results: Bleeding time after tail clipping was increased by 2.5-fold in Panx1−/− mice compared to wild-type controls, suggesting that Panx1 deficiency impairs primary hemostasis. Wire myography on mesenteric arteries revealed diminished vasoconstriction in response to phenylephrine or U446619 in Panx1−/− mice. Mice with platelet-specific deletion of Panx1 (Panx1PDel) displayed 2-fold longer tail bleeding times than Panx1+/+ controls. Moreover, venous thromboembolism (VTE) after injection of collagen/epinephrine in the jugular vein was reduced in Panx1−/− and Panx1PDel mice. Panx1PDel mice also showed reduced FeCl3-induced thrombosis in mesenteric arteries. BrilliantBlue-FCF, a Panx1 channel inhibitor, decreased collagen-induced platelet aggregation in vitro, increased tail bleeding time and reduced VTE in wild-type mice. Furthermore, we developed a specific Panx1 blocking antibody targeting a Panx1 extracellular loop, which reduced ATP release from platelets in vitro. Treating wild-type mice with this antibody increased tail bleeding time and decreased VTE compared to control antibody.

Conclusions: Panx1 channel deletion or inhibition diminishes clot formation during hemostasis and thrombosis in vivo. Blocking Panx1 channels may be an attractive strategy for modulating platelet aggregation in thrombotic disease.

1. Introduction

Pannexins (Panxs) form a three-membered family of glycoproteins, which establish membrane channels at the cell surface. Panx1 is expressed in most mammalian cells and tissues, including skeletal and smooth muscle, endothelium, leukocytes and adipocytes [1,2]. The general topology of Panxs features four transmembrane domains, two extracellular loops (ELs) and an amino-terminus, carboxy-terminus (CT) and intracellular loop all located in the cytoplasm. The CTs differ considerably in both length and composition and are unique to the Panx subtype [1,3]. Panx1 is synthesized, N-glycosylated and oligomerized into hexameric channels in the endoplasmic reticulum prior to further editing and delivery to the Golgi apparatus. Then, Panx1 channels traffic to the plasma membrane where they act as nucleotide release channels but likely serve additional roles in release or uptake of small molecules [3]. Released nucleotides, such as ATP, can signal by targeting surface receptors in a paracrine, autocrine, or even in an endocrine fashion, thus contributing to intercellular signaling and tissue homeostasis. Multiple factors induce Panx1 channel activation, including mechanical stretch, high extracellular K+, metabotropic
receptor activation with associated elevated intracellular Ca\(^{2+}\) (f.e. P2Y receptors), and ionotropic, chemokine-, and glycoprotein receptor activation resulting in Src family kinase (SKF)-mediated phosphorylation (f.e. GPVI receptors) [2].

P2 receptors play a critical role in platelet function. These receptors are subdivided into two groups that are represented by receptors sensitive to ATP (P2X1) and receptors sensitive to ADP (P2Y1 and P2Y12). The ATP-gated P2X1 receptors allow Ca\(^{2+}\) influx into the platelets, resulting in a transient shape change and platelet activation [4,5]. These receptors have been shown to induce and sustain ATP-mediated aggregation in response to collagen after vascular injury [6]. In addition, P2X1 is sensitive to low collagen concentrations in high shear stress conditions, which makes ATP an agonist of platelet aggregation at the early stage of arterial endothelial damage [7]. A role for Panx1 in fine-tuning collagen-induced platelet reactivity was recently shown [8,9]. Specifically, collagen binding to GPVI receptors drives a Src-dependent phosphorylation of Panx1 channels leading to the release of ATP and subsequent activation of P2X1 receptors resulting in human platelet aggregation. Furthermore, a single nucleotide polymorphism of Panx1 (Panx1-400A > C), inducing the expression of a gain-of-function Panx1 channel, was associated with increased collagen-induced aggregation in healthy volunteers [8]. Altogether, these observations suggest that Panx1 sets platelet reactivity. However, direct in vivo involvement of Panx1 in hemostasis and thrombosis remains to be shown.

2. Materials and methods

2.1. Animals

All animal studies were approved by the Swiss Federal Veterinary Office. In vivo tail bleeding, venous thromboembolism (VTE) and FeCl3-induced mesenteric artery thrombosis experiments were performed as previously described [10-12] on age-matched male wild-type (WT), Panx1\(^{-/-}\) [13], Pf4Cre\(^{+}\)Panx1\(^{-/-}\) (Panx1\(^{-/-}\)Fem) and Panx1\(^{-/-}\) blocked mice [14] on a C57Bl6/J background. In some experiments, 100 μg/kg Brilliant-Blue-FCF (BB-FCF; Sigma-Aldrich) [15], vehicle, 1 μg/kg antibody HRB454 or HRB460 were retroorbi tally administered in WT mice 7 min prior to the experiment. Whole blood cell counts were performed using a hematocytometer (Sysmex Digitana). Wire myography was performed on isolated mesenteric arteries using previously established protocols [16,17].

2.2. Immunofluorescent staining, quantitative PCR and platelet aggregation

Murine washed platelets were obtained as described previously [10]. In some experiments, preincubation with 1 mM BB-FCF or 100 μM PP2 (Tocris) was performed for 7 min before activation with 1 μg/mL collagen-I (Nycomed). Then, platelets or RAW264.7 macrophages (ATCC) were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100 for 15 min blocked with 2% bovine serum albumin (BSA) and incubated overnight with an anti-Panx1414 mini-antibody with the antigen-binding scFv fused to a human Fc receptor activation with associated elevated intracellular Ca\(^{2+}\) (f.e. P2Y receptors), and ionotropic, chemokine-, and glycoprotein receptor activation resulting in Src family kinase (SKF)-mediated phosphorylation (f.e. GPVI receptors) [2].

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2.5. Statistical analyses

Data were analyzed using GraphPad Prism6 software and shown as mean ± SEM. Comparisons were performed using t-test, Mann-Whitney U test or ANOVA followed by Bonferroni’s post-tests, where appropriate. P < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Ubiquitous Panx1 deletion decreases hemostasis in mice

Panx1 immunosignal was detected in platelets from WT mice and was absent in Panx1\(^{-/-}\) platelets (Fig. 1A). After vascular injury, local endothelium-derived factors induce a transient vasoconstriction followed by platelet adherence to the exposed extracellular matrix, which induces platelet activation, release of their secretory granules and recruitment of additional platelets to form a hemostatic plug. To study the role of Panx1 in primary hemostasis in vivo, we measured the time to bleeding cessation after transverse amputation of the tail tip. Bleeding time was 2.5-fold higher in Panx1\(^{-/-}\) mice to that of WT mice (Fig. 1B,C).

As endothelial Panx1 has been shown to modulate the severity of ischemic stroke by controlling cerebral inflammation and myogenic tone [21], we compared vasomotor responses in mesenteric arteries of WT and Panx1\(^{-/-}\) mice. We found impaired vasorelaxation in response to phenylephrine or to the thromboxane A2 analogue U446619 in Panx1\(^{-/-}\) mice (Fig. 1D,E). These results support earlier studies showing that Panx1 facilitates the contraction of murine resistance arteries by associating with the α1-adrenergic receptors in smooth muscle cells, whereas vasoconstriction in response to serotonin or endothelin-1 is independent of Panx1 [22,23]. Endothelium-dependent relaxation in response to acetylcholine as well as endothelium-independent relaxation in response to sodium nitroprusside were unaffected in Panx1\(^{-/-}\) mice (Fig. 1F,G).

In addition to platelets and endothelial cells (ECs), erythrocytes and leukocytes may participate in the hemostatic process. Therefore, we quantified the number of these cells in peripheral blood from WT and Panx1\(^{-/-}\) mice. Hematocrit, number of lymphocytes and platelets were not different between the genotypes (Fig. 1H–J).

Altogether, these results show that Panx1\(^{-/-}\) mice have increased bleeding time in vivo, however whether the effect is attributable to differences in initial vasoconstriction or in platelet function remains to be investigated.
3.2. Specific deletion of Panx1 from platelets delays hemostasis in vivo

PF4-Cre transgenic mice allow for the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo [24]. We generated Pf4Cre<sup>−/−</sup>Panx1<sup>−/−</sup> mice (hereafter called Panx1<sup>PDel</sup>) and confirmed the efficiency and specificity of the deletion by quantitative PCR on platelets and lymphocytes. While Panx1<sup>−/−</sup> platelets did not show any difference in Panx1 mRNA levels compared to that of WT platelets, Panx1 was absent from Panx1<sup>PDel</sup> and Panx1<sup>−/−</sup> platelets (Fig. 2A). Furthermore, comparable Panx1 mRNA levels were found in lymphocytes of Panx1<sup>PDel</sup>, Panx1<sup>−/−</sup> and WT mice while Panx1 expression was absent in lymphocytes of Panx1<sup>−/−</sup> mice (Fig. 2B). Immunofluorescent staining confirmed absence of Panx1 protein from Panx1<sup>PDel</sup> platelets and its presence in platelets of Panx1<sup>−/−</sup> control mice (Fig. 2C). Platelet-specific Panx1 deletion increased the time to cessation of tail bleeding by 2-fold (Fig. 2D), suggesting that the increased bleeding time in Panx1<sup>−/−</sup> mice is largely due to impaired platelet function, and that vasoconstriction plays only a minor role in the hemostatic response of Panx1<sup>−/−</sup> mice. Nevertheless, further studies are warranted to evaluate the possible effects of Panx1 deficiency on thrombosis in vivo.

3.3. Platelet-specific Panx1 deletion decreases thrombosis in mice

We have recently demonstrated that collagen binding to GPVI receptors induces Src-dependent phosphorylation and activation of Panx1 channels in vitro. This in turn leads to a Panx1-dependent ATP release and subsequent activation of P2X1 receptors promoting human platelet aggregation [8]. To study the effect of Panx1 deletion in a context where collagen-induced platelet aggregation is independent from ECs, we induced VTE by injecting a mix of collagen/epinephrine in the jugular vein of anesthetized mice and measured the time to respiratory arrest as compared to their respective controls (Fig. 2E,F). Suggesting delayed venous thrombosis and pulmonary embolism upon Panx1 deficiency. Next, we investigated the effects of platelet-specific Panx1 deletion in mice on clot formation in FeCl<sub>3</sub>-injured mesenteric arteries using intravital microscopy. Interestingly, both the time to initial thrombus formation (Fig. 2G) and the time to arterial occlusion (Fig. 2H) tend to increase in Panx1<sup>PDel</sup> mice compared to Panx1<sup>−/−</sup> controls. Altogether, our data support the idea that Panx1 might be a potential in vivo target to modulate platelet aggregation induced by collagen.
3.4. Blocking Panx1 channels delays hemostasis and thrombosis in mice

The lack of chemical compounds that selectively act on Panx1 channel gating hampers Panx1 research. Currently used pharmacological Panx1 blockers, such as carbenoxolone, mefloquine and probenecid, often also block connexin channels or act on purinergic receptors \[25,26\]. The best option seems to be Panx1, an EL1 mimetic peptide that impedes the passage of small molecules and ATP through the Panx1 channel \[27\]. However, such peptides display poor serum stability limiting in vivo applicability.

BB-FCF is a relatively new Panx1 channel blocking compound that inhibits Panx1 channels as shown by patch clamp and ATP release measurements \[28\]. Moreover, it has been used for the marking of saphenous vein grafts, in which it abrogates response to vascular injury \[29\]. Furthermore, Panx1 channel inhibition with BB-FCF reduces collagen-induced aggregation of human platelets without toxic side-effects \[18\]. Thus, we assessed the function of murine platelets by turbidimetry after 7 min preincubation with BB-FCF. A high dose of BB-FCF (1 mM) completely inhibited collagen-induced aggregation (Fig. 3A,B), whereas a lower dose (100 μM) was without effect, pointing to a narrow range of effectiveness of the compound. As inhibition of collagen-induced aggregation of human platelets involved phosphorylation of Panx1 by SFKs, we performed immunostaining on murine platelets using an antibody recognizing the Src kinase phosphorylation site on Panx1 (Panx1Y308) \[30\]. Panx1Y308 phosphorylation was increased after platelet activation with collagen as compared to non-activated platelets (Fig. 3C, upper left panels), illustrating that Panx1-induced inhibition of collagen-induced platelet aggregation involves a similar mechanism in human and mouse platelets, as expected. Although lysates of resting WT and Panx1-deficient platelets contained similar ATP concentrations (95.5 ± 12.3 nM vs. 106.6 ± 5.9 nM, respectively, \(N = 9\)), lymphokines of activated Panx1-deficient platelets contained 38% more ATP than lysates of activated WT platelets (65.9 ± 3.7 nM vs. 47.8 ± 5.2 nM, respectively; \(P = 0.01\)). This reduction in ATP release by Panx1-deficient platelets further underlines that mouse and human platelets use similar activation pathways involving Panx1 upon activation by collagen.

Preincubation of platelets with the Src-kinase inhibitor PP2 abolished Panx1 phosphorylation. Interestingly, preincubation with 1 mM BB-FCF did not inhibit collagen-induced Panx1 phosphorylation, suggesting that BB-FCF acts extracellularly on Panx1 channel permeation rather than by affecting phosphorylation (Fig. 3C, upper right panel).
panel). The specificity of the anti-Panx1_{PhosphoY308} antibody was assured by absence of staining in platelets from Panx1^{−/−} mice (Fig. 3C, lower panels). Next, we performed intravenous injection of BB-FCF in WT mice and tested tail bleeding and VTE 7 min later. Bleeding time and time to respiratory arrest were increased after treatment with BB-FCF (Fig. 3D,E), demonstrating the feasibility of altering the hemostatic balance in vivo by compounds acting on Panx1 channels. Importantly, BB-FCF is rapidly excreted from the body and does not seem to display serious side-effects either [25]. However, similar to other pharmacological Panx1 inhibitors, the Panx1 specificity of BB-FCF has recently been called into question by studies showing that high concentrations of atherothrombosis but their clinical benefit is limited by an increased risk for bleeding. P2X1^{−/−} mice exhibit inhibition of thrombosis with no prolongation of the tail bleeding time [7], indicating a dispensable role of P2X1 function in hemostasis, and the collagen receptor GPVI and P2X1 have therefore been proposed as potential targets for a safe new class of anti-platelet drugs [32,33]. By their role upstream of P2X1 and downstream of GPVI in this signaling cascade, inhibition of Panx1 channels diminishes clot formation only to the Panx1 peptide against which they were raised (Fig. 4A). Flow cytometry on non-permeabilized RAW264.7 macrophages revealed a strong immunofluorescence with α-Panx1EL but not with α-Panx1CT (Fig. 4B). Similarly, α-Panx1EL but not α-Panx1CT revealed Panx1 staining by fluorescence microscopy on non-permeabilized RAW264.7 cells (Fig. 4C). Furthermore, α-Panx1EL recognized Panx1 in platelets of WT mice, whereas Panx1^{−/−} platelets were without signal, thus attesting antibody specificity (Fig. 4D). α-Panx1EL reduced collagen-induced ATP release from human platelets by half compared to α-Panx1CT (Fig. 4E), illustrating the specific blocking properties of α-Panx1EL. Interestingly, tail bleeding time and VTE were delayed in mice injected with α-Panx1EL compared to those of mice injected with α-Panx1CT (Fig. 4F,G).

Currently, antiplatelet drugs are the cornerstone in the prevention of atherothrombosis but their clinical benefit is limited by an increased risk for bleeding. P2X1^{−/−} mice exhibit inhibition of thrombosis with no prolongation of the tail bleeding time [7], indicating a dispensable role of P2X1 function in hemostasis, and the collagen receptor GPVI and P2X1 have therefore been proposed as potential targets for a safe new class of anti-platelet drugs [32,33]. By their role upstream of P2X1 and downstream of GPVI in this signaling cascade, inhibition of Panx1 channels may be an attractive target to further fine-tune the regulation of this signaling pathway. Altogether, our data demonstrate that targeting Panx1 channels diminishes clot formation in vivo in collagen-dependent mouse models of arterial and venous thrombosis. It should
however be kept in mind that the hemostatic response was also affected in the Panx1-knockout mouse models or with a single high dose of the novel specific Panx1 channel blocker. Whether besides ATP-mediated P2X1 channel activation, other pathways are modified in Panx1-deficient platelets or whether the potential interference of different signaling cascades does not occur at lower concentrations of α-Panx1EL remains subject of future studies. Given the current lack of such drugs, research towards new targets that would optimally balance anti-thrombotic effects and hemorrhagic risk remains mandatory.

Author contributions
FM and BRK designed the research, analyzed and interpreted the data, and wrote the manuscript; FM, MJM, GP, AH, YE, SM performed experiments; BAI, PF, SM, ES provided scientiﬁc data, and wrote the manuscript; FM, MJM, GP, AH, YE, SM performed technical assistance and the Geneva Antibody Facility for their help would like to thank Bernard Foglia and Dr. Mahdia Benkhoucha for their help. This work was supported by grants from the Swiss National Science Foundation [310030_162579 and 310030_182573 to B.R. Kwak]. We would like to thank Bernard Foglia and Dr. Mahdia Benkhoucha for technical assistance and the Geneva Antibody Facility for their help with the production of the Panx1 blocking and control antibodies.

Declaration of competing interest
None to report for any of the authors.

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References

Fig. 4. Panx1-blocking antibody delays hemostasis and VTE in mice.
A. ELISA results demonstrating that HRB454 and HRB460 (α-Panx1CT, black) bind in a concentration-dependent manner only to the Panx1 peptide against which they were raised (peptide1 = WRQAAFVDSY; peptide2 = EKNSRQRLNPS). B. Flow cytometry on RAW264.7 macrophages with α-Panx1EL or α-Panx1CT (black) antibodies. Control curve (secondary antibody only) in grey. C. Immunofluorescent staining for Panx1 (in red) on RAW264.7 macrophages. Nuclei were stained with DAPI (in blue). Scale bar represents 20 μm. D. Immunofluorescent staining for Panx1 (in green) on platelets from WT or Panx1−/− mice. Platelets were counterstained with Evans Blue (in red). Negative control (Neg) included omission of first antibody. Scale bar represents 5 μm. E. Collagen-induced ATP release from human platelets (N = 5) after 7 min preincubation with α-Panx1EL or α-Panx1CT antibodies (white). F. Quantification of time to cessation of tail bleeding in WT mice treated for 7 min with α-Panx1EL or α-Panx1CT (white) antibodies (N = 9–10). G. Quantification of time to respiratory arrest in WT mice treated for 7 min with α-Panx1EL or α-Panx1CT antibodies (white) prior to VTE induction (N = 10). *P < 0.05, †P = 0.07. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)


