Dynamic adhesion of eryptotic erythrocytes to immobilized platelets via platelet phosphatidylserine receptors

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1Department of Physiology, University of Tübingen, Tübingen, Germany; 2Department of Cardiology and Cardiovascular Medicine, University of Tübingen, Tübingen, Germany; and 3Department of Gynecology and Obstetrics, University of Tübingen, Tübingen, Germany

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Walker B, Towhid ST, Schmid E, Hoffmann SM, Abed M, Münzer P, Vogel S, Neis F, Brucker S, Gawaz M, Borst O, Lang F. Dynamic adhesion of eryptotic erythrocytes to immobilized platelets via platelet phosphatidylserine receptors. Am J Physiol Cell Physiol 306: C291–C297, 2014. First published November 27, 2013; doi:10.1152/ajpcell.00318.2013.—Glucose depletion of erythrocytes triggers suicidal erythrocyte death or eryptosis, which leads to cell membrane scrambling with phosphatidylserine exposure at the cell surface. Eryptotic erythrocytes adhere to endothelial cells by a mechanism involving phosphatidylserine at the erythrocyte surface and CXCL16 as well as CD36 at the endothelial cell membrane. Nothing has hitherto been known about an interaction between eryptotic erythrocytes and platelets, the decisive cells in primary hemostasis and major players in thrombotic vascular occlusion. The present study thus explored whether and how glucose-depleted erythrocytes adhere to platelets. To this end, adhesion of phosphatidylserine-exposing erythrocytes to platelets under flow conditions was examined in a flow chamber model at arterial shear rates. Platelets were immobilized on collagen and further stimulated with adenosine diphosphate (ADP, 10 μM) or thrombin (0.1 U/ml). As a result, a 48-h glucose depletion triggered phosphatidylserine translocation to the erythrocyte surface and augmented the adhesion of erythrocytes to immobilized platelets, an effect significantly increased upon platelet stimulation. Adherence of erythrocytes to platelets was blunted by coating of erythrocytic phosphatidylserine with annexin V or by neutralization of platelet phosphatidylserine receptors CXCL16 and CD36 with respective antibodies. In conclusion, glucose-depleted erythrocytes adhere to platelets. The adhesive properties of platelets are augmented by platelet activation. Eryptosome adhesion to immobilized platelets requires phosphatidylserine at the erythrocyte surface and CXCL16 as well as CD36 expression on platelets. Thus, platelet-mediated erytho-rocyte adhesion may foster thromboocclusive complications in diseases with stimulated phosphatidylserine exposure of erythrocytes.

cGMP-dependent protein kinase (20), Janus-activated kinase JAK3 (6), casein kinase 1α (30, 57), and p38 kinase (23).

Phosphatidylserine exposure on erythrocytes is observed in a variety of diseases including diabetes mellitus, sepsis, hemolytic uremic syndrome, chronic renal failure, as well as several hereditary anemias (32) and leads to the thrombogenic activation of erythrocytes (14, 42, 47). Phosphatidylserine-exposing erythrocytes adhere to endothelial CXCL16/SR-PSOX (1, 9) and thus presumably interfere with blood flow, resulting in thrombotic vascular occlusion (3, 9, 15, 22, 43, 53).

At least in theory, eryptotic erythrocytes could similarly adhere to platelets, which are critically important for primary hemostasis (45) and by the same token contribute to acute thrombotic occlusion in myocardial infarction (10–12, 33) and ischemic stroke (7). As a matter of fact, in the ferrer chloride thrombosis model erythrocytes have been shown to recruit platelets to endothelial cells (5). Similar to endothelial cells, platelets express CXCL16 in an activation-dependent manner (10, 46). CXCL16 triggers platelet activation and thus translates vascular inflammation into thromboocclusive diseases (10). Platelets further express CD36 (4, 41), a receptor similarly suggested to bind phosphatidylserine (16, 40). Nevertheless, the exact mechanisms underlying phosphatidylserine-mediated adhesion of erythrocytes and especially the role of platelets in recruitment of eryptotic erythrocytes to the vascular wall have remained elusive.

The present study explored whether eryptotic erythrocytes adhere to activated platelets immobilized on collagen. Further experiments explored whether erythrocyte phosphatidylserine as well as platelet phosphatidylserine receptors CXCL16 and/or CD36 are involved in this process. Eryptosis was triggered by glucose depletion, and binding of erythrocytes to immobilized platelets under flow conditions was quantified in a flow chamber.

MATERIALS AND METHODS

Preparation of erythrocytes and induction of eryptosis. Eryptocytes were isolated from healthy male and female volunteers. Viability of erythrocytes may depend on the donor and the storage time, which was not longer than 1 h. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch. The study was approved by the ethics committee of the Eberhard Karls University of Tübingen, and the protocols were performed under a license obtained from this committee.

Eryptocytes were incubated in vitro at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 glucose, and 1 CaCl2, pH 7.4 at 37°C. Where indicated, extracellular...
glucose was removed for 48 h to induce eryptosis with phosphatidylserine exposure at the surface of eryptotic erythrocytes as described previously (9). Where indicated, annexin V (1:200 dilution; Roche) was added to coat and thereby functionally neutralize phosphatidylserine at the surface of eryptotic erythrocytes.

FACS analysis of annexin V-binding and forward scatter of eryptotic erythrocytes. To monitor induction of eryptosis, after incubation under the respective experimental conditions, a 50-μl cell suspension was washed in Ringer solution containing 5 mM CaCl₂ and then stained for 20 min with Annexin V-Fluos (1:500 dilution; Roche) under protection from light (50). Next, the forward scatter (FSC) of the cells was determined and annexin V fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCalibur (BD Biosciences).

Isolation of platelets. Human platelets were isolated as described previously (10). Blood from healthy volunteers was collected in ACD buffer and centrifuged at 200 g for 20 min. The obtained platelet-rich plasma was added to modified Tyrode-HEPES buffer [in mM: 137 NaCl, 2.8 KCl, 12 NaHCO₃, 5 glucose, 0.4 Na₂HPO₄, and 10 HEPES, with 0.1% bovine serum albumin (BSA), pH 6.5]. After centrifugation at 900 g for 10 min and removal of the supernatant, the resulting platelet pellet was resuspended in Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl₂).

Immobilization and preparation of platelets. Coverslips were degemdered with 70% alcohol, dried in the flame, and coated with 20 μg/ml Horm collagen (Nycomed) for 2 h at 37°C. The coated coverslips were washed once in PBS (pH 7.4) (GIBCO) and coated with 0.2% PBS-BSA for 1 h. The coverslips were washed twice in PBS and dried in laminar airflow for 30 min. A platelet suspension of 10⁸/ml PBS was applied to the coated coverslips and incubated at 37°C for 1 h to immobilize the platelets on the coated coverslips. Collagen itself induced a platelet shape change. If <10⁸ platelets/ml PBS were used to coat the collagen layer, a change in platelet morphology was visible. The concentration of 10⁸ platelets/ml PBS created an immobile platelet layer. At this concentration the platelets were satisfactorily immobilized under perfusion conditions. The coverslips with immobilized platelets were washed with PBS and observed under a microscope to ensure sufficient adherence for a perfusion experiment. Immobilized platelets were stimulated with 0.1 U/ml thrombin (Roche) or 10 μM adenosine diphosphate (ADP) (Sigma) for 15 min at 37°C, which changed the morphology of the platelets. Where indicated, platelet surface phosphatidylserine receptors were neutralized by incubation for 30 min at 37°C with anti-CXCL16 (4 μg/ml) (R&D Systems), anti-CD36 (R&D Systems), or, as control, isotype antibodies.

Dynamic erythrocyte adhesion to immobilized platelets in vitro. Platelets were attached on sterile coverslips coated with collagen (0.04% or 0.08%). Erythrocyte suspensions prepared as indicated were perfused on a human umbilical vein endothelial cell (HUVEC) monolayer in a flow chamber model (Oligene) at shear rates of 1,000 s⁻¹ with a Harvard PHD Ultra perfusor (Harvard). The interaction events between perfused erythrocytes and immobilized platelets were observed through an Axio observer A1 video microscope (Carl Zeiss) and recorded with a CCD camera (Carl Zeiss) at ×20 magnification, followed by analysis of the number of adherent erythrocytes per high-power field. For each perfusion experiment, one micrograph was taken at the beginning of the perfusion and the second was taken 10 min after erythrocytes had been perfused over the platelet layer and the number of adherent erythrocytes were enumerated.

Analysis of results and statistics. The numbers of erythrocytes adhering to platelets after 10 min of perfusion were counted and expressed as arithmetic means ± SD or SE, as indicated. Statistical analysis was made with t-test or paired ANOVA with Tukey’s test as posttest, as appropriate; n denotes the number of different erythrocyte specimens studied. The batches of erythrocytes differed moderately in their susceptibility to eryptosis. Thus the control values were not identical in all series of experiments. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch.

RESULTS

Energy depletion by glucose deprivation has previously been shown to trigger eryptosis, suicidal erythrocyte death (Table 1). A hallmark of eryptosis is cell membrane scrambling with phosphatidylserine exposure at the cell surface. The percentage of phosphatidylserine-exposing erythrocytes was estimated from annexin V-binding in erythrocytes incubated for 48 h in
either the presence or the absence of glucose. As illustrated in Fig. 1, glucose removal significantly increased the percentage of annexin V-binding erythrocytes.

Phosphatidylserine-exposing erythrocytes have been shown to adhere to endothelial cells of the vascular wall (1, 2, 9). To test whether eryptotic erythrocytes similarly adhere to blood platelets, platelets were immobilized on collagen in flow chambers with arterial flow rates. As illustrated in Fig. 2, eryptotic erythrocytes bound to platelets and glucose depletion significantly enhanced the number of adhering erythrocytes to collagen-immobilized platelets. In the presence of collagen only, erythrocyte adhesion was low and was not significantly modified by glucose depletion. Immobilized platelets appeared as a layer of small dark corpuscles in the background. Adherent erythrocytes were visible as larger white streaks because of dynamic conditions and resulting shear stress. Nonadherent erythrocytes moving at a speed of 1,000 s$^{-1}$ were virtually invisible in a micrograph taken under dynamic conditions. Scale bar, 100 μm.

Fig. 2. Adherence of glucose-depleted erythrocytes to immobilized platelets. A: arithmetic means ± SD (n = 10) of adherent erythrocyte binding to surfaces coated with collagen or platelets immobilized on collagen under flow conditions after exposure of the erythrocytes for 48 h to glucose-containing (gray bars) or glucose-depleted (black bars) solutions. Platelets were exposed to Tyrode buffer (untreated) or to Tyrode buffer containing platelet agonists ADP (10 μM) or thrombin (0.1 U/ml). *P < 0.05 and **P < 0.01, statistically significant difference between glucose-depleted erythrocytes; *P < 0.05, statistically significant difference between glucose-containing erythrocytes. B: time course of adherence of glucose-depleted erythrocytes to untreated platelets (left) in comparison to adherence of glucose-depleted erythrocytes to thrombin-stimulated platelets (right). The immobilized platelets appear as a layer of small dark corpuscles in the background. Adherent erythrocytes are visible as larger white streaks because of dynamic conditions and resulting shear stress of a perfusion experiment. Nonadherent erythrocytes moving at a speed <1,000 s$^{-1}$ appear as thin diffuse streaks. Erythrocytes flowing at 1,000 s$^{-1}$ are virtually invisible in a micrograph taken under dynamic conditions. Scale bar, 100 μm.
moving slowly. The slowly moving cells produced thin light streaks. Nonadhering erythrocytes moving at the fluid speed were virtually invisible.

The next series of experiments was performed to test whether adherence of erythrocytes to platelets under flow conditions was modified by platelet stimulation. To this end, platelets either were left unstimulated or were stimulated with ADP (10 μM) or thrombin (0.1 U/ml). As shown in Fig. 3, the number of both glucose-replete and glucose-deprived erythrocytes adhering to collagen-immobilized platelets was significantly upregulated by both ADP (10 μM) and thrombin (0.1 U/ml).

To explore whether binding of glucose-depleted erythrocytes to blood platelets involved phosphatidylserine at the erythrocyte surface, the phosphatidylserine at the erythrocyte surface was coated with annexin V. As illustrated in Fig. 4, the coating of phosphatidylserine at the erythrocyte surface with annexin V indeed significantly decreased the number of adhering erythrocytes. Annexin V decreased the binding to both thrombin-stimulated and unstimulated platelets.

A further series of experiments tested whether the binding of phosphatidylserine-exposing erythrocytes to platelets involved platelet phosphatidylserine receptors CXCL16 and CD36. As illustrated in Fig. 5, the number of adherent glucose-depleted erythrocytes binding to both unstimulated and thrombin-stimulated platelets was significantly decreased by neutralizing antibodies against either CXCL16 and CD36. As illustrated in Fig. 6, blocking CXCL16 and CD36 are additive. As illustrated in Fig. 6, blocking CXCL16 and CD36 with neutralizing antibodies was again followed by a significant decrease of the number of glucose-depleted erythrocytes adhering to platelets. Coating of phosphatidylserine at
the erythrocyte surface with annexin V significantly decreased the number of adhering erythrocytes in the absence of neutralizing antibodies against CXCL16 and CD36 but did not further decrease the number of glucose-depleted erythrocytes adhering to platelets in the presence of neutralizing antibodies against CXCL16 and CD36.

**DISCUSSION**

Increased adhesiveness of erythrocytes has been described in different diseases with markedly increased risk of thromboembolic complications (14, 42, 47, 52). It is well known that phosphatidylserine-exposing erythrocytes become more adhesive to the vascular wall and are prone to cause erythrocyte aggregation, suggesting the contribution of phosphatidylserine-exposing erythrocytes to development of thrombus formation (42). Platelet adhesion to the vascular wall followed by platelet activation is essential for primary hemostasis but is also critically important for acute arterial thrombotic occlusion at regions of atherosclerotic plaque rupture, the major pathophysiological mechanism underlying myocardial infarction and ischemic stroke (10, 11, 33). At sites of vascular injury, activated platelets interact with various cell types recruiting particularly inflammatory cells by direct cellular interactions via specific surface receptors (24, 39). Nevertheless, platelet-mediated recruitment of erythrocytes and underlying mechanisms have not been elucidated so far.

The present study reveals that glucose-depleted erythrocytes bind to platelets. The binding is augmented by platelet activation with either ADP or thrombin and apparently involves phosphatidylserine at the erythrocyte surface and CXCL16 as well as CD36 at the platelet surface. Thus phosphatidylserine-exposing erythrocytes not only interact with endothelial cells (9) but similarly interact with platelets. The interaction could contribute to the previously observed stimulation of blood clotting and thrombosis by phosphatidylserine-exposing erythrocytes (3, 14, 59). For their interaction with the endothelium, erythrocytes with enhanced phosphatidylserine exposure have been identified to establish specific interactions via several receptors including thrombospondin, αvβ3, and CD36 (47, 54).

Since coating of erythrocyte phosphatidylserine was not found to further decrease the interaction of erythrocytes and platelets with neutralized CXCL16 and CD36, we speculate that CXCL16 and CD36 are the critical platelet receptors for phosphatidylserine-driven interaction between eryptotic erythrocytes and platelets. Nevertheless, blocking phosphatidylserine with annexin V or neutralizing CXCL16 or CD36 by antibodies did not fully prevent binding of phosphatidylserine-exposing erythrocytes to platelets, an observation possibly pointing to the involvement of additional adhesion molecules. Further molecules implicated in the binding of phosphatidylserine include thrombospondin (51). Besides involving phosphatidylserine, erythrocyte adhesion may be accomplished by interaction of erythrocytic ICAM-4 with αvβ3 integrins (25) or erythrocyte Lutheran blood group/basal cell adhesion molecule (Lu/BCAM) with laminin α5 (52).

According to the present observations, any trigger of cell membrane scrambling in erythrocytes is expected to foster interaction of the affected erythrocytes with platelets. Eryptosis is stimulated by a wide variety of xenobiotics (2, 18, 26, 31, 32, 56, 58) and is observed in several clinical disorders (32), such as diabetes (13, 38), renal insufficiency (44), hemolytic uremic syndrome (34), sepsis (27), sickle cell disease (35), malaria (19), Wilson’s disease (36), iron deficiency (28), phosphate depletion (8), and presumably metabolic syndrome (55). The interaction of suicidal erythrocytes with platelets is expected to foster thromboembolic complications and the pathophysiology of the respective diseases.

In conclusion, glucose-depleted suicidal erythrocytes adhere to blood platelets under flow conditions. Interaction of the two blood cells critically involves phosphatidylserine at the erythrocyte surface and CXCL16 or CD36 at the platelet surface. The interaction of the two major blood cells is expected to foster thromboembolic disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: B.W., S.T.T., E.S., S.M.H., M.A., P.M., S.V., and F.N. performed experiments; B.W., O.B., and F.L. interpreted results of experiments; B.W. prepared figures; B.W., E.S., P.M., O.B., and F.L. edited and revised manuscript; B.W., S.T.T., E.S., S.M.H., M.A., P.M., S.V., F.N., S.B., M.P.G., O.B., and F.L. approved final version of manuscript; P.M. analyzed data; S.B., M.P.G., O.B., and F.L. conception and design of research; O.B. and F.L. drafted manuscript.

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