Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells

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AM J Physiol Cell Physiol 303: C991–C999, 2012. First published September 5, 2012; doi:10.1152/ajpcell.00239.2012. —Eryptosis, the suicidal erythrocyte death or eryptosis is characterized by cell shrinkage and cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface (29). Eryptosis may be triggered by activation of Cytoplasmic calcium (Ca2+) concentration (29). Increased cytosolic Ca2+ activates Ca2+-sensitive K+ channels (29) resulting in subsequent exit of KCl with osmotically obliged water and thus in cell shrinkage (29). Increased cytosolic Ca2+ further triggers phospholipid scrambling of the cell membrane (29). The sensitivity of erythrocytes to the scrambling effect of Ca2+ may be enhanced by ceramide (28; 30), which may be generated by sphingomyelinase (33).

Eryptosis contributes to the pathophysiology of several diseases (29), such as diabetes mellitus, renal insufficiency, iron deficiency, phosphate depletion, hemolytic uremic syndrome, sepsis, sickle cell disease, malaria, Wilson’s disease, and presumably metabolic syndrome (7, 16, 29, 32, 49, 59). Ceramide formation significantly contributes to the stimulation of eryptosis in several of these diseases including hemolytic uremic syndrome, sepsis, fever, and Wilson disease (16, 29). Moreover, ceramide-dependent and -independent eryptosis is triggered by a wide variety of small molecules (4–7, 14, 15, 18, 19, 27, 29, 41, 44, 45, 49).

Phosphatidylserine-exposing cells may activate coagulant enzymes (12) and adhere to the vascular wall (11), thus triggering thrombosis and thrombo-occlusive diseases (2, 12, 13, 17, 43, 55, 60). However, the mechanisms possibly underlying erythrocyte-endothelial cell interaction, especially in diseases with excessive eryptosis, are not well defined.

The adhesion of phosphatidylserine-exposing erythrocytes to endothelial cells involves the transmembrane CXC chemokine ligand 16 (CXCL16) (Ref. 11), a scavenger receptor protein (20, 48). CXCL16 is expressed at the surface of inflammatory cells (39). Binding to CXCL16 fosters internalization of the bound particles (20).

The present study explored whether exposure of erythrocytes to sphingomyelinase results in adherence to human umbilical vein endothelial cells (HUVEC) under flow conditions and, if so, whether the adherence is blunted by coating the phosphatidylserine at the surface of erythrocytes with annexin-V or by coating endothelial CXCL16 with neutralizing antibodies.

MATERIALS AND METHODS

Erythrocytes, solutions, and chemicals. Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. Viability of erythrocytes may depend on the donor and the storage time thus causing some interindividual variability. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparisons were always made within a given erythrocyte batch. The study was approved by the Ethics Committee of the Eberhard Karls University of Tübingen.

Erythrocytes were incubated in vitro at a hematocrit of 0.4% in Ringer solution containing the following (in mM): 125 NaCl, 5 KCl, 1 MgSO4, 32 HEPES, 5 glucose, and 1 CaCl2, pH 7.4 at 37°C. Where indicated, 0.01 U/ml bacterial sphingomyelinase (Sigma) was added, glucose removed (replaced by mannitol), cytosolic Ca2+ concentration increased by exposure to 1 μM Ca2+ ionophore ionomycin.
(Sigma, Taufkirchen, Germany). Ca\textsuperscript{2+} replaced by EGTA, annexin-V (1:200 dilution, Roche) added to coat and thereby functionally neutralize phosphatidylserine at the surface of eryptotic erythrocytes, or anti-CXCL16 antibody (4 \mu\text{g}/ml, R&D Systems) added 2 h before perfusion experiments to neutralize endothelial CXCL16.

Cell culture and silencing of HUVEC. HUVEC from early passage of culture were grown to confluency in complete endothelial cell basal medium (PAA) containing growth factors and 10\% FBS.

For silencing CXCL16, HUVEC were cultured on six-well plates (2.5 \times 10^5 cells). The cells were subsequently transfected with 10 nM validated small interfering (si)RNA for CXCL16 (ID no. s33807; Ambion) or with 10 nM negative control siRNA (ID no. 4390843; Ambion) using siPORT amine transfection agent (Ambion) according to the manufacturer’s protocol. The cells were used 48 h after transfection.

FACS analysis. For determination of annexin V-binding and forward scatter, the cells were incubated under the respective experimental conditions, and a 50-\mu l cell suspension was washed in Ringer solution containing 5 mM CaCl\textsubscript{2} and then stained for 20 min with annexin-V-Fluos (1:500 dilution; Roche) under protection from light (51). In the following, the forward scatter of the cells was determined and annexin-V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS-Calibur (BD Biosciences).

For determination of CXCL16 abundance at the surface utilizing anti-human CXCL16-phycocerythrin, HUVEC were incubated 6 h at 37\% CO\textsubscript{2}, 5\% CO\textsubscript{2}, with or without 0.01 U/ml sphingomyelinase. The cells were washed three times in isotonic phosphate buffer PBS (supplemented with 0.5\% BSA) by centrifugation at 1,600 g for 5 min to remove any residual growth factors that may be present in the culture medium. Cells were then resuspended in the same buffer at a final concentration of 4 \times 10^6 cells/ml. A volume of 25 \mu l of cells (1 \times 10^5) was transferred to 5 ml tube for staining. A volume of 10 \mu l of PE-conjugated anti-CXCL16 antibody (R&D Systems) was added and incubated for 45 min at 2–8\°C. Following incubation, unreacted anti-CXCL16 reagent was removed by washing the cells twice in 4 ml of same PBS buffer and resuspending in 200 \mu l of PBS buffer for final flow cytometric analysis.

Determination of ceramide formation. For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without 10 \mu M sphingomyelinase for 6 h, cells were stained for 1 h at 37\°C with 1 \mu g/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1\% BSA at a dilution of 1:5. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 min with polyclonal FITC-conjugated goat anti-mouse IgG- and IgM-specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis in FL-1.

Dynamic erythrocyte adhesion to endothelium in vitro. Cultured HUVEC (5 \times 10^5) were attached on sterile coverslips coated with

\hspace{1cm} Fig. 1. Effect of sphingomyelinase on erythrocyte forward scatter. A: original histogram of forward scatter of erythrocytes following exposure for 6 h to normal Ringer solution without (grey shadow) or with presence of bacterial sphingomyelinase at a concentration of 1 \mu M (broken black line), 5 \mu M (grey solid line), or 10 \mu M (solid black line). B: arithmetic means ± SE (n = 4) of the normalized erythrocyte forward scatter (FSC) following incubation for 6 h to Ringer solution without (white bars), or with 1, 5, and 10 \mu M sphingomyelinase (black bars). ***P < 0.001, statistically significant difference from absence of sphingomyelinase by ANOVA. C: original histogram of FSC of erythrocytes following exposure for 6 h to normal Ringer solution with 1 mM CaCl\textsubscript{2} (broken black line) or in Ringer with nominal absence (grey shadow) of Ca\textsuperscript{2+} or presence of bacterial sphingomyelinase (10 \mu M/ml) in absence (grey solid line) or presence (solid black line) of Ca\textsuperscript{2+}. D: arithmetic means ± SE (n = 4) of the normalized erythrocyte FSC following incubation for 6 h to Ringer solution without (left) or with (right) bacterial sphingomyelinase (10 \mu M/ml) in the nominal absence (white bars) or presence (black bars) of Ca\textsuperscript{2+}. *P < 0.05, ***P < 0.001, statistically significant difference from absence of sphingomyelinase; ###P < 0.01, statistically significant difference from presence of Ca\textsuperscript{2+} by ANOVA.
0.2% gelatine (Sigma-Aldrich) by overnight incubation in complete endothelial cell basal medium under cell culture conditions for 24 h. Erythrocytes prepared as indicated were perfused on a HUVEC monolayer in a flow chamber model (Harvard) at arterial shear rates (1,200 s⁻¹). The interaction events were recorded with a CCD camera (Carl Zeiss) with ×20 magnification, followed by analysis of the number adherent erythrocytes per high powerfield.

Western blot analysis. HUVEC were lysed with ice cold RIPA lysis buffer (Cell Signaling, Danvers, MA) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL). After centrifugation at 10,000 rpm for 5 min, 60 μg of proteins were boiled in nonreducing protein loading buffer (Thermo Fisher Scientific) at 100°C for 10 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with rabbit horseradish peroxidase-conjugated antibody (diluted 1:1,000; Cell Signaling) or secondary anti-rabbit horseradish peroxidase-conjugated antibody (diluted 1:1,000; Cell Signaling) and then with secondary anti-rabbit horseradish peroxidase-conjugated antibody (diluted 1:1,000; Cell Signaling) or secondary anti-rabbit horseradish peroxidase-conjugated antibody (diluted 1:1,000; Cell Signaling) for 1 h at room temperature. For loading controls, the membranes were stripped in stripping buffer (Thermo Fisher Scientific) and bands were quantified using Quantity One Software (Bio-Rad, München, Germany).

Quantitative real-time PCR. Gene expression and silencing efficiency of CXCL16 in HUVEC was verified by quantitative real-time PCR (RT-PCR). To this end, total RNA was isolated using the Trifast Reagent (Peglab). Reverse transcription of 2 μg RNA was performed using oligo(dT)₁₂-₁₈ primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). cDNA samples were treated with RNase H (Invitrogen). Quantitative RT-PCR was performed with the iCycler iQ RT-PCR Detection System (Bio-Rad) and iTag Sybr Green Supermix with ROX (Bio-Rad) according to the manufacturer’s instructions. The following primers were used (5’→3’ orientation): CXCL16 fwd: GACATGCTTACTCQGGGATTC; CXCL16 rev: CAGTGATCTCATTGAGGATT; GAPDH fwd: GAGTCAACGGATTTGGTCGT and GAPDH rev: GACAAGCTTCCCGTTCGCAG. The specificity of the PCR products was confirmed by analysis of the melting curves and by

![Image](http://ajpcell.physiology.org/)

**Fig. 2. Effect of sphingomyelinase on erythrocyte phosphatidylserine exposure.** A: original histogram of annexin-V binding erythrocytes exposed for 6 h to normal Ringer solution (grey shadow) or with 1 mM CaCl₂ (broken black line), 5 mM (solid grey line), or 10 mM (solid black line) bacterial sphingomyelinase. B: arithmetic means ± SE (n = 4) of the percentage of annexin-V binding erythrocytes exposed for 6 h to Ringer solution without (white bars) or with 1, 5, and 10 mM sphingomyelinase (black bars). ***p < 0.001, statistically significant difference from absence of sphingomyelinase. C: original histogram of annexin-V binding erythrocytes exposed for 6 h to normal Ringer solution with 1 mM CaCl₂ (broken black line) or in Ringer with nominal absence of Ca²⁺ (grey shadow) or with bacterial sphingomyelinase (10 μM/ml) in the nominal absence (solid grey line) or presence (solid black line) of Ca²⁺. D: arithmetic means ± SE (n = 4) of the percentage of annexin-V binding erythrocytes exposed for 6 h to Ringer solution without (left) or with bacterial sphingomyelinase 0.01 U/ml (right) in the nominal absence (white bars) or presence (black bars) of Ca²⁺. ***p < 0.001, statistically significant difference from absence of sphingomyelinase; ###p < 0.001, statistically significant difference from presence of Ca²⁺ by ANOVA.
Sphingomyelinase further stimulated cell membrane scrambling with phosphatidylserine exposure at the cell surface. As illustrated in Fig. 2, A and B, sphingomyelinase (≥1 mU/ml) treatment was followed by a significant increase of the percentage of annexin-V binding erythrocytes pointing to the appearance of erythrocytes exposing phosphatidylserine at their surface. The phosphatidylserine exposure was abrogated in the nominal absence of Ca^{2+}, and thus phosphatidylserine exposure was dependent on the presence of Ca^{2+} (Fig. 2, C and D).

**Effect of sphingomyelinase treatment on erythrocyte adhesion.** Phosphatidylserine-exposing erythrocytes might adhere to the vascular wall. To test this possibility, the effect of sphingomyelinase on adherence of erythrocytes to HUVEC was determined under in vitro flow conditions at arterial shear rates of 1,200−s⁻¹. As illustrated in Fig. 3A, treatment of erythrocytes with sphingomyelinase (10 mU/ml) was followed by marked increase of the percentage of erythrocytes adhering to HUVEC.

Additional experiments were performed to investigate whether enhanced vascular adhesion of sphingomyelinase-treated erythrocytes requires phosphatidylserine exposure at
the cell surface. To this end, phosphatidylserine at the erythrocyte surface was coated with annexin-V, which firmly binds to and thus masks phosphatidylserine. As illustrated in Fig. 3B, the increased adhesion of sphingomyelinase-treated erythrocytes to HUVEC under flow at shear rates of 1,200/s was significantly attenuated in the presence of annexin-V (1:200 dilution).

Since CXCL16 has been shown to bind phosphatidylserine and to be involved in cell adhesion processes (11, 20, 48), further experiments were performed to test whether the binding of sphingomyelinase-treated erythrocytes to endothelium under flow conditions involves CXCL16. To this end, HUVEC were exposed to an antibody directed against CXCL16 (4/μg/ml). As shown in Fig. 3C, the adhesion to endothelium of sphingomyelinase-treated erythrocytes was significantly decreased following exposure of HUVEC cells to CXCL16-blocking antibody compared with treatment with isotype control antibody of the same concentration.

To further test for a role of CXCL16 in adhesion of eryptotic erythrocytes to endothelial cells, endothelial CXCL16 expression was decreased by transfection of HUVEC with CXCL16 siRNA. As shown in Fig. 3D, CXCL16 silencing significantly decreased the adhesion of ionomycin-treated phosphatidylserine-exposing erythrocytes to endothelial cells at arterial shear rates compared with endothelium transfected with negative control siRNA of the same concentration.

Effect of sphingomyelinase treatment on endothelial CXCL16 expression and ceramide production. According to the experiments presented thus far, spingomyelinase exposure fosters erythrocyte adhesion to endothelial cells by triggering eryptosis. At least in theory, sphingomyelinase could be additionally effective by modifying CXCL16 expression in endothelial cells. To explore that possibility, HUVEC were incubated for 6 h without or with sphingomyelinase (1, 5, and 10 mU/ml) and CXCL16 protein abundance was determined by both Western blotting and FACS analysis. As illustrated in Fig. 4, A–C, the exposure of HUVEC to sphingomyelinase was followed by a significant increase of CXCL16 protein abundance. Figure 4D illustrates the formation of ceramide in response to sphingomyelinase (10 mU/ml) in HUVEC after 6 h of stimulation.

To check the transcript levels of CXCL-16 in HUVEC cells, RT-PCR was performed on mRNA of HUVEC without treatment or following treatment with 100 mU/ml sphingomyelinase. The CXCL-16 mRNA abundance was not significantly different (P = 0.63) between untreated (1.01 ± 0.05) and sphingomyelinase-treated (0.95 ± 0.11) HUVEC.

**Fig. 4.** Effect of sphingomyelinase on CXCL16 protein expression. A: arithmetic means ± SE (n = 4) of Western blot densitometric analysis of CXCL16/GAPDH ratio showing protein abundance in HUVEC incubated for 6 h without (control, white bar) and with (black bar) 10 mU/ml sphingomyelinase. *P < 0.05, statistically significant difference from absence of sphingomyelinase. B: original Western blot showing CXCL16 protein abundance in HUVEC. C: arithmetic means ± SE (n = 4) of CXCL16 positive HUVEC in the absence (white bar) and presence (black bars) of 1, 5, and 10 mU/ml sphingomyelinase. *P < 0.05, **P < 0.001, ***P < 0.001, statistically significant difference from absence of sphingomyelinase. D: arithmetic means ± SE (n = 4) of ceramide abundance in HUVEC exposed for 6 h to Ringer solution without (white bar) or with (black bars) 10 mU/ml sphingomyelinase. **P < 0.01, statistically significant difference from absence of sphingomyelinase.
Erythrocyte adhesion following sphingomyelinase treatment of HUVEC. Additional experiments were performed to elucidate whether the enhanced expression of CXCL16 in sphingomyelinase-treated endothelial cells influenced the adhesion of phosphatidylserine exposing erythrocytes. To this end, eryptosis was induced by a 30-min treatment of erythrocytes with the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) and endothelial adhesion was tested utilizing HUVEC, which were either left untreated or were treated for 6 h with sphingomyelinase (10 nU/ml). As illustrated in Fig. 5A, the treatment with ionomycin was followed by a marked increase of erythrocyte adhesion to HUVEC. The additional treatment of HUVEC with sphingomyelinase was followed by a further significant increase of erythrocyte adhesion to the endothelial cells.

In an additional series of experiments, eryptosis was induced by glucose deprivation of erythrocytes for 48 h. Again, endothelial adhesion was tested utilizing HUVEC, which were either left untreated or were treated for 6 h with sphingomyelinase (10 nU/ml). Glucose deprivation was followed by a marked increase of erythrocyte adhesion to HUVEC. The additional treatment of HUVEC with sphingomyelinase was again followed by a further significant increase of erythrocyte adhesion to the endothelial cells (Fig. 5B).

Effect of C6-ceramide treatment on adhesion of erythrocytes to HUVEC cells. In a final series of experiments, the effect of C6-ceramide on eryptosis and erythrocyte adhesion to HUVEC was explored. As illustrated in Fig. 6A, a 6-h treatment with C6-ceramide (50 \(\mu\)M) was followed by a significant increase of the percentage of annexin-V binding erythrocytes pointing to the appearance of erythrocytes exposing phosphatidylserine at their surface. The phosphatidylserine exposure was significantly reduced in the nominal absence of Ca\(^{2+}\). C6-ceramide further fostered the adhesion of erythrocytes to HUVEC under in vitro flow conditions at arterial shear rates of 1,200 s\(^{-1}\). As illustrated in Fig. 6B, treatment of erythrocytes with C6-ceramide (50 \(\mu\)M) was followed by marked increase of the percentage of erythrocytes adhering to HUVEC.

Additional experiments were performed to elucidate whether the enhanced expression of CXCL16 in C6-ceramide-treated endothelial cells influenced the adhesion of phosphatidylserine exposing erythrocytes. To this end, eryptosis was induced by a 30-min treatment of erythrocytes with the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) and endothelial adhesion was tested utilizing HUVEC that were either left untreated or were treated for 6 h with C6-ceramide (50 \(\mu\)M). As illustrated in Fig. 6C, the treatment with ionomycin was followed by a marked increase of erythrocyte adhesion to HUVEC.

In an additional series of experiments, eryptosis was induced by glucose deprivation of erythrocytes for 48 h. Again, endothelial adhesion was tested utilizing HUVEC that were either left untreated or were treated for 6 h with C6-ceramide (50 \(\mu\)M). Glucose deprivation was followed by a marked increase of erythrocyte adhesion to HUVEC (Fig. 6D).

DISCUSSION

The present observations confirm previous studies (29) demonstrating that exposure of erythrocytes to sphingomyelinase leads to cell shrinkage and cell membrane scrambling, both hallmarks of suicidal erythrocyte death or eryptosis. The present observations further reveal that exposure to sphingomyelina-

![Fig. 5. Effect of sphingomyelinase on HUVEC (CXCL16) in dynamic adhesion of erythrocytes to endothelial cells under arterial shear stress. A: arithmetic means ± SE (n = 4) of erythrocytes binding to HUVEC under flow following exposure to Ringer solution without (white bars) or with (black bars) 1 \(\mu\)M ionomycin for 30 min with (left) or with (right) treatment of HUVEC with 10 nU/ml sphingomyelinase for 6 h. ***P < 0.001, statistically significant difference from absence of sphingomyelinase; ###P < 0.001, statistically significant difference from absence of ionomycin. B: arithmetic means ± SE (n = 4) of erythrocytes binding to HUVEC under flow following exposure to normal Ringer solution (white bar) or glucose deficient Ringer (black bar) without (left) or with (right) treatment of HUVEC with 10 nU/ml sphingomyelinase for 6 h. ***P < 0.001, statistically significant difference from absence of sphingomyelinase; ###P < 0.001, statistically significant difference from absence of glucose.](http://ajpcell.physiology.org/)

nase is followed by phosphatidylserine- and SR-POX/CXCL16-dependent binding of affected erythrocytes to endothelial cells.

The effect of sphingomyelinase on both eryptosis and adhesion to endothelial cells is dependent on the presence of Ca\(^{2+}\). As shown earlier (29), ceramide sensitizes the erythrocytes to the effects of Ca\(^{2+}\). An increase of cytosolic Ca\(^{2+}\) activity stimulates erythrocyte cell membrane scrambling leading to phosphatidylserine exposure at the cell surface (29). An increase of cytosolic Ca\(^{2+}\) activity further activates Ca\(^{2+}\)-sensitive K\(^+\) channels (29) with subsequent exit of K\(^+\), hyperpo-
lization of the cell membrane, exit of Cl⁻, and thus cellular loss of KCl together with osmotically obliged water, effects eventually leading to cell shrinkage (29). Sphingomyelinase is primarily effective by increasing Ca²⁺ sensitivity of the erythrocyte (29).

Eryptosis is followed by adhesion of the eryptotic erythrocytes to endothelial cells. Erythrocyte membrane proteins are known to participate in the pathophysiology of thrombosis, which may involve increased adhesion of erythrocytes to endothelial cells (2). The adhesion is expected to interfere with microcirculation, which presumably contributes to cardiovascular complications in several diseases such as diabetes or chronic renal failure (10, 37, 42).

As reported earlier (11), eryptotic erythrocytes bind at least partially to CXCL16 or SR-PSOX, a scavenger receptor binding phosphatidylserine and oxidized low-density lipoprotein (24). CXCL16 is produced by several cell types including dendritic cells (21), lymphocytes (56), vascular smooth muscle cells (52), bone marrow stromal cells (40), tumor cells (35, 36), and cardiac tissue (58). SR-PSOX/CXCL16 expression in endothelial cells is upregulated by proinflammatory cytokines, such as TNF-α or IFN-γ (1, 25), known mediators of inflammation and ischemia (46, 50). TNFα stimulates CXCL16 production via a signaling cascade involving NF-κB, p38MAPK, and protein kinase A (22). We show here that CXCL16 expression is further upregulated by exposure of endothelial cells to sphingomyelinase.

Endothelial CXCL16 mediates not only adhesion of erythrocytes but also adhesion of leukocytes (25, 47). CXCL16 promotes the adhesion of monocytes to the endothelium during atherogenesis (24). CXCL16 is further expressed in macrophages of atherosclerotic plaques (39) and presumably contributes to thrombotic vascular complications. Further studies will be needed to clarify whether the expression of CXCL16 in leukocytes is similarly regulated by sphingomyelinase.

**Fig. 6. Effect of C6-ceramide on erythrocyte phosphatidylserine exposure and adhesion of erythrocytes to endothelial cells under flow conditions.** A: arithmetic means ± SE (n = 6) of the percentage of annexin-V binding erythrocytes following exposure for 6 h to Ringer solution without (left) or with (right) C6-ceramide (50 μM) in the nominal absence (white bars) or presence (black bars) of Ca²⁺. ***P < 0.001, statistically significant difference from absence of sphingomyelinase; #P < 0.05, statistically significant difference from presence of Ca²⁺ by ANOVA. B: arithmetic means ± SE (n = 6) of erythrocytes binding to HUVEC under flow conditions following exposure for 6 h to Ringer solution without (left) or with (right) C6-ceramide in the nominal absence (white bars) or presence (black bars) of Ca²⁺. ***P < 0.001, statistically significant difference from absence of sphingomyelinase; ###P < 0.01, statistically significant difference from presence of Ca²⁺. C: arithmetic means ± SE (n = 6) of erythrocytes binding to HUVEC under flow following exposure to Ringer solution without (white bars), or with (black bars) 1 μM ionomycin for 30 min without (left) or with (right) prior treatment of HUVEC with C6-ceramide (50 μM) for 6 h. ***P < 0.001, statistically significant difference from absence of sphingomyelinase; ###P < 0.01, statistically significant difference from absence of ionomycin. D: arithmetic means ± SE (n = 6) of erythrocytes binding to HUVEC under flow following exposure to normal Ringer solution (white bar) or glucose-deficient Ringer (black bar) without (left) or with (right) prior treatment of HUVEC with C6-ceramide (50 μM) for 6 h. ***P < 0.001, statistically significant difference from absence of glucose.
Adhesion of eryptotic erythrocytes to phagocytosing cells fosters phagocytosis and thus clearance from circulating blood (29). The phagocytosis could be suppressed by coating of eryptotic erythrocytes with annexin-V (32).

The HUVEC utilized in the present study express several adhesion molecules including integrins αv (e.g., αvβ3, or αvβ5) and β1, E- and P-selectins, laminin α5, vWF, and the adhesion molecules ICAM-1, PECAM-1, or VCAM-1 (3, 8) and are thus widely used as model cells for adhesion to the vascular wall (3, 8, 9, 54). Similar to what was shown in earlier studies (57) blocking phosphatidylserines or neutralizing CXCL16 by antibodies decreased but did not fully abrogate adhesion. Thus, binding of cryptic erythrocytes to the vascular wall may involve further mechanisms. Phosphatidylserines could interact with subendothelial thrombospondin (53) or endothelial CD36 (38), and erythrocytes may express further molecules participating in erythrocyte adhesion to HUVEC, such as ICAM-4 adhering to endothelial αvβ3 integrins (23) or the erythrocyte Lutheran blood group/basal cell adhesion molecule (Lu/BCAM) binding to endothelial cell laminin α5 (54).

The present observations strongly suggest that acid sphingomyelinase stimulates eryptosis with cell membrane scrambling leading to adhesion of externalized phosphatidylserine to endothelial CXCL16. It should be pointed out, however, that those observations do not rule out further mechanisms involved in the stimulation of erythrocyte adhesion by acid sphingomyelinase operative in addition to phosphatidylserine and CXCL16. Clearly, additional experimentation is required to fully unravel the complex machinery underlying erythrocyte adhesion to the vascular wall.

In conclusion, the present observations show that treatment of erythrocytes with sphingomyelinase is followed by triggering of eryptosis with partially phosphatidylserine- and CXCL16-dependent binding of the cryptic erythrocytes to the vascular wall. Sphingomyelinase-dependent eryptosis with subsequent adhesion of erythrocytes to the vascular wall may contribute to the pathophysiology of several diseases involving ceramide formation including hemolytic uremic syndrome (31), sepsis (26), fever (16), and Wilson disease (34).

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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