Lipopeptides in the Triggering of Erythrocyte Cell Membrane Scrambling

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Key Words

Apoptosis • Eryptosis • Calcium • Pam3Cys • Pam3CSK4

by bacterial cell wall components. Thus, lipoprotein-dependent suicidal erythrocyte death may contribute to the pleiotropic effects of sepsis.

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Abstract

Sepsis is paralleled by anemia, an effect partially resulting from eryptosis, the suicidal death of erythrocytes. Eryptosis is characterized by cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface. Pathogen-induced eryptosis may partially result from interaction of bacterial cell wall components such as lipoproteins with the erythrocyte cell membrane. The present study explored, whether the synthetic lipopeptide Pam3CSK4 mimicking the acylated amino terminus of bacterial lipoproteins triggers eryptosis. According to annexin-V-binding in FACS analysis, Pam3CSK4 (1 µg/ml) stimulated phosphatidylserine exposure, an effect significantly blunted in the nominal absence of Ca²⁺. According to Fluo3 fluorescence, Pam3CSK4 increased cytosolic Ca²⁺ activity and moderately stimulated erythrocytic ceramide formation, both major triggers of eryptosis. In conclusion, bacterial lipoproteins participate in the stimulation of erythrocyte cell membrane scrambling

Introduction

Activation of toll-like receptors may lead to pleiotropic effects participating in a variety of disorders including sepsis [1], atherosclerosis [2], insulin resistance and obesity-related metabolic dysfunction [3, 4]. Sequelae of overwhelming infection or sepsis include stimulation of eryptosis, the suicidal erythrocyte death [5]. Eryptosis is characterized by scrambling of the cell membrane with phosphatidylserine exposure at the cell surface [6]. Eryptosis may be stimulated by bacterial or host sphingomyelinase [5, 7] and/or by activation of Ca²⁺-permeable erythrocyte cation channels with subsequent Ca²⁺ entry [8-10]. Further bacterial components described to stimulate eryptosis include hemolysin from *Vibrio parahaemolyticus* [11], listeriolysin [12] and peptidoglycans [13]. Phosphatidylserine exposure in erythrocytes could further be elicited by ligation of specific surface antigens, such as glycophorin-C [14], of CD47,

the critical RBC antigenic marker that exhibits an inhibitory role in macrophage activation [15], and of the death receptor CD95/Fas [16]. Moreover, eryptosis is triggered by paclitaxel [17], amantadine [18], azathioprine [19], retinoic acid [20], chlorpromazine [21], cyclosporine [22], methylglyoxal [23], amyloid peptides [24], anandamide [25], Bay-Y5884 [26] and curcumin [27]. Mechanisms underlying eryptosis may not only affect circulating erythrocytes but may contribute to the limitation of erythrocytes during storage [28-30].

The present study explored, whether exposure to the synthetic lipopeptide Pam3CSK4 [31] stimulates eryptosis and, if so, to elucidate the underlying signaling.

Materials and Methods

Erythrocytes, solutions, and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V). For this study, 11 blood samples were studied.

Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂; pH 7.4. In Ca²⁺-free Ringer, 1 mM CaCl₂ was substituted for 1 mM ethylene glycol tetraacetic acid (EGTA). The Ca²⁺ ionophore ionomycin (Sigma, Schnellendorf, Germany; dissolved in dimethyl sulfoxide [DMSO]) was used at a concentration of 1 µM. The final concentration of the solvent DMSO was 0.1%.

The synthetic tripalmitoylated lipopeptide Pam3CSK4 (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 HCl) that mimics the acylated amino terminus of bacterial lipoproteins was purchased from Invivogen (San Diego, USA) and has an endotoxin level of <0.125 EU/mg according to the manufacturer. In some experiments Pam3Cys from EMC Microcollections GmbH (Sindelfinger Str.3, 72070 Tübingen) was used yielding similar results. Pam3CSK4 and Pam3Cys are the same compounds sold under different names by different manufacturers.

FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described [32]. After incubation, cells were washed in Ringer solution containing 5 mM CaCl₂. Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ measurements were performed as described previously [33]. Briefly, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM CaCl₂ and 2 µM Fluo-3-AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured in fluorescence channel FL-1.

Determination of ceramide formation

To determine formation of ceramide, which is exposed at the cell surface, a monoclonal antibody-based assay [34, 35] was used in FACS analysis. After incubation, cells were stained for 1 hour at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (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. Samples were then analysed by flow cytometric analysis on a FACS-Calibur in FL-1.

Statistics

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by unpaired t-test or ANOVA using Tukey's test as post hoc test, as appropriate.

Results

Annexin V-binding was determined to identify phosphatidylserine (PS)-exposing erythrocytes. The percentage of erythrocytes exposing annexin V at their surface was low after incubation in plain Ringer solution (Fig. 1B). A 48 hours treatment with Pam3CSK4 (5 µg/ml) at 37°C significantly increased annexin V-binding (Fig. 1B). At 1 µg/ml Pam3CSK4, annexin V binding similarly tended to be increased, but in the series displayed in 1B, the effect escaped statistical significance due to large scatter of the data. To further explore whether Pam3CSK4 could be effective at this low concentration, an additional series was performed analysing the effect of 1 µg/ml at different exposure times. As shown in Fig. 1 C, Pam3CSK4 increased PS exposure of erythrocytes significantly in this series.

Forward scatter in FACS analysis was employed to detect alterations of cell volume. As illustrated in Fig. 2A, B, a 48 hours treatment with Pam3CSK4 (5 µg/ml), was followed by an increase in cell volume. At least in

Fig. 1. Stimulation of phosphatidylserine exposure of erythrocytes by Pam3CSK4. A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (-, black line) or to 5 $\mu\text{g/ml}$ Pam3CSK4 (+, red line). B. Effect of Pam3CSK4 (1, 5, 10 $\mu\text{g/ml}$) on phosphatidylserine exposure. Arithmetic means $\pm$ SEM (n = 4-20) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars).

Fig. 2. Effects of Pam3CSK4 on erythrocyte forward scatter. A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (-, black line) or to 5 $\mu\text{g/ml}$ Pam3CSK4 (+, red line). B. Effect of Pam3CSK4 (1, 5, 10 $\mu\text{g/ml}$) on forward scatter. Arithmetic means $\pm$ SEM (n = 4-16) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars). *, ** ($P < 0.05$, $P < 0.01$) indicate significant difference from values in control Ringer solution (ANOVA). C. Arithmetic means $\pm$ SEM (n = 6) of forward scatter of erythrocytes exposed for 120 min to plain Ringer solution or to 5 $\mu\text{g/ml}$ Pam3CSK4 and further 30 min in the absence (left bars) or presence (right bars) of 1 μM ionomycin. *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA). ### ($P < 0.001$) indicates significant difference between absence and presence of Pam3CSK4.

Fig. 3. Effects of Pam3CSK4 on cytosolic Ca^{2+} activity. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (-, black line) or to 5 $\mu\text{g/ml}$ Pam3CSK4 (+, red line). B. Dose dependence of the effect of Pam3CSK4 on Fluo3 fluorescence. Arithmetic means $\pm$ SEM (n = 4-13) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars) at the indicated concentrations. **, *** ($P < 0.01$, $P < 0.001$) indicate significant difference from values in control Ringer solution (ANOVA).

theory, Pam3CSK4 might inhibit the erythrocyte Ca^{2+} -dependent K^+ (Gardos) channels which, upon increase in intracellular Ca^{2+} , leads to an efflux of K^+ , Cl^- and osmotically obliged water resulting in cell shrinkage. To check this possibility, erythrocytes were exposed to the Ca^{2+} ionophore ionomycin, which within 30 min led to the expected decrease of cell volume (Fig. 2C). Addition of Pam3CSK4 significantly blunted ionomycin-induced cell shrinkage indeed pointing to an inhibition of the Gardos channels (Fig. 2C).

Additional experiments were performed to elucidate the underlying mechanisms for the observed phosphatidylserine scrambling following exposure to Pam3CSK4. Fluo3 fluorescence was employed to determine the cytosolic Ca^{2+} activity. As illustrated in Fig. 3, a 48 hours exposure to Pam3CSK4 (5 $\mu\text{g/ml}$) significantly increased Fluo3 fluorescence.

To determine, whether the increase in cytosolic Ca^{2+} indeed contributes to or even accounts for the increase in phospholipid scrambling, experiments were performed

Fig. 4. Role of Ca^{2+} in the stimulation of phosphatidylserine exposure by Pam3CSK4. A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to 1 $\mu\text{g}/\text{ml}$ Pam3CSK4 in the absence (4.1% annexin V-binding cells, -, red line) and presence (6.9% annexin V-binding cells, +, black line) of 1 mM Ca^{2+} . B. Arithmetic means $\pm$ SEM ($n = 8$) of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bars) or to Pam3CSK4 (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA). ### ($P < 0.001$) indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

in the presence and absence of extracellular Ca^{2+} . As shown in Fig. 4, 1 $\mu\text{g}/\text{ml}$ led to a statistically significant increase in annexin V binding. Removal of extracellular Ca^{2+} indeed significantly blunted the stimulation of annexin V-binding following a 48 hours exposure to Pam3CSK4.

Similar to Pam3CSK4, Pam3Cys stimulated annexin V-binding, increased forward scatter, and enhanced Fluo3 fluorescence (data not shown).

The second major stimulus of eryptosis is ceramide. Therefore, further experiments were performed to investigate whether Pam3CSK4 also affects ceramide formation. As illustrated in Fig. 5A, B, 1 $\mu\text{g}/\text{ml}$ Pam3CSK4 within 48 hours significantly stimulated the generation of erythrocytic ceramide.

Discussion

The present study reveals that exposure of erythrocytes to Pam3CSK4, a known stimulator of Toll-like receptors [36, 37], leads to scrambling of the cell membrane with subsequent phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages equipped with phosphatidylserine receptors [38] and thus eliminated from circulating blood [39]. Thus, eryptosis results in disposal of affected erythrocytes [6]. Moreover, phosphatidylserine-exposing erythrocytes adhere to the vascular wall and could thus compromise microcirculation [40]. As a matter of fact, ischemia leads to enhanced trapping of annexin V-binding erythrocytes in the renal medulla [11]. It is noteworthy that Toll-like receptor triggering could contribute to atherosclerosis [2].

As shown earlier [41], an increase in cytosolic Ca^{2+} activity further activates Ca^{2+} -sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, and exit of Cl^- together with osmotically obliged water, thus eventually

Fig. 5. Effect of Pam3CSK4 on ceramide formation. A. Histogram of ceramide abundance in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (-, black line) or with (+, red line) 1 $\mu\text{g}/\text{ml}$ Pam3CSK4. B. Arithmetic means $\pm$ SEM ($n = 6-8$) of ceramide abundance in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) 1 $\mu\text{g}/\text{ml}$ Pam3CSK4. ** ($P < 0.01$) indicates significant difference from values in control Ringer solution (t-test).

leading to cell shrinkage [41]. The cell shrinkage then contributes to the triggering of cell membrane scrambling [42]. However, surprisingly, Pam3CSK4 had no significant effect on the forward scatter. Possibly, Pam3CSK4 inhibited the Ca^{2+} -sensitive K^+ channel. Accordingly, ionomycin-induced cell shrinkage was indeed blunted in the presence of Pam3CSK4. The observations suggest that Pam3CSK4 increases Ca^{2+} entry but at the same time abrogates the stimulating effect of Ca^{2+} on the Ca^{2+} sensitive K^+ channels. In dendritic cells Toll-like receptor activation has been shown to rather upregulate K^+ channels [43].

Similar to the effect of peptidoglycans [13], the effect of Pam3CSK4 is blunted by removal of extracellular Ca^{2+} . In polymorphonuclear leukocytes, peptidoglycans have been shown to trigger the arachidonic acid cascade with subsequent formation of prostaglandin E_2 [44].

In erythrocytes PGE₂ has been shown to stimulate Ca²⁺ entry by activation of the Ca²⁺-permeable cation channels [33]. Activation of toll-like receptors may similarly stimulate the formation of PGE₂ [45-47]. Hitherto an effect of Pam3CSK4 on prostaglandin formation has, however, not been shown.

Further mechanisms may be involved in the stimulation of Ca²⁺ entry and cell membrane scrambling following exposure to Pam3CSK4. It is noteworthy that cytosolic Ca²⁺ activity may be increased by bacterial hemolysin pneumolysin, an effect more pronounced in cells expressing a Cu/Zn SOD1 mutant and blunted by the anti-oxidant N-acetylcysteine [48]. Oxidative stress is known to stimulate the erythrocyte cation channels [49].

The present study does not address the functional consequences of Pam3CSK4 induced eryptosis for circulation in sepsis. Sepsis is known to cause severe hypotension, an effect at least partially due to nitric oxide (NO) formation [1]. Accordingly, animals do not become hypotensive after an LPS exposure if they are deficient

in inducible NO synthase (iNOS), an enzyme critical for the excessive production of NO [1]. On the other hand, Toll-like receptor activation has been shown to suppress the formation of nitric oxide [50], which in turn inhibits eryptosis [51] at least partially through G-kinase [52].

In conclusion, the Toll-like receptor agonist Pam3CSK4 stimulates Ca²⁺ entry and leads to moderate formation of ceramide in erythrocytes, resulting in cell membrane scrambling and subsequent exposure of phosphatidylserine at the cell surface.

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