Triggering of Suicidal Erythrocyte Death by Amphotericin B

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Key Words

Phosphatidylserine • Scrambling • Calcium • Cell volume • Eryptosis • Apoptosis

to the clearance of circulating erythrocytes and thus to anemia.

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Abstract

Amphotericin B is widely used as antifungal drug. Side effects include anemia. A variety of drugs and diseases associated with anemia has recently been shown to trigger suicidal erythrocyte death or eryptosis, i.e. cell membrane scrambling and cell shrinkage. Eryptosis may be triggered by increased cytosolic Ca^{2+} activity and by lack of ATP. The present study explored whether amphotericin B stimulates eryptosis. Cell membrane scrambling was estimated from annexin V-binding to phosphatidylserine exposed at the cell surface, cell shrinkage from forward scatter in FACS analysis, cytosolic Ca^{2+} activity from Fluo3 fluorescence and the cytosolic ATP concentration from a luciferase-based assay. Exposure to amphotericin B (0.1 - 1 $\mu\text{g/ml}$) within 48 hours significantly increased annexin V-binding, decreased forward scatter, increased cytosolic Ca^{2+} activity and decreased cytosolic ATP content. In conclusion, amphotericin B stimulates suicidal cell death of erythrocytes, which may in turn contribute

Introduction

Amphotericin B is widely used against infection with fungi or parasites [1-9]. Side effects of amphotericin B may include anemia [10]. Amphotericin B is known to generate cation channels in the plasma membrane [11]. The channels mediate entry of Na^+ and may, at least in nucleated cells, eventually increase intracellular Ca^{2+} concentration [12]. Amphotericin B has further been shown to stimulate a signaling cascade involving TLR-2, Btk, PLC, PKC, c-Src and NF-kappaB [13]. The amphotericin B-induced increase in the cytosolic Na^+ concentration activity may be further followed by enhanced insertion of the Na^+/K^+ ATPase into the cell membrane [14]. In erythrocytes, Amphotericin B has previously been reported to trigger lysis, which could contribute or even account for the amphotericin B-induced anemia [15]. On the other hand, in several clinical conditions leading to hemolytic anemia the accelerated loss of erythrocytes has been shown to be the result of

enhanced suicidal erythrocyte death or eryptosis rather than hemolysis [16].

Eryptosis may be triggered by Ca^{2+} entry into erythrocytes through Ca^{2+} -permeable cation channels [17-24]. The activation of those channels is a major mechanism stimulating eryptosis [16]. Specifically, enhanced cytosolic Ca^{2+} concentration activates Ca^{2+} -sensitive K^+ channels [25, 26], which in turn results in exit of KCl with osmotically obliged water and thus in cell shrinkage [27]. Moreover, Ca^{2+} stimulates scrambling of the erythrocyte membrane with exposure of phosphatidylserine at its surface, which is apparent from annexin V-binding of the erythrocytes [24, 28-31]. The Ca^{2+} sensitivity of phospholipid scrambling is increased by ceramide [32]. Phosphatidylserine-exposing erythrocytes are rapidly cleared by phagocytosis from circulating blood [33-35].

The present study explored whether Amphotericin B stimulates Ca^{2+} entry into and subsequent suicidal death of erythrocytes.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4 at 37°C for 48 hours. Where indicated, amphotericin B (Sigma, Schnelldorf, Germany) was added at the indicated concentrations. In Ca^{2+} -free Ringer, 1 mM CaCl_2 was substituted for 1 mM ethylene glycol tetraacetic acid (EGTA).

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, 50 μl cell suspension were washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-Fluo3 (1:500 dilution; Roche, Mannheim, Germany) in this solution for 20 min under protection from light. 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, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation 50 μl erythrocyte suspension were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were

Fig. 1. Effect of amphotericin B on cytosolic Ca^{2+} concentration in erythrocytes. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 hours to Ringer solution without (-, black line) and with (+, red line) 0.2 $\mu\text{g/ml}$ amphotericin B. B. Arithmetic means $\pm$ SEM ($n = 6$) of the geo means of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) amphotericin B. *** ($p < 0.001$) indicates significant difference from the respective value in the absence of amphotericin B. C. Dependence of the effect of amphotericin B on the storage period of the erythrocytes. Arithmetic means $\pm$ SEM ($n = 6$) of the geo means of Fluo3 fluorescence of erythrocytes treated for 48 hours without (squares) or with (triangles) 1.0 $\mu\text{g/ml}$ amphotericin B. The experiment was performed on day 0 and repeated 2 days, 4 days, and 6 days later using erythrocytes from the same sample.

Fig. 2. Effect of amphotericin B on phosphatidylserine exposure of erythrocytes. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to Ringer solution without (-, black line) and with (+, red line) 0.2 µg/ml amphotericin B. B. Arithmetic means $\pm$ SEM (n = 6) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) amphotericin B. *** (p < 0.001) indicates significant difference from the respective value without exposure to amphotericin B. C. Dependence of the effect of amphotericin B on the storage period of the erythrocytes. Arithmetic means $\pm$ SEM (n = 6) of the percentage of phosphatidylserine-exposing erythrocytes treated for 48 hours without (squares) or with (triangles) 1.0 µg/ml amphotericin B. The experiment was performed on day 0 and repeated 2 days, 4 days, and 6 days later using erythrocytes from the same sample. D. Arithmetic means $\pm$ SEM (n = 4) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution in the presence (left bars) or absence (right bars) of extracellular Ca²⁺ without (white bars) or with (black bars) 0.1 µg/ml amphotericin B (ATB). ** (p < 0.001) indicates significant difference from the respective value without exposure to amphotericin B. ## (p < 0.001) indicates significant difference from the respective value in the presence of extracellular Ca²⁺.

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 in FACS analysis.

Determination of ceramide formation

To determine ceramide, a monoclonal antibody-based assay was used. 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 in FL-1.

Measurement of hemolysis

After 48 hours of incubation at 37°C, the samples were centrifuged (3 min at 400 g, RT), and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Determination of intracellular ATP concentration

For determination of erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without Amphotericin B (final hematocrit 5%). All manipulations were then performed at 4°C to avoid ATP degrada-

Fig. 3. Analysis of the integrity of the erythrocyte membrane under the influence of amphotericin B. A. Transmission microphotograph (left panel) and fluorescence microphotograph (right panel) of an erythrocyte stained with fluorescent annexin V. Prior to microscopy, the erythrocytes were exposed for 48 h to 0.5 µg/ml amphotericin B in Ringer solution. B. Arithmetic means $\pm$ SEM (n= 4) of the percentage of hemolysed erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) amphotericin B at the indicated concentrations.

tion. Cells were lysed in distilled water, and proteins were precipitated by addition of HClO_4 (5%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated KHCO_3 solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations refer to the cytosol of erythrocytes.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to eryptotic effects, paired comparison was employed.

Results

Activation of cation channels is expected to increase cytosolic Ca^{2+} activity. Fluo3 fluorescence was employed to test, whether amphotericin B modifies the cytosolic Ca^{2+} concentration. As illustrated in Fig. 1, a 48 hours incubation of erythrocytes in the presence of 0.5 µg/ml amphotericin B was followed by a significant increase in Fluo3 fluorescence. Accordingly, exposure to amphotericin B increases cytosolic Ca^{2+} activity of human erythrocytes. To check whether the effect of amphotericin B on Fluo3 fluorescence of human erythrocytes is dependent on the storage period of the cells, erythrocytes were incubated in the presence or absence of 1.0 µg/ml

amphotericin B on day 0. The same experiment was repeated 2 days, 4 days, and 6 days later using the same erythrocyte specimens. As shown in Fig. 1C, the effect of amphotericin B was more pronounced following prolonged storage of erythrocytes.

An increase in cytosolic Ca^{2+} -activity is expected to stimulate cell membrane scrambling, which could be quantified by determination of annexin V-binding. As shown in Fig. 2A,B, the percentage of phosphatidylserine-exposing erythrocytes was low in the absence of amphotericin B. Exposure of erythrocytes for 48 hours to Ringer solution containing amphotericin B (0.1 µg/ml to 1 µg/ml) was followed by a significant increase in annexin V-binding (Fig. 2A,B). As shown in Fig. 2C, the percentage of PS-exposing cells increased following prolonged storage of both, untreated and amphotericin B-treated cells.

To test whether amphotericin B-induced increase in the cytosolic Ca^{2+} concentration indeed accounts for phosphatidylserine exposure, erythrocytes were exposed to amphotericin B in the absence of extracellular Ca^{2+} . As shown in Fig. 2D, amphotericin B-induced phosphatidylserine exposure was significantly blunted in the absence of extracellular Ca^{2+} .

At the applied concentrations amphotericin B did not disrupt the integrity of the cell membrane (Fig. 3A) and did not induce significant hemolysis (Fig. 3B).

An increase in cytosolic Ca^{2+} -activity is further expected to induce cell shrinkage, which should be reflected by a decrease of forward scatter in FACS analysis. Exposure of erythrocytes for 48 hours to Ringer

Fig. 4. Effect of amphotericin B on erythrocyte forward scatter. A. Original histogram of the forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) 0.1 µg/ml amphotericin B. B. Arithmetic means $\pm$ SEM (n = 6) of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) amphotericin B. ** (p<0.01) indicates significant difference from the respective value without exposure to amphotericin B.

solution with amphotericin B (0.1 µg/ml) was indeed followed by a significant decrease of erythrocyte forward scatter, an effect reversed at higher concentrations of amphotericin B (1 µg/ml; Fig. 4).

Cell membrane scrambling and cell shrinkage could be triggered by ceramide, which could thus, in theory, have contributed to the effects of amphotericin B. Immune fluorescence was employed to determine ceramide formation. A 48 hours exposure of erythrocytes to 0.1 - 1 µg/ml amphotericin B did, however, not result in a significant increase in the ceramide abundance at the erythrocyte surface (data not shown).

Cellular depletion of ATP is further known to stimulate eryptosis [36]. The intracellular ATP concentration of erythrocytes was therefore determined upon incubation in the presence or absence of amphotericin B. As shown in Fig. 5, a 48 hours exposure of human erythrocytes to 0.5 - 1 µg/ml amphotericin B resulted in a significant decrease of the cytosolic ATP concentration. Incubation in glucose-free solution served as a positive control (Fig. 5, grey bar).

Discussion

According to the present observations, amphotericin B increases cytosolic Ca^{2+} -activity and decreases the intracellular ATP concentrations, effects, which in turn

Fig. 5. Effect of amphotericin B on erythrocyte ATP content. Arithmetic means $\pm$ SEM (n = 8) of the ATP concentration after a 48 hours incubation in Ringer solution without (white bar) or with (black bars) amphotericin B at the indicated concentrations, or in energy-depleted Ringer solution (-glu, grey bar) as a positive control. **, *** indicate significant difference (p<0.01, p<0.001) from control (absence of amphotericin B and presence of glucose).

trigger cell membrane scrambling and cell shrinkage. Accordingly, amphotericin B stimulates suicidal erythrocyte death or eryptosis. At higher concentrations the amphotericin B induced cell shrinkage is reversed, which may be due to excessive entry of Na^+ along with Cl^- and osmotically obliged water.

The concentrations required to elicit eryptosis are well within the range of those reached under treatment with amphotericin [37, 38]. Eryptosis clearly exceeds hemolysis and thus, the observed phosphatidylserine exposure is not secondary to hemolysis. Higher levels of hemolysis have been reported earlier following exposure of erythrocytes to amphotericin B formulations [15].

In nucleated cells amphotericin B has been demonstrated to enhance the cation conductance in the plasma membrane [11] with subsequent Na^+ entry and increase in the intracellular Ca^{2+} concentration [12]. It has further been shown to trigger a signaling cascade involving TLR-2, Btk, PLC, PKC, c-Src and NF-kappaB [13]. Activation of PKC has in turn been shown to stimulate eryptosis [39].

Phosphatidylserine-exposing cells are recognized by macrophages [40], which engulf and degrade the affected cells [41]. Accordingly, phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood [35], which may lead to anemia. As a matter of fact, eryptosis contributes to the anemia of several clinical disorders, including iron deficiency [35], phosphate deple-

tion [42], Hemolytic Uremic Syndrome [43], sepsis [44], malaria [45-48], or Wilson's disease [49]. Furthermore, several anemia producing xenobiotics and endogeneous substances are at least partially effective through stimulation of eryptosis, such as cordycepin [50], antibodies [51], amyloid peptides [52], lipopeptides [53], retinoic acid [54], amantadine [55], amiodarone [56], anandamide [57], ciglitazone [58], curcumin [59], valinomycin [60], listeriolysin [61], copper [49], bismuth [62], tin [63], cadmium [64], selenium [65], vanadate [66], gold [67] and arsenic [68]. Amphotericin B treatment has similarly been shown to cause anemia [10]. The comparison between the rate of eryptosis and the rate of hemolysis strongly suggests that the anemia following amphotericin B treatment is due to eryptosis rather than hemolysis. It should be kept in mind, though, that at lower levels of eryptosis the accelerated loss of erythrocytes could be compensated by enhanced erythropoiesis. Thus, increase of reticulocyte count rather than overt anemia may parallel a moderate stimulation of eryptosis.

Phosphatidylserine-exposing erythrocytes further adhere to the vascular wall [69-73], and stimulate blood

clotting [69, 74, 75]. Thus, excessive eryptosis may compromise microcirculation. Along those lines, enhanced eryptosis may participate in the vascular injury of metabolic syndrome [76]. Eryptosis might further promote the release of pro-inflammatory cytokines [76], which may in turn contribute to the in vivo toxicity of amphotericin B [77]. Amphotericin B stimulates cytokine release further by stimulation of gene expression in nucleated cells [78].

In conclusion, amphotericin B stimulates suicidal erythrocyte death or eryptosis. The effect may account for the anemia during amphotericin B treatment.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. This study was supported by the Carl-Zeiss-Stiftung, the Deutsche Forschungsgemeinschaft, Nr. La 315/4-3 and La 315/6-1 and the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research).

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