Original Paper

Embelin-Induced Phosphatidylserine Translocation in the Erythrocyte Cell Membrane

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Key Words

Phosphatidylserine • Cell volume • Ceramide • ROS • p38 kinase • SB203580 • Staurosporine • Eryptosis

Abstract

Background/Aims: The antihelminthic, contraceptive, anti-inflammatory and anticancer phytochemical embelin is at least in part effective against malignancy by inducing suicidal death or apoptosis of tumor cells. Erythrocytes are similarly able to enter suicidal death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Signaling of eryptosis includes increase of cytosolic Ca^{2+} -activity ($[Ca^{2+}]_i$), ceramide formation, oxidative stress as well as activation of p38 kinase and protein kinase C (PKC). The present study tested, whether and how embelin induces eryptosis. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, ceramide abundance utilizing specific antibodies and reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. **Results:** A 48 hours exposure of human erythrocytes to embelin ($\geq 25 \mu M$) significantly increased the percentage of annexin-V-binding cells and hemolysis. Embelin did not significantly modify $[Ca^{2+}]_i$. The effect of embelin on annexin-V-binding was not blunted by removal of extracellular Ca^{2+} , by p38 kinase inhibitor SB203580 ($2 \mu M$) or by PKC inhibitor staurosporine ($1 \mu M$). Embelin did, however, significantly increase the ceramide abundance. **Conclusions:** Embelin stimulates phospholipid scrambling of the erythrocyte cell membrane, an effect involving ceramide formation.

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Introduction

Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), a phytochemical isolated from *Embelia ribes* [1], has antihelminthic [2], contraceptive [2], anti-inflammatory [3-6] and anticancer [1-4, 6-11] potency, as well as photosensitizing [1] and radiation sensitizing [12]

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property. Embelin has further been shown to decrease cardiac injury following experimental myocardial infarction [13] and to protect cells against UVB-induced oxidative damage [14], but to aggravate the encephalopathy following neonatal hypoxia-ischemia [15].

The anticancer activity of embelin has in part been attributed to stimulation of apoptosis [6-8, 11, 16-32]. Mechanisms mediating the effects of embelin include mitochondrial depolarization [22, 23, 27, 29, 31], induction of oxidative stress [17], inhibition of X-linked inhibitor of the apoptosis protein (XIAP) [3, 5, 10, 11, 16, 19, 20, 24, 30, 32-36], of PTEN/Akt/mTOR/S6K1 signaling [37, 38] and of the transcription factors NF- κ B [2, 11, 16, 25, 39] and STAT3 [3, 6, 18, 37], increase of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [40], upregulation of TRAIL receptors DR4/DR5 [19] and Bax [7, 23, 31], as well as activation of p38 kinase [17, 32], Jun kinase [17], p53 transcription factor [6, 22, 28, 32] and caspases [7, 8, 11, 17, 19, 20, 22, 23, 28, 31, 34, 38]. Moreover, embelin may sensitize tumor cells to the anticancer effect of peroxisome proliferator-activated receptor- γ (PPAR γ) ligands [41].

Erythrocytes may, similar to apoptosis of nucleated cells, enter suicidal death or eryptosis, which is characterized by cell shrinkage [42] and phospholipid scrambling of the cell membrane leading to phosphatidylserine translocation to the cell surface [43]. Signaling involved in the triggering of eryptosis include oxidative stress [43], Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [43], ceramide [44], energy depletion [43], activated caspases [43, 45, 46], stimulation of casein kinase 1 α [47], Janus-activated kinase JAK3 [48], protein kinase C [49] and p38 kinase [50], as well as decreased activity of AMP activated kinase AMPK [51], cGMP-dependent protein kinase [52], PAK2 kinase [53] and sorafenib/sunitinib sensitive kinases [43, 54, 55]. A wide variety of xenobiotics have previously been shown to trigger eryptosis [43, 56-84].

The present study tested the influence of embelin on eryptosis. To this end, human erythrocytes from healthy volunteers were treated with embelin and phosphatidylserine surface abundance and cell volume determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples 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 (184/2003 V). The blood was centrifuged at 120 x g for 20 min at 21°C and the platelets and leukocytes-containing supernatant was disposed. 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, erythrocytes were exposed to embelin (Enzo Life Sciences, Lörrach, Germany) which was dissolved in DMSO (Carl Roth, Karlsruhe, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μL cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin V binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin V-binding cells and control cells. The same threshold was used for untreated and embelin treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 5 μM Fluo-3/AM. The cells were

incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μL Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, a 100 μL cell suspension was stained for 1 hour at 37°C with 1 $\mu\text{g/mL}$ anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. Subsequently, the 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. The samples were then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Reactive oxygen species (ROS)

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μL suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnelldorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μM . Erythrocytes were incubated at 37°C for 30 min in the dark and then washed three times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μL Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Hemolysis

In order to determine hemolysis, the samples were centrifuged (3 min at 1600 rpm, room temperature) after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the haemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100 % hemolysis. Hemolysis is expressed in % in order to allow comparison with % annexin V binding cells.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and t test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored, whether embelin stimulates eryptosis, the suicidal erythrocyte death. Hallmarks of eryptosis include cell membrane scrambling with phosphatidylserine translocation to the cell surface. In order to identify erythrocytes with phosphatidylserine at their surface, the cells were incubated with phosphatidylserine-binding FITC-labeled annexin-V, which was determined by flow cytometry. The erythrocytes were analyzed following incubation for 48 hours in Ringer solution without or with embelin (10 - 50 μM). As illustrated in Fig. 1B, a 48 hours exposure to embelin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 25 μM embelin.

The suicidal erythrocyte death could involve hemolysis. In order to estimate the effect of embelin on hemolysis, the haemoglobin concentration in the supernatant was determined. As a result, a 48 hours incubation with embelin resulted in hemolysis, an effect reaching statistical significance at 25 μM embelin (Fig. 2).

Fig. 1. Effect of embelin on phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 50 μM embelin. M1 indicates the annexin-V-fluorescence defining the percentage of annexin-V-binding erythrocytes. (B) Arithmetic means ± SEM (n = 12) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of embelin (10 - 50 μM) or of the solvent DMSO (grey bar). *** (P < 0.001) indicates significant difference from the absence of embelin (ANOVA).

Fig. 2. Effect of embelin on hemolysis. Arithmetic means ± SEM of erythrocyte hemolysis (n = 7) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of embelin (10-50 μM) or of the solvent DMSO (grey bar). ** (P < 0.01), *** (P < 0.001) indicate significant difference from the absence of embelin (ANOVA).

Forward scatter was determined in order to determine whether embelin modifies cell volume. As a result, the forward scatter was similar following a 48 hours incubation without (510 ± 5, a.u., n = 12) and with 10 μM (520 ± 5, a.u., n = 12), 15 μM (522 ± 4, a.u., n = 12), 25 μM (518 ± 6, a.u., n = 12) and 50 μM embelin (506 ± 6, a.u., n = 12). Accordingly, embelin did not significantly modify average forward scatter.

In order to determine whether embelin modifies cytosolic Ca²⁺ activity ([Ca²⁺]_i), Fluo3 fluorescence was taken as measure of [Ca²⁺]_i. As illustrated in Fig. 3A, B, the Fluo3 fluorescence was similar following a 48 hours incubation without and with embelin (10 - 50 μM). Accordingly, embelin did not significantly modify [Ca²⁺]_i. An additional series of experiments explored whether embelin-induced translocation of phosphatidylserine to the cell surface was dependent on entry of extracellular Ca²⁺. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 50 μM embelin, either in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 3C, removal of extracellular Ca²⁺ did not significantly blunt the effect of embelin on annexin-V-binding. Instead, embelin significantly increased the percentage of annexin-V-binding erythrocytes to similarly high levels in the absence and in the presence of extracellular Ca²⁺. Thus, triggering of phosphatidylserine translocation by embelin was due to mechanisms other than entry of extracellular Ca²⁺.

Triggering of phosphatidylserine translocation to the erythrocyte surface may further involve oxidative stress. Reactive oxygen species (ROS) was thus quantified utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, the DCFDA fluorescence

Fig. 3. Erythrocyte Ca^{2+} activity and Ca^{2+} insensitivity of embelin-induced phosphatidylserine exposure. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of embelin (50 μM). (B) Arithmetic means $\pm$ SEM ($n = 12$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) presence of embelin (10 - 50 μM) or of the solvent DMSO (grey bar). (C) Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) embelin (50 μM) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *** ($P < 0.001$) indicates significant difference from the absence of embelin (ANOVA).

Fig. 4. Effect of embelin on ceramide abundance. (A) Original histogram of ceramide abundance at the erythrocyte surface following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 50 μM embelin. (B) Arithmetic means $\pm$ SEM ($n = 9$) of erythrocyte ceramide abundance following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of embelin (50 μM). * ($P < 0.05$) indicates significant difference from the absence of embelin (t test).

was similar in the absence (16.9 ± 1.5 a.u., $n = 14$) and presence (15.6 ± 1.0 a.u., $n = 14$) of $50 \mu\text{M}$ embelin. For comparison, a 10 min exposure of erythrocytes to 0.3 mM *tert*-butylhydroperoxide (TBOOH) was followed by a significant increase of DCFDA fluorescence (224.6 ± 32.5 a.u., $n = 6$).

An additional series of experiments explored whether the embelin-induced phosphatidylserine translocation was dependent on activation of p38 kinase. To this end, erythrocytes were exposed for 48 hours to $50 \mu\text{M}$ embelin in the absence or presence of the p38 kinase inhibitor SB203580 ($2 \mu\text{M}$). As a result, the effect of embelin ($50 \mu\text{M}$) on annexin-V-binding was similar in the presence ($5.4 \pm 0.9 \%$, $n = 9$) and absence ($5.8 \pm 0.8 \%$, $n = 9$) of p38 kinase inhibitor SB203580.

An additional series of experiments explored whether the embelin-induced phosphatidylserine translocation was dependent on activation of protein kinase C. To this end, erythrocytes were exposed for 48 hours to $50 \mu\text{M}$ embelin in the absence or presence of the PKC inhibitor staurosporine ($1 \mu\text{M}$). As a result, the effect of embelin on annexin-V-binding was similar in the presence ($8.2 \pm 0.3 \%$, $n = 7$) and absence ($8.1 \pm 0.4 \%$, $n = 7$) of PKC inhibitor staurosporine.

Stimulators of eryptosis in the absence of increased $[\text{Ca}^{2+}]_i$ include ceramide. Thus, specific antibodies were utilized to quantify ceramide abundance at the erythrocyte surface. As illustrated in Fig. 4, a 48 hours exposure to embelin ($50 \mu\text{M}$) significantly increased the ceramide abundance at the erythrocyte surface.

Discussion

The present observations uncover a novel effect of embelin, i.e. the stimulation of erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The embelin concentration ($25 \mu\text{M}$) required for stimulation of erythrocyte cell membrane scrambling was within the range of concentrations observed *in vivo* [85] and of those previously shown to trigger tumor cell apoptosis *in vitro* [86]. Embelin did not significantly modify erythrocyte forward scatter and had thus little effect on cell volume.

Embelin further did not appreciably modify cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). An increase of $[\text{Ca}^{2+}]_i$ would be expected to trigger cell shrinkage by activating Ca^{2+} sensitive K^+ channels with K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [42]. The failure of embelin to increase $[\text{Ca}^{2+}]_i$ provides an explanation for the lack of cell shrinkage following embelin treatment. The present observations do not exclude minor alterations of $[\text{Ca}^{2+}]_i$. However, the embelin induced phosphatidylserine translocation does not require entry of extracellular Ca^{2+} with subsequent increase of $[\text{Ca}^{2+}]_i$.

In contrast to observations in nucleated cells [17], embelin did not increase the abundance of reactive oxygen species (ROS), a well known trigger of eryptosis [43]. The embelin induced eryptosis was further not significantly blunted by p38 kinase inhibitor SB203580, an observation again contrasting observations in nucleated cells [17, 32]. Moreover, embelin induced cell membrane scrambling was insensitive to the PKC inhibitor staurosporine.

The stimulation of cell membrane phosphatidylserine translocation was paralleled by and at least in part due to increased abundance of ceramide, which may trigger erythrocyte cell membrane scrambling even in the absence of increased $[\text{Ca}^{2+}]_i$ [43].

Besides its effect on eryptosis, embelin triggered hemolysis. The rate of hemolysis even exceeded that of eryptosis. A purpose of eryptosis is the clearance of defective erythrocytes prior to hemolysis [43], thus preventing release of haemoglobin, which is otherwise filtered in renal glomeruli, precipitates in renal tubular fluid and thus occludes nephrons [87]. Eryptosis further serves the clearance of *Plasmodium* infected erythrocytes in malaria and thus favourably influences the clinical course of the disease [88]. As a matter of fact, by increasing the erythrocyte susceptibility to triggers of eryptosis, sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency protect against a severe course of malaria [43,

89-91]. Along those lines, iron deficiency [92] and treatment with lead [92], chlorpromazine [93] or NO synthase inhibitors [93] counteract parasitemia by increasing the erythrocyte susceptibility to triggers of eryptosis [88]. It is tempting to speculate that embelin may accelerate phosphatidylserine translocation in *Plasmodium* infected erythrocytes.

On the other hand, embelin may aggravate anaemia in clinical conditions with enhanced phosphatidylserine translocation, including dehydration [72], hyperphosphatemia [83], chronic kidney disease (CKD) [72, 94-96], Hemolytic-uremic syndrome [97], diabetes [98], liver failure [99], malignancy [100], sepsis [101] and Wilsons disease [102]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [103], trigger blood clotting and elicit thrombosis [104-106]. By stimulating phosphatidylserine translocation, embelin may thus impair microcirculation [44, 104, 107-110].

Conclusion

Embelin triggers phosphatidylserine translocation in the erythrocyte cell membrane, an effect paralleled by and in part due to ceramide formation.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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