Estramustine-Induced Suicidal Erythrocyte Death

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Abstract
Background: The nitrogen mustard derivative of estradiol-17β-phosphate estramustine is used for the treatment of prostate cancer. Estramustine may trigger suicidal death of cancer cells. Side effects of estramustine include anemia. At least in theory, estramustine could cause anemia by stimulation of eryptosis, the suicidal death of erythrocytes. Hallmarks of eryptosis include cell shrinkage, increased cytosolic Ca2+ activity ([Ca2+]i), ceramide formation and phosphatidylserine translocation to the outer leaflet of the cell membrane with phosphatidylserine exposure at the erythrocyte surface. Eryptosis is stimulated by increase of cytosolic Ca2+ activity ([Ca2+]i). The present study explored whether estramustine triggers eryptosis. Methods: [Ca2+]i was estimated from Fluo3 fluorescence, cell volume from forward scatter, phosphatidylserine exposure from annexin V binding, and hemolysis from hemoglobin release. Results: A 24 h exposure to estramustine (≤ 100 µM) significantly increased [Ca2+]i, increased annexin V binding and increased hemoglobin release. The effect of estramustine on annexin V binding was significantly blunted by removal of extracellular Ca2+. Conclusions: Estramustine stimulates both, eryptosis and hemolysis. The estramustine induced translocation of phosphatidylserine to the cell surface is at least partially due to increase of cytosolic Ca2+ activity.
Introduction

Estramustine, a nitrogen mustard derivative of estradiol-17β-phosphate, is widely used alone or in combination with other anticancer drugs for the treatment of prostate cancer [1, 2]. Estramustine is effective by suppressing the dynamics of microtubules [1] thus interfering with mitosis [3]. Moreover, estramustine has been shown to trigger apoptosis [1, 4, 5]. Side effects of treatment with combinations containing estramustine include anemia [6-10], which is a risk factor of patient survival [11].

At least in theory, anemia following cytostatic treatment could involve suicidal erythrocyte death or eryptosis, which is characterized by erythrocyte shrinkage and breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane [12, 13]. Eryptosis may be triggered by Ca$^{2+}$ entry through Ca$^{2+}$ permeable cation channels with subsequent increase of cytosolic Ca$^{2+}$ activity ([Ca$^{2+}]_c$) [14, 15]. The increase of [Ca$^{2+}]_c$ leads to activation of Ca$^{2+}$ sensitive K$^+$ channels [16], K$^+$ exit, hyperpolarization, Cl$^-$ exit and thus cell shrinkage due to cellular loss of KCl with osmotically obliged water [17]. The increase of [Ca$^{2+}]_c$ further triggers translocation of phosphatidylserine to the outer leaflet of the erythrocyte cell membrane with exposure of phosphatidylserine at the erythrocyte surface [18]. Ca$^{2+}$ sensitivity of eryptosis is increased by ceramide [19]. Eryptosis can further be stimulated by energy depletion [20] and caspase activation [21-25]. Regulation of eryptosis further involves AMP activated kinase AMPK [15], cGMP dependent protein kinase [26], Janus activated kinase JAK3 [27], casein kinase 1α [28, 29], p38 kinase [30], PAK2 kinase [31] as well as sorafenib [32] and sunitinib [33] sensitive kinases.

Eryptosis may be stimulated by a wide variety of xenobiotics [33-64]. Moreover, excessive eryptosis contributes to the pathophysiology of several clinical disorders [12] including diabetes [25, 65, 66], renal insufficiency [67], hemolytic uremic syndrome [68], sepsis [69], malaria [70-74], sickle cell disease [75], Wilson’s disease [73], iron deficiency [76], malignancy [77], phosphate depletion [78], and metabolic syndrome [60].

The present study explored, whether estramustine triggers eryptosis, and if so, whether the effect involves alterations of erythrocyte [Ca$^{2+}]_c$.

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 h. Where indicated, erythrocytes were exposed to estramustine (Sigma-Aldrich, Germany) at the indicated concentrations. Estramustine was dissolved in H$_2$O and added at concentrations of up to 100 µM. The addition of estramustine increased osmolarity by < 0.05%, an effect not leading to appreciable cell shrinkage and not sufficient to trigger eryptosis [79]. In Ca$^{2+}$ free Ringer solution, 1 mM CaCl$_2$ was substituted by 1 mM glycol bis(2 aminoethylether) N,N',N’ tetraacetic acid (EGTA).

FACS analysis of annexin V binding and forward scatter

After incubation under the respective experimental condition, 50 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. In the following, the forward scatter (FSC) 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 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 2 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 in fluorescence channel FL 1 in FACS analysis.

**Measurement of hemolysis**

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (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.

**Determination of ceramide formation**

For the determination of ceramide, a monoclonal antibody-based assay was used. To this end, 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. 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 (Pharimagen, 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. As control, secondary antibody alone was used.

**Statistics**

Data are expressed as arithmetic means ± 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 was designed to explore, whether estramustine stimulates eryptosis, the suicidal death of erythrocytes. As eryptosis is triggered by increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]), Fluo3 fluorescence was employed to estimate [Ca\(^{2+}\)]. To this end, the erythrocytes were incubated in Ringer solution without or with estramustine (≤ 100 µM), loaded with Fluo3 AM and Fluo3 fluorescence quantified by FACS analysis. As illustrated in Fig. 1, a 24 hours exposure of human erythrocytes to estramustine was followed by an increase of Fluo3 fluorescence, an effect reaching statistical significance at 100 µM estramustine concentration. Thus, estramustine treatment was followed by increase of [Ca\(^{2+}\)] in human erythrocytes.

An increase of [Ca\(^{2+}\)] is expected to activate Ca\(^{2+}\) sensitive K\(^{+}\) channels leading to cellular loss of KCl together with osmotically obliged water and thus to cell shrinkage. Accordingly, cell volume was estimated from forward scatter in FACS analysis. As shown in Fig. 2, a 24 hours treatment with estramustine did not result in a decrease of forward scatter.

An increase of [Ca\(^{2+}\)] is further expected to trigger cell membrane scrambling with breakdown of phosphatidylserine asymmetry of the cell membrane and appearance of phosphatidylserine at the cell surface. Accordingly, phosphatidylserine abundance at the cell surface was estimated utilizing annexin V binding in FACS analysis. As shown in Fig. 3, a 24 h estramustine treatment increased the percentage of annexin V binding erythrocytes, an effect reaching statistical significance at 50 µM estramustine concentration. Accordingly, estramustine triggered cell membrane scrambling.

In order to quantify the effect of estramustine exposure on hemolysis, the percentage of hemolysed erythrocytes was determined from hemoglobin concentration in the supernatant.
According to hemoglobin concentration in the supernatant, estramustine treatment resulted in hemolysis, an effect reaching statistical significance at 75 µM estramustine concentration (Fig. 4).

Further experiments were performed to test, whether the stimulation of cell membrane scrambling following estramustine treatment was partially or even fully explained by Ca$^{2+}$ entry from the extracellular space. To this end, erythrocytes were exposed to 100 µM...
estramustine for 24 hours in either the presence of 1 mM extracellular Ca$^{2+}$ or in the absence of extracellular Ca$^{2+}$ and presence of the Ca$^{2+}$ chelator EGTA (1 mM). As shown in Fig. 4, removal of extracellular Ca$^{2+}$ significantly blunted the effect of estramustine on annexin V binding. However, in the absence of extracellular Ca$^{2+}$ the percentage annexin V binding erythrocytes was still slightly, but significantly increased by estramustine treatment (Fig. 5). Thus, estramustine induced cell membrane scrambling was in large part but not completely dependent on the presence of extracellular Ca$^{2+}$.

A final series of experiments explored the effect of estramustine treatment on the formation of ceramide, which would be similarly expected to trigger cell membrane scrambling. Ceramide formation was quantified utilizing FITC-labeled anti-ceramide
antibodies. As illustrated in Figure 6, the ceramide-dependent fluorescence was significantly higher following a 24 h exposure to Ringer containing 100 µM estramustine than following exposure to Ringer solution without estramustine. Utilizing the secondary antibody alone, the fluorescence did not change significantly upon treatment with estramustine (-3.8 ± 1.4%, n = 5). Thus, estramustine indeed significantly enhanced ceramide formation.

**Discussion**

The present study explored, whether estramustine triggers eryptosis, the suicidal death of erythrocytes. The results reveal that estramustine treatment of erythrocytes drawn from healthy volunteers is followed by breakdown of phosphatidylserine asymmetry of the cell membrane, a hallmark of eryptosis. The concentrations required to trigger eryptosis are in the range of those concentrations encountered *in vivo*, which may exceed 100 µM [80].

The erythrocyte shrinkage following estramustine treatment is most likely the result of increased cytosolic Ca²⁺ activity, which activates Ca²⁺ sensitive K⁺ channels [16, 81] leading to cell membrane hyperpolarization. The increased electrical driving force drives Cl⁻ exit and thus leads to cellular loss of KCl with osmotically obliged water [17].

The breakdown of phosphatidylserine asymmetry of the erythrocyte cell membrane was significantly blunted in the absence of extracellular Ca²⁺ and was thus again at least in part due to increase of cytosolic Ca²⁺ activity ([Ca²⁺]). An increase of [Ca²⁺] is well known to stimulate cell membrane scrambling with phosphatidylserine translocation from the inner leaflet of the cell membrane to the outer leaflet of the cell membrane [12]. Mechanisms underlying Ca²⁺ entry include Ca²⁺ permeable non selective cation channels involving the transient receptor potential channel TRPC6 [14]. The Ca²⁺ permeable erythrocyte cation channels are activated by oxidative stress [82].

Estramustine-induced phosphatidylserine translocation is blunted but not abolished in the nominal absence of extracellular Ca²⁺, pointing to involvement of additional mechanisms. Those mechanisms include formation of ceramide, which is known to increase Ca²⁺ sensitivity of cell membrane scrambling. Moreover, at least in theory, estramustine may influence the
activity of eryptosis regulating kinases such as AMP activated kinase AMPK [15], casein kinase 1α [28, 29], cGMP dependent protein kinase [26], Janus activated kinase JAK3 [27], p38 kinase [30] and/or PAK2 kinase [31].

Triggering of eryptosis has been observed following treatment of erythrocytes with further anticancer drugs [64], including carmustine [83], sorafenib [32], sunitinib [33], cisplatin [84], and paclitaxel [85]. Moreover, eryptosis is enhanced in malignancy [77].

Consequences of enhanced eryptosis include anemia. In vivo, eryptotic erythrocytes are mainly trapped in the spleen and thus rapidly removed from from circulating blood [12]. As soon as the loss of erythrocytes by triggering of eryptosis is not matched by a similar enhancement of erythropoiesis, anemia develops [12].

Consequences of enhanced eryptosis further include adhesion of phosphatidylserine exposing erythrocytes to endothelial CXCL16/SR PSO [86]. The adhesion of erythrocytes to the vascular wall could at least in theory compromise microcirculation and thus interfere with blood flow [86-91]. The effect may be compounded by the stimulating effect of phosphatidylserine exposure on blood clotting, which may foster the development of thrombosis [87, 92, 93]. Along those lines, the treatment with estramustine may be associated with an increased risk of thromboembolic events [2].

In conclusion, estramustine triggers Ca\textsuperscript{2+} entry with subsequent suicidal erythrocyte death or eryptosis. The stimulation of eryptosis may contribute to the development of anemia following estramustine treatment.

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References


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59 Weiss E, Cytak UM, Rees DC, Osei A, Gibson JS: Deoxygenation-induced and Ca\(^{2+}\) dependent phosphatidylserine externalisation in red blood cells from normal individuals and sickle cell patients. Cell Calcium 2012;51:51-56.
60 Zappulla D: Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO\(_2\) increases? J Cardiometab Syndr 2008;3:30-34.
71 Foller M, Bobbala D, Koka S, Huber SM, Gulbins E, Lang F: Suicide for survival—death of infected erythrocytes as a host mechanism to survive malaria. Cell Physiol Biochem 2009;24:133-140.
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84 Mahmud H, Foller M, Lang F: Suicidal erythrocyte death triggered by cisplatin. Toxicology 2008;249:40-44.


