Original Paper

Programmed Erythrocyte Death Following *in Vitro* Treosulfan Treatment

Thomas Peter^a Rosi Bissinger^a Sigrid Enkel^b Kousi Alzoubi^a Gergely Oswald^a
Florian Lang^a

Department of Physiology^a and Blood Transfusion^b, University of Tübingen, Tübingen, Germany

Key Words

Phosphatidylserine • Reactive oxidant species • Calcium • Cell volume • Eryptosis

Abstract

Background/Aims: The cytotoxic drug Treosulfan is clinically used for the treatment of malignancy. A common side effect of Treosulfan treatment is anemia. Treosulfan is at least partially effective by triggering apoptosis of tumor cells. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, a suicidal death characterized by cell shrinkage and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Triggers of eryptosis include oxidative stress, Ca^{2+} -entry and increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$). The present study explored whether Treosulfan stimulates eryptosis, which may contribute to development of anemia. **Methods:** Erythrocyte volume was estimated from forward scatter, phosphatidylserine abundance at the erythrocyte surface from Fluorescein isothiocyanate (FITC)-annexin-V-binding, $[\text{Ca}^{2+}]_i$ from Fluo3 fluorescence and reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA)-fluorescence. **Results:** A 48 hours exposure of human erythrocytes to Treosulfan (800 $\mu\text{g}/\text{ml}$) significantly decreased erythrocyte forward scatter, increased the percentage of annexin-V-binding cells, increased $[\text{Ca}^{2+}]_i$, and increased ROS. The effect of Treosulfan on annexin-V-binding was virtually abrogated by removal of extracellular Ca^{2+} . **Conclusion:** Treosulfan stimulates suicidal erythrocyte death or eryptosis at least in part by inducing oxidative stress and stimulating Ca^{2+} entry.

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Introduction

Treosulfan, a hydrophilic analogue of busulfan [1], is in clinical use for the treatment of ovarian cancer and of hematologic malignancies [1]. The substance is at least in part effective by triggering apoptosis [2-9]. The most common side effect of Treosulfan treatment is anemia, affecting as many as 88% of the patients [10].

Prof. Dr. Florian Lang

Physiologisches Institut der Universität Tübingen,
Gmelinstr. 5, D-72076 Tübingen (Germany)
Tel. +49 7071 29 72194, Fax +49 7071 29 5618, E-Mail florian.lang@uni-tuebingen.de

We hypothesized that the anemia following Treosulfan treatment could in part result from accelerated loss of erythrocytes due to stimulation of apoptosis-like suicidal cell death or eryptosis, which is characterized by cell shrinkage [11] and by translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane [12].

Signaling participating in the triggering of eryptosis includes oxidative stress [12] and increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [12]. Eryptosis is further triggered by ceramide [13], energy depletion [12], caspase activation [14-17], activation of casein kinase 1 α [18, 19], Janus-activated kinase JAK3 [20], protein kinase C [21] or p38 kinase [22], as well as inhibition or knockout of AMP-activated kinase AMPK [23], cGMP-dependent protein kinase [15], PAK2 kinase [24], as well as sorafenib- and sunitinib-sensitive kinases [25, 26].

Eryptosis is triggered by a myriad of xenobiotics [27-63] and enhanced eryptosis contributes to the pathophysiology of several clinical conditions including fever, malaria, sepsis, iron deficiency, malignancy, metabolic syndrome, diabetes, hepatic and renal insufficiency, hemolytic uremic syndrome, dehydration, hyperphosphatemia, phosphate depletion, sickle cell disease, thalassemia, glucose phosphate dehydrogenase deficiency and Wilson's disease [12, 63-65].

The present study tested, whether Treosulfan triggers eryptosis. To this end, erythrocytes drawn from healthy volunteers were exposed to Treosulfan and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$ and reactive oxygen species were determined.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Lithium-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 g for 20 min at room temperature 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 h. Where indicated, erythrocytes were exposed to Treosulfan (DocMorris, Aachen, Germany) at the indicated concentrations by dilution of a 200 mg/ml stock solution prepared in DMSO. For comparison, the effect of 4 μl DMSO/ml Ringer was tested.

Analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, 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 15 min under protection from light. The cell suspension was measured on a FACS Calibur (BD, Heidelberg, Germany). A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. A FSC vs. counts histogram plot was established, small particles cut off to eliminate cell debris, and the geomean value of fluorescence intensity calculated. Annexin 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 treosulfan treated erythrocytes.

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 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 150 μl Ringer. Then, Ca^{2+} -dependent 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 and the geomean of the Ca^{2+} -dependent fluorescence was determined.

Quantification of reactive oxidant 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 μ M. 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 μ l Ringer solution, ROS-dependent fluorescence intensity was measured in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD) and the geomean of the ROS-dependent fluorescence was determined.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. The number of experiments (n) denotes the number of blood donors tested. 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.

Results

The present study explored whether Treosulfan triggers eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and translocation of phosphatidylserine to the erythrocyte surface. To this end, erythrocytes drawn from healthy individuals were incubated for 48 hours in Ringer solution without or with Treosulfan (100 - 800 μ g/ml).

In order to estimate cell volume, forward scatter was determined in flow cytometry. As shown in Fig. 1, a 48 hours incubation in Treosulfan-containing Ringer was followed by a decrease of average forward scatter, an effect reaching statistical significance at 800 μ g/ml Treosulfan concentration. Close inspection of the histogram reveals, however, that a large portion of cells actually increases forward scatter (Fig. 1A).

FITC-annexin-V binding was utilized to quantify phosphatidylserine abundance at the erythrocyte surface. As shown in Fig. 2, 48 hours exposure to Treosulfan was followed by an increase of the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 800 μ g/ml Treosulfan concentration.

Stimulators of cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Accordingly, further experiments addressed the effect of Treosulfan on $[\text{Ca}^{2+}]_i$. To this end, erythrocytes were loaded with Fluo3-AM and the Fluo3 fluorescence quantified by flow cytometry. Measurements were made following 48 hours incubation in Ringer solution without or with Treosulfan (100 - 800 μ g/ml). As shown in Fig. 3, exposure of the erythrocytes to Treosulfan

Fig. 1. Effect of Treosulfan on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 800 μ g/ml Treosulfan. B. Arithmetic means $\pm$ SEM (n = 17) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (4 μ l DMSO/ml Ringer alone, white bar) or with (black bars) Treosulfan (100 - 800 μ g/ml). * (p<0.05) indicates significant difference from the absence of Treosulfan [presence of DMSO alone] (ANOVA).

Fig. 2. Effect of Treosulfan 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 800 µg/ml Treosulfan. M1 indicates the annexin-V-fluorescence defining the percentage of annexin-V-binding erythrocytes. B. Arithmetic means $\pm$ SEM ($n = 17$) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (4 µl DMSO/ml Ringer alone, white bar) or with (black bars) presence of Treosulfan (100 - 800 µg/ml). *** ($p < 0.001$) indicates significant difference from the absence of Treosulfan [presence of DMSO alone] (ANOVA).

Fig. 3. Effect of Treosulfan on erythrocyte Ca^{2+} activity. 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 800 µg/ml Treosulfan. B. Arithmetic means $\pm$ SEM ($n = 17$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (4 µl DMSO/ml Ringer alone, white bar) or with (black bars) presence of Treosulfan (100 - 800 µg/ml). ** ($p < 0.01$), *** ($p < 0.001$) indicate significant differences from the absence of Treosulfan [presence of DMSO alone] (ANOVA).

was followed by an increase of Fluo3 fluorescence, an effect reaching statistical significance at 400 µg/ml Treosulfan concentration.

In order to test whether the Treosulfan-induced cell membrane scrambling required entry of extracellular Ca^{2+} , erythrocytes were exposed to 800 µg/ml Treosulfan for 48 hours either in the presence of 1 mM Ca^{2+} or in the absence of Ca^{2+} and the presence of Ca^{2+} chelator EGTA (1 mM). As illustrated in Fig. 4, the effect of Treosulfan on annexin-V-binding was significantly decreased in the nominal absence of Ca^{2+} . In the absence of extracellular Ca^{2+} Treosulfan still tended to increase slightly annexin-V-binding. The effect, however, did not reach statistical significance.

Fig. 4. Effect of Ca^{2+} withdrawal on Treosulfan-induced phosphatidylserine exposure. Arithmetic means $\pm$ SEM ($n = 10$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bar) or with (black bars) 800 $\mu\text{g}/\text{ml}$ Treosulfan in the presence (left bars, + Calcium) and absence (right bars, - Calcium) of calcium in the Ringer solution. ** ($p < 0.01$) indicates significant difference from the respective values in the absence of Treosulfan, ## ($p < 0.01$) indicates significant difference from the respective value in the presence of Ca^{2+} (ANOVA).

Fig. 5. Effect of Treosulfan on reactive oxygen species. A. Original histogram of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 800 $\mu\text{g}/\text{ml}$ Treosulfan. B. Arithmetic means $\pm$ SEM ($n = 12$) of erythrocyte DCFDA fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bar) presence of 800 $\mu\text{g}/\text{ml}$ Treosulfan. ** ($p < 0.01$) indicates significant difference from the absence of Treosulfan (t -test).

Ca^{2+} entry and eryptosis may be triggered by oxidative stress. In order to test whether Treosulfan increased the abundance of reactive oxygen species (ROS), ROS was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As illustrated in Fig. 5, a 48 hours exposure to Treosulfan (800 $\mu\text{g}/\text{ml}$) significantly increased the DCFDA fluorescence, indicating that Treosulfan triggered oxidative stress.

Discussion

The present study reveals a novel effect of Treosulfan, i.e. the triggering of eryptosis, the suicidal death of erythrocytes. Treatment of human erythrocytes with Treosulfan is followed by decrease of average erythrocyte volume and by cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, both hallmarks of eryptosis [12]. The Treosulfan concentrations (800 $\mu\text{g}/\text{ml}$) required for stimulation of eryptosis was well in the range of those encountered *in vivo* [66].

The stimulation of cell membrane scrambling and cell shrinkage of erythrocytes following Treosulfan treatment is at least partially due to stimulation of Ca^{2+} entry with subsequent

increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Accordingly, removal of extracellular Ca^{2+} virtually abolished the effect of Treosulfan treatment on phosphatidylserine abundance at the cell surface. The increase of $[\text{Ca}^{2+}]_i$ triggers cell membrane scrambling [12] and is followed by activation of Ca^{2+} sensitive K^+ channels, K^+ exit, hyperpolarization of the cell membrane, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [11]. The increase of $[\text{Ca}^{2+}]_i$ is presumably due to opening of Ca^{2+} permeable non-selective cation channels, which are known to be activated by oxidative stress [12]. However, cell shrinkage is seen only in a subset of the erythrocytes and forward scatter actually increases in a large erythrocyte population. The cell swelling may result from entry of Na^+ through the cation channels.

The stimulation of eryptosis contributes to or even accounts for the anemia observed in the vast majority of patients following Treosulfan treatment [10]. Eryptotic erythrocytes are phagocytosed and rapidly cleared from circulating blood [12]. The development of clinically overt anemia results if the accelerated loss of erythrocytes by eryptosis is not matched by similarly increased formation of new erythrocytes [12]. The formation of new erythrocytes is reflected by the reticulocyte number [12]. The present data do not allow, however, safe predictions about the relative contribution of eryptosis to Treosulfan-induced anemia as compared to other mechanisms, such as Treosulfan-induced inhibition of progenitor cell proliferation.

As erythrocytic phosphatidylserine binds to endothelial CXCL16/SR-PSO [34], eryptotic erythrocytes adhere to the vascular wall and thus interfere with microcirculation [13, 67-71]. Phosphatidylserine exposing erythrocytes further stimulate blood clotting and thrombosis [67, 72, 73]. Thus, excessive stimulation of eryptosis may foster vascular occlusion. Thrombosis has indeed been observed under Treosulfan therapy [74, 75].

In conclusion, Treosulfan treatment leads to erythrocyte shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface, effects due to oxidative stress and increase of cytosolic Ca^{2+} activity. The effect presumably contributes to or even accounts for the anemia observed following Treosulfan treatment.

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