Original Paper

Sunitinib-sensitive Suicidal Erythrocyte Death

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Key WordsPhosphatidylserine • Sunitinib • Ca^{2+} • Cell volume • Eryptosis • Red blood cells • Erythrocytes**Abstract**

Sunitinib, a multikinase inhibitor, stimulates apoptosis and is thus utilized for the treatment of malignancy. Even though lacking mitochondria and nuclei, critical elements in apoptosis of nucleated cells, erythrocytes may undergo eryptosis, an apoptosis-like suicidal death, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine-exposure at the cell surface. Triggers of eryptosis include activation of Ca^{2+} permeable cation channels with subsequent increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$), ceramide formation, ATP-depletion, stimulation of p38 kinase and caspase activation. The present study explored, whether sunitinib stimulates eryptosis. $[\text{Ca}^{2+}]_i$ was estimated from Fluo-3-fluorescence, cell volume from forward scatter, phosphatidylserine-exposure from annexin-V-binding, hemolysis from hemoglobin release, ceramide abundance from anti-ceramide antibody binding, and cytosolic ATP from luciferin-luciferase activity. A 48 h exposure to sunitinib (10 μM) significantly decreased forward scatter and increased annexin-V-binding, effects paralleled by significant increase of $[\text{Ca}^{2+}]_i$. Sunitinib exposure was followed by a slight but significant increase of hemolysis. Sunitinib induced annexin-V-binding was slightly, but significantly blunted by removal of extracellular Ca^{2+} , by p38 kinase inhibitor SB203580 (10 μM) and by the pancaspase inhibitor zVAD (10 μM). Sunitinib, however, did not significantly modify cytosolic ATP and ceramide abundance. The present observations reveal that sunitinib is able to trigger suicidal death in erythrocytes even in the absence of nuclei and mitochondria.

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Introduction

Sunitinib is a multikinase inhibitor interfering with the signaling of several growth factor receptors and used in the treatment of several malignancies such as metastatic renal cell carcinoma and unresectable gastrointestinal stromal tumors [1-7]. Sunitinib is effective by triggering necrotic cell death [8] and apoptosis [9-18] paralleled by cellular ATP depletion and glutathione oxidation [19].

In analogy to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling [20]. Triggers of eryptosis include increase of cytosolic Ca^{2+} activity due to Ca^{2+} entry through Ca^{2+} -permeable cation channels [21-24]. Cytosolic Ca^{2+} activates Ca^{2+} -sensitive K^{+} channels [25] resulting in K^{+} exit, hyperpolarization, Cl^{-} exit and thus cellular KCl loss, which drives parallel loss of osmotically obliged water leading to cell shrinkage [26]. Increased cytosolic Ca^{2+} concentration further leads to cell membrane scrambling with phosphatidylserine exposure at the cell surface [27]. Erythrocytes are sensitized to the cell membrane scrambling effect of Ca^{2+} by ceramide [28]. Eryptosis could be further elicited by ATP depletion [29] and activation of caspases [30-34]. Moreover, eryptosis may result from dysregulation of several kinases including p38 kinase [35], AMP activated kinase AMPK [22], cGMP-dependent protein kinase [36], casein kinase CK1 [37] and Janus-activated kinase JAK3 [38].

The present study explored, whether sunitinib stimulates eryptosis and, if so, to uncover underlying mechanisms.

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 sunitinib (Enzo, Lörrach, Germany) at the indicated concentrations. In Ca^{2+} -free Ringer solution, 1 mM CaCl_2 was substituted by 1 mM glycol-bis(2-aminoethylether)-N,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.

Determination of intracellular ATP concentration

For determination of intracellular erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without sunitinib and in Ringer solution with or without extracellular Ca^{2+} (final hematocrit 5%). Additionally, erythrocytes were also incubated in glucose depleted Ringer solution

as a positive control. All subsequent manipulations were performed at 4°C to avoid ATP degradation. 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 are expressed in mmol/l cytosol of erythrocytes.

Determination of ceramide formation

For determination of ceramide, a monoclonal antibody-based assay was used. After incubation with and without sunitinib, cells were stained for 1 h at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in phosphate buffered saline (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 (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 in FL-1.

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.

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. $P < 0.05$ was considered statistically significant. The number of different erythrocyte specimens studied is indicated by *n*. Since individual erythrocyte specimens may differ in their susceptibility to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

Suicidal erythrocyte death or eryptosis is characterized by cell shrinkage and cell membrane scrambling. In order to test, whether sunitinib exposure leads to cell shrinkage, cell volume was estimated utilizing forward scatter in FACS analysis. As illustrated in Fig. 1, sunitinib treatment decreased forward scatter, an effect statistically significant at 10 μM sunitinib.

In order to test whether sunitinib treatment leads to cell membrane scrambling, phosphatidylserine exposing erythrocytes were identified from binding of fluorescent annexin-V in FACS analysis. As shown in Fig. 2, a 48 h exposure to sunitinib increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 10 μM sunitinib.

Further experiments explored whether sunitinib treatment resulted in hemolysis. The hemolysis was quantified from hemoglobin release into the supernatant. As shown in Fig. 2, exposure of erythrocytes for 48 h to sunitinib triggered hemolysis, an effect, however, reaching statistical significance only at 20 μM . Moreover, following sunitinib treatment the percentage of hemolysed erythrocytes was one order of magnitude smaller than the percentage of phosphatidylserine exposing cells.

As both, cell shrinkage and cell membrane scrambling could be triggered by increase of cytosolic Ca^{2+} activity in erythrocytes, the erythrocytes were loaded with Fluo 3 and the Fluo3 fluorescence determined in FACS analysis. As shown in Fig. 3, treatment of human erythrocytes with sunitinib increased Fluo3 fluorescence, an effect reaching statistical

significance at 10 μ M sunitinib concentrations. As a result, sunitinib increases cytosolic Ca^{2+} concentration.

Additional experiments were performed exploring whether sunitinib induced cell membrane scrambling required Ca^{2+} entry. To this end, erythrocytes were exposed to 20 μ M sunitinib in the presence or in the nominal absence of extracellular Ca^{2+} . As a result, Ca^{2+} removal significantly decreased sunitinib-induced cell membrane scrambling (Fig. 4). However, in the nominal absence of extracellular Ca^{2+} sunitinib still tended to enhance annexin-V-binding. Accordingly, the stimulation of erythrocyte membrane scrambling by sunitinib presumably involves mechanisms in addition to Ca^{2+} entry.

As eryptosis could be triggered by energy depletion, a luciferin-luciferase assay kit was utilized to determine cytosolic ATP concentration. As a result, following a 48 hours incubation in the absence or presence of sunitinib, the erythrocyte ATP content was 0.34 ± 0.01 mM (n = 4) in the absence and 0.40 ± 0.03 mM (n = 4) in the presence of sunitinib (20 μ M), values not significantly different. Glucose depletion, however, significantly (p<0.001) decreased the erythrocyte ATP content to 0.07 ± 0.01 mM (n = 4). Thus, in contrast to glucose removal, sunitinib exposure did not lead to significant energy depletion.

Fig. 3. Effect of sunitinib on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (-, black line) and with (+, red line) presence of 20 μM sunitinib. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized geo means (geometric mean of the histogram in arbitrary units) of Fluo-3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) sunitinib (0–20 μM). * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of sunitinib (ANOVA).

Fig. 4. Effects of Ca^{2+} withdrawal, p38 kinase- and caspase-inhibition on sunitinib-induced annexin-V-binding. A. Arithmetic means $\pm$ SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 20 μM sunitinib 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 sunitinib (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the presence of Ca^{2+} . B. Arithmetic means $\pm$ SEM ($n = 6$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 20 μM sunitinib in the absence (left bars) and presence (right bars) of 10 μM SB203580. * ($p < 0.05$), *** ($p < 0.001$) indicates significant difference from the absence of sunitinib (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the absence of SB203580. C. Arithmetic means $\pm$ SEM ($n = 8$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 20 μM sunitinib in the absence (left bars) and presence (right bars) of zVAD (10 μM). *** ($p < 0.001$) indicates significant difference from the absence of sunitinib (ANOVA), # ($p < 0.05$) indicates significant difference from the respective values in the absence of zVAD.

Further experiments explored the effect of sunitinib treatment on the formation of ceramide, which is known to sensitize the cell for the scrambling effects of cytosolic Ca^{2+} activity. Ceramide abundance was determined utilizing FITC-labeled anti-ceramide antibodies. As a

result, following a 48 hours incubation in the absence or presence of sunitinib, the ceramide-dependent fluorescence intensity was 21.4 ± 1.7 a.u. ($n = 4$) in the absence and 27.3 ± 5.1 a.u. ($n = 4$) in the presence of sorafenib ($10 \mu\text{M}$). Accordingly, sunitinib tended to increase ceramide formation, an effect, however, not reaching statistical significance.

As eryptosis following osmotic shock is paralleled by activation of p38 kinase and blunted in the presence of the p38 kinase inhibitor SB203580 [35], additional experiments were performed to elucidate cell membrane scrambling in the presence and absence of SB203580 ($10 \mu\text{M}$). As shown in Fig. 4B, the effect of sunitinib on annexin-V-binding was significantly blunted in the presence of SB203580 ($10 \mu\text{M}$). However, even in the presence of SB203580, sunitinib significantly stimulated annexin-V-binding. Thus, p38 kinase activation contributes to but does not fully account for the stimulation of erythrocyte membrane scrambling by sunitinib.

Additional experiments were performed exploring whether sunitinib induced cell membrane scrambling was sensitive to pancaspase inhibitor zVAD ($10 \mu\text{M}$). As illustrated in Fig. 4C, the effect of sunitinib on annexin-V-binding was slightly, but significantly blunted in the presence of zVAD ($10 \mu\text{M}$). Nevertheless, even in the presence of zVAD, sunitinib still significantly stimulated annexin-V-binding. Accordingly, the stimulation of erythrocyte membrane scrambling by sunitinib is in part due to caspase activation but may involve caspase independent additional mechanisms.

Discussion

According to the present observations, sunitinib stimulates suicidal erythrocyte death or eryptosis, which is characterized by erythrocyte membrane scrambling (Fig. 2) and erythrocyte shrinkage (Fig. 1). The concentration required for the effect on cell membrane scrambling is in the range of those required for kinase inhibition [39, 40] but an order of magnitude higher than plasma concentrations published during sunitinib treatment [39, 41]. Accordingly, relevant eryptosis is not expected during treatment with sunitinib. The present observations, however, show that sunitinib may trigger suicidal death in cells lacking mitochondria and nuclei, key elements in the machinery leading to apoptosis [42-46].

Sunitinib increases cytosolic Ca^{2+} activity (Fig. 3), an effect presumably due to activation of non-selective cation channels. As shown earlier [21], the Ca^{2+} permeable erythrocyte cation channels may be partially composed by TRPC6 [21]. The cation channels are known to be activated by oxidative stress [47]. Sunitinib has been reported to oxidize glutathione [19]. Thus, at least in theory, sunitinib could induce oxidative stress, which in turn activates the cation channels.

The increase of cytosolic Ca^{2+} activity is known to activate Ca^{2+} -sensitive K^+ channels [25, 48] leading to exit of K^+ following its chemical gradient, cell membrane hyperpolarization, Cl^- exit and thus cellular KCl loss, which is followed by osmotically obliged water and thus results in cell shrinkage [26].

An increase of cytosolic Ca^{2+} activity further triggers cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface [26, 27, 49]. Removal of extracellular Ca^{2+} significantly decreased, but did not fully abrogate the cell membrane scrambling effect of sunitinib (Fig. 4A). Thus, the effect of sunitinib on cell membrane scrambling must have been due to additional mechanism(s).

Sunitinib has previously been shown to lower ATP concentration in nucleated cells [19], which is in turn known to trigger eryptosis [29]. However, in the present study, we could not observe a significant effect of sunitinib on erythrocyte ATP levels. Sunitinib tended to increase ceramide formation, an effect failing, however, to reach statistical significance. Ceramide is known to sensitize the cell for the effects of cytosolic Ca^{2+} and to stimulate cell membrane scrambling in erythrocytes [28, 50]. Ceramide may further trigger apoptosis of

nucleated cells [51] and ceramide formation contributes to the pathophysiology of lung inflammation, fibrosis and infection [52], cystic fibrosis [53], cardiovascular disease [54, 55], Wilson's disease [56], multiple sclerosis [57], major depression [51], Parkinson's disease [58], Alzheimer's disease [51, 59, 60] and diabetes [61-63].

Sunitinib-induced eryptosis was sensitive to SB203580 (Fig. 4B), an inhibitor of p38 kinase, which has previously been shown to be involved in the regulation of eryptosis following osmotic shock [35]. Thus, sunitinib may, at least partially, be effective by increasing p38 kinase activity. In the presence of the p38 kinase inhibitor SB203580, the effect of sunitinib was blunted, but not abrogated, indicating that some additional mechanisms must be involved in sunitinib induced eryptosis. Besides Ca^{2+} and p38 kinase, mechanisms triggering cell membrane scrambling following sunitinib exposure may include activation of caspases, which have previously been shown to participate in the triggering of eryptosis. [30-34]. Accordingly, sunitinib induced eryptosis is blunted in the presence of pancaspase inhibitor zVAD (Fig. 4C).

Sunitinib triggers hemolysis, an effect affecting far less erythrocytes, however, than eryptosis. Moreover, the concentration required to yield significant hemolysis is higher than that resulting in significant eryptosis (Fig. 2).

A wide variety of further xenobiotics have been shown to trigger eryptosis [30, 35, 38, 64-73]. Moreover, eryptosis has been shown or suggested to participate in the pathophysiology of several clinical disorders [20], such as diabetes [34, 74, 75], renal insufficiency [76], hemolytic uremic syndrome [77], sepsis [78], sickle cell disease [79], malaria [56, 80-83], Wilson's disease [56], iron deficiency [84], phosphate depletion [85] and metabolic syndrome [86].

Phosphatidylserine exposing erythrocytes are phagocytosed and thus rapidly cleared from circulating blood [20]. Accordingly, excessive eryptosis may lead to anemia. Phosphatidylserine exposing erythrocytes may further stimulate blood clotting [87-89] and adhere to endothelial CXCL16/SR-PSO [90]. The adhesion of phosphatidylserine exposing erythrocytes to the vascular wall may interfere with microcirculation [87, 90-94]. Thus, at least in theory, excessive eryptosis may foster thrombosis [95] and the triggering of erythrocyte cell membrane scrambling could contribute to the known risk of thromboembolic events during sunitinib treatment [96].

In conclusion, sunitinib triggers Ca^{2+} entry, cell shrinkage as well as Ca^{2+} -, SB203580- and zVAD-sensitive cell membrane scrambling. Thus, similar to its effect on nucleated cells, sunitinib stimulates the suicidal death of erythrocytes.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by T. Loch. This study was supported by the Deutsche Forschungsgemeinschaft.

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