Triggering of Suicidal Erythrocyte Death by Ruxolitinib

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The JAK1/JAK2 tyrosine kinase inhibitor ruxolitinib is widely used for the treatment of myeloproliferative neoplasm-associated myelofibrosis and other malignancies. Most important side effects include anemia. A common cause of anemia is accelerated suicidal death of erythrocytes or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Mechanisms contributing to the triggering of eryptosis include oxidative stress, Ca\textsuperscript{2+} entry with increase of cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]\textsubscript{i}), and activation of distinct kinases, such as p38 mitogen activated protein (MAP) kinase. The present study explored whether and how ruxolitinib induces eryptosis. Methods: Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, hemolysis from hemoglobin release, [Ca\textsuperscript{2+}] from Fluo3-fluorescence, and ROS formation from DCFDA-dependent fluorescence. Results: A 48 hours exposure of human erythrocytes to ruxolitinib (25 \textmu M) significantly increased the percentage of annexin-V-binding cells and significantly decreased forward scatter. Ruxolitinib did not significantly modify Fluo3-fluorescence and DCFDA fluorescence and the effect of ruxolitinib on annexin-V-binding was not significantly modified by removal of extracellular Ca\textsuperscript{2+}. The effect of ruxolitinib on annexin-V-binding was, however, significantly blunted by the p38 MAP kinase inhibitor SB203580 and virtually abolished by the p38 MAP kinase inhibitor skepinone. Conclusion: Ruxolitinib triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect in part requiring p38 MAP kinase activity.

Introduction

Ruxolitinib, a JAK1/JAK2 tyrosine kinase inhibitor [1-15], is now widely used for the treatment of myeloproliferative neoplasm-associated myelofibrosis [1, 2, 5-11, 13, 14, 16-25], further myeloproliferative neoplasms [26], and refractory cancer [27]. Common side
effects of ruxolitinib treatment include anaemia and thrombocytopenia [3, 5, 6, 8-10, 12-14, 18]. On the other hand, ruxolitinib is successfully used for the treatment of polycythemia vera [28-31].

At least in theory, the decline of circulating erythrocytes following ruxolitinib treatment could result in part from stimulation of eryptosis, the suicidal erythrocyte death characterized by cell shrinkage [32] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [33]. Cellular mechanisms involved in the execution of eryptosis include oxidative stress, Ca\(^{2+}\) entry with increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)), ceramide [34], decline of cytosolic ATP [33], caspaces [33, 35, 36], stimulated activity of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [33], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [33].

The present study explored whether ruxolitinib triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with ruxolitinib and phosphatidylserine surface abundance, cell volume as well as [Ca\(^{2+}\)], and ROS formation, 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 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; pH 7.4), 5 glucose, 1 CaCl\(_2\) at 37°C for 24 h. Where indicated, erythrocytes were exposed to ruxolitinib (Selleckchem, Houston, USA) at the indicated concentrations.

**Annexin-V-binding and forward scatter**

After incubation under the respective experimental condition, 100 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). 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.

**Hemolysis**

For the determination of hemolysis, the samples were centrifuged (10 min at 2000 rpm, room temperature) after incubation under the respective experimental conditions 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.

**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 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed once 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.

**Reactive oxygen species (ROS)**

Oxidative stress was determined utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 100 µl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA.
(Sigma, Schnelldorf, Germany) in PBS containing DCFDA at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed in PBS. 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).

**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 explored whether and how ruxolitinib may trigger eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine at the erythrocyte surface was detected utilizing annexin-V-binding quantified by flow cytometry.

![Fig. 1](image1.png)

**Fig. 1.** Effect of ruxolitinib 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 25 µM ruxolitinib. (B) Arithmetic means ± SEM (n = 12) of erythrocyte annexin-V-binding (black bars) following incubation for 48 hours to Ringer solution without or with presence of ruxolitinib (1 - 25 µM). For comparison, arithmetic means ± SEM (n = 8) of hemolysis are shown (grey bars). # (p<0.05), *** (p<0.001) indicate significant difference from the absence of ruxolitinib (ANOVA).

![Fig. 2](image2.png)

**Fig. 2.** Effect of ruxolitinib 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 25 µM ruxolitinib. (B) Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) ruxolitinib (1 - 25 µM). ** (p<0.01), *** (p<0.001) indicate significant difference from the absence of ruxolitinib (ANOVA).
Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with ruxolitinib (1 - 25 µM). As illustrated in Fig. 1, a 48 hours treatment with 25 µM ruxolitinib significantly increased the percentage of phosphatidylserine exposing erythrocytes. For comparison, hemolysis was estimated from the hemoglobin concentration in the supernatant which was determined by photometry. As a result, 48 hours incubation with 25 µM ruxolitinib significantly increased the percentage of hemolytic erythrocytes (Fig. 1). The percentage of hemolytic erythrocytes remained, however, one order of magnitude lower than the percentage of annexin-V-binding erythrocytes (Fig. 1).

In order to estimate erythrocyte volume, forward scatter was quantified utilizing flow cytometry following 48 hours incubation in Ringer solution without or with ruxolitinib (1 – 25 µM). As shown in Fig. 2, ruxolitinib decreased erythrocyte forward scatter, an effect reaching statistical significance at 10 µM ruxolitinib concentration.

Fluo3 fluorescence was taken as measure of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)). As illustrated in Fig. 3, a 48 hours exposure to ruxolitinib up to 25 µM did not significantly modify the Fluo3 fluorescence. In order to test whether ruxolitinib-induced translocation of phosphatidylserine required entry of extracellular Ca\(^{2+}\), erythrocytes were incubated for 48 hours in the absence or presence of 25 µM ruxolitinib in the presence or nominal absence of extracellular Ca\(^{2+}\). As illustrated in Fig 3, removal of extracellular Ca\(^{2+}\) did not significantly interfere with the effect of ruxolitinib on annexin-V-binding. Instead, the effect of ruxolitinib
on the percentage of annexin-V-binding erythrocytes was virtually identical in the absence and presence extracellular Ca\textsuperscript{2+}. Thus, ruxolitinib-induced cell membrane scrambling did not lead to and did not require entry of extracellular Ca\textsuperscript{2+}.

In order to test for an effect of ruxolitinib on oxidative stress, reactive oxygen species (ROS) was quantified utilizing 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA). As a result, a 48 hours exposure to ruxolitinib (25 µM) did not significantly increase the DCFDA fluorescence, which approached 15.6 ± 2.5 (n = 4) without and 7.3 ± 0.4 (n = 4) with prior ruxolitinib (25 µM) treatment. Thus, ruxolitinib did not induce oxidative stress.

A next series of experiments explored whether ruxolitinib-induced translocation of phosphatidylserine or erythrocyte shrinkage required activity of p38 MAP kinase. To this end, erythrocytes were incubated for 48 hours in the absence or presence of p38 kinase inhibitors SB203580 (25 µM) or skepinone (2 µM). As illustrated in Fig 4, the effect of ruxolitinib on annexin-V-binding was significantly blunted by SB203580 (25 µM) and was virtually abolished by skepinone (2 µM). As illustrated in Fig. 5, the effect of ruxolitinib on forward scatter was slightly blunted by the p38 kinase inhibitors, an effect reaching statistical significance for skepinone. Collectively, the data suggest that p38 kinase activity is required for the stimulation of eryptosis by ruxolitinib. Additional experiments were performed to test whether the stimulation of hemolysis by ruxolitinib is similarly sensitive to p38 inhibition. As a result, the hemolysis following treatment with 25
µM ruxolitinib was similar in the absence (2.1 ± 0.4%, n = 8) and in the presence (2.2 ± 0.4%, n = 8) of p38 inhibitor skepinone (2 µM).

Discussion

The present observations reveal a novel effect of ruxolitinib, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to ruxolitinib results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentration required for the effect (10-25 µM) is only slightly higher than the peak concentrations observed in human plasma (approximately 8 µM) following the administration of 100 mg of the substance [37]. Thus, the observed stimulation of eryptosis may well contribute to the anemia following ruxolitinib intoxication. It must be further considered that the susceptibility to eryptosis is enhanced by several clinical conditions, including dehydration [38], hyperphosphatemia [39] chronic kidney disease (CKD) [40-43], hemolytic-uremic syndrome [44], diabetes [45], hepatic failure [46], malignancy [33], sepsis [47], sickle-cell disease [33], beta-thalassemia [33], Hb-C and G6PD-deficiency [33], as well as Wilsons disease [48]. Moreover, eryptosis is triggered by a wide variety of xenobiotics [33, 38, 39, 41, 49-71].
The effect of ruxolitinib on cell membrane scrambling and cell shrinkage was not due to increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(i\)) and apparently did not involve induction of oxidative stress (data not shown). Instead, ruxolitinib induced cell membrane scrambling and cell shrinkage apparently required the activity of p38 kinase, which has previously been shown to trigger eryptosis [33]. The mechanism leading to activation of p38 kinase following ruxolitinib treatment remained illdefined, but may well involve activation or inhibition of other kinases known to modify eryptosis [33], erythrocyte shrinkage and/or vesiculation [72], such as phosphoinositide 3-kinase (PI3K)-Akt (protein kinase B), Raf-MEK (mitogen-activated protein kinase kinase)-ERK (extracellular signal-regulated kinase), casein kinase 2 (CK2) Janus-activated kinase isoforms, protein kinase C, AMP activated kinase, cGMP-dependent protein kinase, and PAK2 kinase.

Eryptosis leads to clearance of defective erythrocytes from circulating blood prior to hemolysis [33] thus preventing release of hemoglobin, which is otherwise filtered in renal glomerula with subsequent precipitation in the acidic lumen of renal tubules and thus occlusion of nephrons [73]. Eryptosis further contributes to the clearance of erythrocytes infected with the malaria pathogen \textit{Plasmodium}, which activates eryptosis inducing Ca\(^{2+}\)-permeable erythrocyte cation channels [33, 74]. Eryptosis is enhanced by sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency, which thus lead to accelerated clearance of infected erythrocytes, decreased parasitemia and protection against a severe course of malaria [33, 75-77]. Enhanced eryptosis and blunted increase of parasitemia are further observed in iron deficiency [78], and treatment with lead [78], chlorpromazine [79] or NO synthase inhibitors [79]. Possibly ruxolitinib may similarly enhance the susceptibility of \textit{Plasmodium} infected erythrocytes to eryptosis.

Phosphatidylserine exposing erythrocytes may further adhere to the vascular wall [80], stimulate blood clotting, trigger thrombosis [81-83], and thus impair microcirculation [34, 81, 84-87].

In conclusion, ruxolitinib triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect requiring the activity of p38 mitogen activated protein (MAP) kinase.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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Cellular Physiology and Biochemistry
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