Original Paper

Oxaliplatin Induced Suicidal Death of Human Erythrocytes

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Oxidative stress • Calcium

Abstract

Background/Aims: The alkylating drug oxaliplatin is widely used for chemotherapy of malignancy. Oxaliplatin is effective by inducing both, necrosis and apoptosis. Similar to necrosis or apoptosis of nucleated cells, erythrocytes may enter hemolysis, which is apparent from hemoglobin release or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include oxidative stress and/or Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The present study explored, whether and how oxaliplatin induces eryptosis. **Methods:** Phosphatidylserine exposure at the cell surface was quantified utilizing annexin-V-binding, cell volume estimated from forward scatter, hemolysis deduced from hemoglobin release, $[\text{Ca}^{2+}]_i$ determined utilizing Fluo-3 fluorescence, and reactive oxygen species (ROS) abundance visualized using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) dependent fluorescence. **Results:** A 48 hours exposure of human erythrocytes to oxaliplatin (10 $\mu\text{g}/\text{ml}$) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter, significantly increased Fluo-3 fluorescence, and significantly increased DCFDA fluorescence. The effect of oxaliplatin on annexin-V-binding and forward scatter was rather augmented by removal of extracellular Ca^{2+} , but was significantly blunted in the presence of the antioxidant N-acetyl-cysteine (1 mM). **Conclusions:** Oxaliplatin triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect partially dependent on ROS formation.

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Introduction

Oxaliplatin, a platinum-type alkylating drug, is widely used for chemotherapy of malignancy [1-13]. Oxaliplatin is effective by inducing both, necrosis [14, 15] and apoptosis [4, 14-56]. Mechanisms involved include TRAIL [48], mitochondrial depolarization [15, 22,

32, 34, 37, 40, 56], caspase activation [15, 26, 53], receptor interacting protein 1 (RIP1) [15], p38 MAP kinase activity [17, 19] and transcription factor NF- κ B [20, 47].

In analogy to apoptosis of nucleated cells, erythrocytes may experience eryptosis [57], the suicidal death of erythrocytes characterized by cell shrinkage [58] and phospholipid scrambling of the cell membrane with translocation of phosphatidylserine to the cell surface [57]. Eryptosis could be triggered by oxidative stress [57] and opening of Ca^{2+} permeable unselective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [57]. Eryptosis is further triggered by activated caspases [57, 59, 60], stimulated activity of casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [57], as well as impaired activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, and sorafenib/sunitinib sensitive kinases [57]. Moreover, eryptosis is triggered by a wide variety of small molecules [57, 61-82].

The present study explored whether oxaliplatin triggers eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to oxaliplatin and phosphatidylserine abundance at the erythrocyte surface, cell volume, $[\text{Ca}^{2+}]_i$ and ROS abundance quantified utilizing 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 48 h. Where indicated, erythrocytes were exposed to oxaliplatin (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

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 20 min under protection from light. The annexin-V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). For identification of PS positive erythrocytes, annexin-V-FITC binding has been determined. 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".

Measurement of hemolysis

For the determination of 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 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 mM CaCl_2 and 5 $\mu\text{g}/\text{ml}$ 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 containing 1 mM CaCl_2 . 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 μ l suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in PBS containing DCFDA at a final concentration of 10 μ g/ml, leading to trapping of deacetylated derivative of DCFDA in the cells. 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 μ l 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). PBS was used instead of Ringer to stop the reaction.

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 oxaliplatin triggers eryptosis, the suicidal erythrocyte death characterized by erythrocyte shrinkage and phospholipid scrambling of the cell membrane with translocation of phosphatidylserine to the cell surface. Phosphatidylserine at the erythrocyte surface was detected utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements the erythrocytes were incubated for 48 hours in Ringer solution without or with oxaliplatin (1 - 10 μ g/ml). As illustrated in Fig. 1, a 48 hours treatment with oxaliplatin increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 10 μ g/ml oxaliplatin. For comparison hemolysis was estimated by photometric measurement of hemoglobin concentration in the supernatant. As shown in Fig. 1, following a 48 hours incubation, the

Fig. 1. Effect of oxaliplatin 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 10 μ g/ml oxaliplatin. (B) Arithmetic means $\pm$ SEM (*n* = 9) of erythrocyte annexin-V-binding (black bars) following incubation for 48 hours to Ringer solution without or with presence of oxaliplatin (1 - 10 μ g/ml). For comparison, arithmetic means $\pm$ SEM (*n* = 9) of hemolysis are shown (grey bars). *** (*p* < 0.001) indicate significant difference from the absence of oxaliplatin (ANOVA). (C) Arithmetic means $\pm$ SEM (*n* = 16) of the percentage annexin-V-binding erythrocytes as a function of exposure time to Ringer without (open circles) or with (closed circles) oxaliplatin (10 μ g/ml). * (*p* < 0.05), ** (*p* < 0.01) indicate significant differences from absence of oxaliplatin (ANOVA).

Fig. 2. Effect of oxaliplatin 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 10 µg/ml oxaliplatin. (B) Arithmetic means $\pm$ SEM ($n = 9$) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) oxaliplatin (1 - 10 µg/ml). * ($p < 0.05$) indicates significant difference from the absence of oxaliplatin (ANOVA).

Fig. 3. Effect of oxaliplatin on erythrocyte Ca^{2+} activity. (A) Original histogram of Fluo-3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of oxaliplatin (10 µg/ml). (B) Arithmetic means $\pm$ SEM ($n = 14$) of the Fluo-3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) oxaliplatin (1 - 10 µg/ml). * ($p < 0.05$), *** ($p < 0.001$) indicate significant difference from the absence of oxaliplatin (ANOVA).

percentage of hemolytic erythrocytes increased slightly, an effect, however, not reaching statistical significance. In order to define the effect of oxaliplatin on erythrocyte volume, forward scatter was determined utilizing flow cytometry. The measurements were made following a 48 hours incubation in Ringer solution without or with oxaliplatin (1 - 10 µg/ml). As illustrated in Fig. 2, oxaliplatin decreased erythrocyte forward scatter, an effect reaching statistical significance at 10 µg/ml oxaliplatin concentration. Fluo-3 fluorescence was determined in order to estimate cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 3, a 48 hours exposure to oxaliplatin increased the Fluo-3 fluorescence, an effect reaching statistical significance at 5 µg/ml oxaliplatin. In order to test, whether oxaliplatin -induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+} , erythrocytes were incubated for 48 hours in the absence or presence of 10 µg/ml oxaliplatin in the presence or nominal absence of extracellular Ca^{2+} . As shown in Fig. 4, removal of extracellular Ca^{2+} significantly increased the effect of oxaliplatin on annexin-V-binding. Removal of extracellular Ca^{2+} further significantly enhanced the effect of oxaliplatin on forward scatter (Fig. 5).

In order to test, whether oxaliplatin induces oxidative stress, reactive oxygen species (ROS) was quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). As

Fig. 4. Ca²⁺ sensitivity of oxaliplatin-induced phosphatidylserine exposure. (A, B) 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 oxaliplatin (10 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. (C) Arithmetic means ± SEM (n = 17) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) oxaliplatin (10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. *** (p < 0.001) indicates significant difference from the absence of oxaliplatin, ##(p < 0.01) indicate significant difference from the presence of Ca²⁺ (ANOVA).

Fig. 5. Ca²⁺ sensitivity of oxaliplatin-induced erythrocyte shrinkage. (A, B) Original histogram of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of oxaliplatin (10 µg/ml) in the presence (A) and absence (B) of extracellular Ca²⁺. (C) Arithmetic means ± SEM (n = 12) of erythrocyte forward scatter after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) oxaliplatin (10 µg/ml) in the presence (left bars, +Ca²⁺) and absence (right bars, -Ca²⁺) of Ca²⁺. * (P < 0.05) *** (P < 0.001) indicates significant difference from the absence of oxaliplatin, ### (p < 0.001) indicate significant difference from the presence of Ca²⁺ (ANOVA).

illustrated in Fig. 6 a 48 hours exposure to oxaliplatin (1 - 10 µg/ml) increased the DCFDA fluorescence, an effect reaching statistical significance at 10 µg/ml oxaliplatin concentration. Accordingly, oxaliplatin induced oxidative stress. A next series of experiments explored whether oxaliplatin-induced translocation of phosphatidylserine or erythrocyte shrinkage required oxidative stress. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 10 µg/ml oxaliplatin in the absence or presence of the antioxidant N-acetylcysteine (1 mM). As illustrated in Fig. 7, addition of N-acetylcysteine (1 mM) significantly blunted the effect of oxaliplatin on annexin-V-binding. N-acetylcysteine (1 mM) did not significantly blunt the slight effect of oxaliplatin on forward scatter. The decrease of forward scatter following oxaliplatin treatment was similar in the absence (from 412 ± 12 to 393 ± 19, n = 5) and presence (from 399 ± 16 to 391 ± 14, n = 5) of N-acetylcysteine. The difference was not significant.

Fig. 6. Effect of oxaliplatin on erythrocyte ROS formation. (A) Original histogram of DCFDA fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of oxaliplatin (10 µg/ml). (B) Arithmetic means $\pm$ SEM ($n = 14$) of the DCFDA fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) oxaliplatin (1 – 10 µg/ml). ** ($p < 0.01$) indicate significant difference from the absence of oxaliplatin (ANOVA).

Fig. 7. Sensitivity of oxaliplatin -induced phosphatidylserine exposure to antioxidant N-acetyl-cysteine. (A, B) 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 oxaliplatin (10 µg/ml) in the absence (A) and presence (B) of 1 mM N-acetylcysteine. (C) Arithmetic means $\pm$ SEM ($n = 12$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) oxaliplatin (10 µg/ml) in the absence (left bars, -NAC) and presence (+NAC 0.5mM, +NAC 1mM, +NAC 2mM) of N-acetylcysteine. ** ($P < 0.01$) indicates significant difference from the absence of oxaliplatin, # ($p < 0.05$) indicate significant difference from the presence of N-acetylcysteine (ANOVA).

Discussion

The present observations reveal that oxaliplatin stimulates the suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to oxaliplatin is followed by the two hallmarks of eryptosis, i.e. cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for the toxic effect are only slightly higher than those reached in plasma following chemotherapy with oxaliplatin [83]. The present study was performed with erythrocytes from healthy individuals. The sensibility to stimulators of eryptosis is enhanced in diverse clinical conditions, such as dehydration [70], hyperphosphatemia [80] chronic kidney disease (CKD) [65, 84-86], hemolytic-uremic syndrome [87], diabetes [88], hepatic failure [89], malignancy [57], sepsis [90], sickle-cell disease [57], beta-thalassemia [57], Hb-C and G6PD-deficiency [57], as well as Wilsons

disease [91]. In patients with those disorders eryptosis may be triggered by lower oxaliplatin concentrations.

In addition to triggering cell membrane scrambling and cell shrinkage oxaliplatin increased cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), which is known to trigger cell membrane scrambling [57] and to trigger erythrocyte shrinkage [58]. Surprisingly, however, cell membrane scrambling and cell shrinkage were augmented by removal of extracellular Ca^{2+} , indicating that the presence of extracellular Ca^{2+} could attenuate oxaliplatin induced eryptosis. The eryptosis following exposure to a wide variety of xenobiotics is blunted or even abolished following removal of extracellular Ca^{2+} , indicating that their effect partially or fully depended on entry of extracellular Ca^{2+} [57]. The mechanism underlying the opposite role of Ca^{2+} following oxaliplatin exposure, remained elusive.

Oxaliplatin treatment further increased the abundance of reactive oxygen species. Moreover, oxaliplatin induced cell membrane scrambling was attenuated by the antioxidant N-acetylcysteine. Accordingly oxaliplatin induced eryptosis at least in part due to oxidative stress.

Stimulation of eryptosis leads to rapid clearance of affected erythrocytes from circulating blood and thus to anemia as soon as the loss of erythrocytes is not matched by a similar increase of erythropoiesis [57]. Moreover, phosphatidylserine exposing erythrocytes adhere to the vascular wall [92], stimulate blood clotting and thrombosis [93-95] and thus interfere with microcirculation [93, 96-100]. At least in theory those effects may contribute to the side effects of oxaliplatin.

Conclusion

Oxaliplatin stimulates eryptosis with cell shrinkage and cell membrane scrambling, an effect in part due to oxidative stress.

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Disclosure Statement

The authors declare that they have nothing to disclose.

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