Original Paper

Inhibition of Suicidal Erythrocyte Death by Reversine

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Key Words

Phosphatidylserine • Eryptosis • Reversine • Energy depletion • Oxidative stress • Calcium

Abstract

Background/Aims: The A3 adenosine receptor antagonist reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine) influences cellular differentiation, inhibits cell proliferation, induces cell-cycle arrest, triggers apoptosis, causes cell swelling with polyploidy and stimulates autophagy. The effect on apoptosis involves mitochondria and caspases. Erythrocytes are lacking mitochondria but express caspases and are, similar to apoptosis of nucleated cells, able to enter suicidal erythrocyte death or eryptosis. Stimulators of eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), energy depletion and oxidative stress. The present study explored, whether reversine influences eryptosis. **Methods:** Flow cytometry was employed to quantify phosphatidylserine exposure at the cell surface from annexin-V-binding and cell volume from forward scatter. Measurements were made without or with energy depletion (glucose deprivation for 48 hours), Ca^{2+} loading (30 minutes treatment with $1\ \mu\text{M}$ Ca^{2+} ionophore ionomycin), or oxidative stress (15 min exposure to $0.3\ \text{mM}$ tert-butylhydroperoxide). **Results:** A 48 hours exposure of human erythrocytes to reversine ($1\text{--}10\ \mu\text{M}$) did not significantly modify the percentage of annexin-V-binding cells and forward scatter. Energy depletion, Ca^{2+} loading, and oxidative stress were each followed by profound and significant increase of the percentage annexin-V-binding erythrocytes and a significant decrease of forward scatter. The effects of each, Ca^{2+} loading, energy depletion and oxidative stress on annexin-V-binding were significantly blunted in the presence of reversine ($1\text{--}10\ \mu\text{M}$). The effect of ionomycin, but not the effects of energy depletion and oxidative stress on forward scatter were again significantly blunted in the presence of reversine ($\geq 1\ \mu\text{M}$). **Conclusions:** Reversine is a powerful inhibitor of cell membrane scrambling following energy depletion, Ca^{2+} loading and oxidative stress.

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Introduction

The purine analog [1-9] and A3 adenosine receptor antagonist [10, 11], reversine (2-(4-morpholinoanilino)-6-cyclohexylaminopurine) reverses differentiation of lineage-committed cells to mesenchymal stem cells (MSCs) allowing them to undergo differentiation

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into other various lineages [1, 4, 6-10, 12-21]. Reversine has been shown to inhibit cell proliferation [18, 22], to induce cell-cycle arrest at the G2/M phase [1, 3, 22-24], to trigger apoptosis [3, 5, 10, 23-27], to induce cell swelling with polyploidy [1, 9, 23, 27], and to stimulate autophagy [3, 10, 24].

Cellular mechanisms sensitive to reversine include mitochondria [22], caspases [5, 22], Monopolar spindle 1 (Mps1) kinase [27-29], aurora kinase A and B [17, 25, 27, 28], and Akt/mTOR [3, 30].

Erythrocytes are devoid of mitochondria but express caspases [31-33] and may enter suicidal death or eryptosis, which is characterized by cell membrane scrambling with phosphatidylserine translocation to the cell surface [33]. The cell membrane scrambling is typically paralleled by cell shrinkage [34]. Triggers of eryptosis include oxidative stress [33], energy depletion [33], and exposure to a wide variety of xenobiotics [33, 35-88]. Cellular mechanisms involved include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [33], ceramide [89], G-protein Galphai2 [90], and several kinases including activated casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [33], or inactivated AMP activated kinase AMPK, cGMP-dependent protein kinase, PAK2 kinase, mitogen and stress activated kinase MSK1/2 [91] and sorafenib/sunitinib sensitive kinases [33].

The present study explored, whether reversine is able to trigger eryptosis and/or is able to modify eryptosis triggered by cellular Ca^{2+} load, energy depletion or oxidative stress. To this end, human erythrocytes from healthy volunteers were exposed to Ca^{2+} ionophore ionomycin, glucose deprivation or the oxidant tert-butyl-hydroperoxide and phosphatidylserine surface abundance as well as cell volume 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 48 hours. Where indicated, erythrocytes were exposed for 48 hours to glucose containing or glucose depleted Ringer solution, for 30 minutes to Ca^{2+} ionophore ionomycin (1 μM , Merck Millipore, Darmstadt, Germany) or for 15 minutes to the oxidant tert-butyl-hydroperoxide (0.3 mM, Sigma Aldrich, Hamburg, Germany), each in the absence and presence of reversine (1-10 μM , Sigma Aldrich, Hamburg, Germany). We decided to use the 1, 5 and 10 μM range of concentration. Previous studies used reversine within the range of 0.1 to 100 μM on human colon carcinoma [27], thyroid cancer cells [5], breast cancer cells [23], hepatic stellate cells [22], lung cancer cells [10], oral squamous carcinoma [24] and myeloid leukemia [25].

Annexin-V-binding, forward scatter and side scatter

After incubation under the respective experimental condition, a 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 annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-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 reversine treated erythrocytes. 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".

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 reversine stimulates or interferes with eryptosis, the suicidal erythrocyte death. The two hallmarks of eryptosis, i.e. cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface were determined by flow cytometry. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding to phosphatidylserine, cell shrinkage was quantified utilizing forward scatter. Moreover, we explored the effect of reversine on cell granularity using the side scatter.

In a first series of experiments erythrocytes were incubated for 48 hours in the absence or presence of reversine. As illustrated in Fig. 1, the percentage of annexin-V-binding erythrocytes was low following a 48 hours exposure to glucose containing Ringer solution with or without addition of reversine (1 – 10 μ M). Accordingly, up to 48 hours, reversine did not appreciably modify phosphatidylserine abundance at the erythrocyte surface.

As further shown in Fig. 1, a 48 hours exposure to glucose depleted Ringer solution was followed by a marked increase of the percentage of annexin-V-binding erythrocytes. The addition of reversine (1-10 μ M) significantly blunted the increase of the percentage of annexin-V-binding erythrocytes following energy depletion. However, even in the presence of reversine, energy depletion significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 1).

Fig. 1. Reversine sensitivity of phosphatidylserine exposure following energy depletion. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to glucose containing Ringer solution (grey area), Ringer solution without glucose (black line) and Ringer solution without glucose in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM (n = 10) of the percentage annexin-V-binding erythrocytes after a 48 hours treatment with Ringer solution with (left bars) or without (right bars) glucose in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the presence of glucose, ###($p < 0.001$) indicates significant difference from the absence of reversine (ANOVA).

Fig. 2. Reversine sensitivity of cell shrinkage following energy depletion. A. Original histograms of erythrocyte forward scatter following exposure for 48 hours to Ringer solution (grey area), Ringer solution without glucose (black line) and Ringer solution without glucose in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM (n = 10) of erythrocyte forward scatter after a 48 hours treatment with Ringer solution with (left bars) or without (right bars) glucose in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the presence of glucose (ANOVA).

The erythrocyte forward scatter was, following a 48 hours exposure to Ringer solution in the presence of glucose, virtually identical in the absence and presence of reversine (1 – 10 μ M).

Moreover, the erythrocyte side scatter in arbitrary units (a.u.) was virtually identical in the absence of reversine (201 ± 15 a.u., $n = 20$), at 1 μ M reversine (195 ± 17 a.u., $n = 20$), at 5 μ M reversine (203 ± 20 a.u., $n = 20$) and at 10 μ M reversine (188 ± 18 a.u., $n = 20$).

Accordingly, up to 48 hours, reversine did not appreciably modify erythrocyte volume (Fig. 2). A 48 hours exposure to glucose-depleted Ringer solution was followed by a marked decrease of forward scatter.

Again, the erythrocyte forward scatter was, following a 48 hours exposure to Ringer solution in the absence of glucose, virtually identical in the absence and presence of reversine (1 – 10 μ M). Accordingly, neither in energy replete nor in energy depleted erythrocytes did reversine influence significantly the erythrocyte volume.

Exposure of the erythrocytes for 30 minutes to 1 μ M ionomycin was followed by a sharp increase of the percentage of annexin-V-binding erythrocytes (Fig. 3). The effect was blunted in the presence of reversine, an effect reaching statistical significance at 5 μ M reversine. However, even in the presence of 10 μ M reversine, ionomycin significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 3).

Exposure of the erythrocytes for 30 minutes to 1 μ M ionomycin was followed by a sharp decrease of forward scatter (Fig. 4). The cell shrinkage was blunted in the presence of reversine, an effect reaching statistical significance at 5 μ M reversine. However, even in the presence of 10 μ M reversine, ionomycin significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 4).

Fig. 3. Reversine sensitivity of phosphatidylserine exposure following Ca^{2+} loading. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 30 minutes to Ringer solution (grey area), Ringer solution with 1 μ M ionomycin (black line) and Ringer solution with 1 μ M ionomycin in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM ($n = 10$) of the percentage annexin-V-binding erythrocytes after a 30 minutes treatment with Ringer solution without (left bars) or with (right bars) 1 μ M ionomycin in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the absence of ionomycin, ###($p < 0.001$) indicates significant difference from the absence of reversine (ANOVA).

Fig. 4. Reversine sensitivity of cell shrinkage following Ca^{2+} loading. A. Original histograms of erythrocyte forward scatter following exposure for 30 minutes to Ringer solution (grey area), Ringer solution with 1 μ M ionomycin (black line) and Ringer solution with 1 μ M ionomycin in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM ($n = 10$) of erythrocyte forward scatter after a 30 minutes treatment with Ringer solution without (left bars) or with (right bars) 1 μ M ionomycin in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the absence of ionomycin, ###($p < 0.001$) indicates significant difference from the absence of reversine (ANOVA).

Fig. 5. Reversine sensitivity of phosphatidylserine exposure following oxidative stress. A. Original histograms of annexin-V-binding of erythrocytes following exposure for 15 minutes to Ringer solution (grey area), Ringer solution with 0.3 mM tert-butyl-hydroperoxide (t-BOOH) (black line) and Ringer solution with 0.3 mM tert-butyl-hydroperoxide (t-BOOH) in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM (n = 10) of the percentage annexin-V-binding erythrocytes after a 15 minutes treatment with Ringer solution without (left bars) or with (right bars) 0.3 mM tert-butyl-hydroperoxide (t-BOOH) in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the absence of t-BOOH, ##($p < 0.01$) indicates significant difference from the absence of reversine (ANOVA).

Figure 5 consists of two panels. Panel A is a flow cytometry histogram showing cell counts (y-axis, 0 to 500) versus Annexin V fluorescence (arb. units) on a logarithmic x-axis (10⁰ to 10⁴). It displays four curves: Ionomycin + Reversine (red line), Ionomycin (grey area), DMSO (black line), and Annexin V + (dotted line). Panel B is a bar graph showing the percentage of Annexin V binding (y-axis, 0 to 30) for different concentrations of Reversine (0, 1, 5, 10 μ M) under two conditions: -tBOOH and +tBOOH. The bars are white for -tBOOH and black for +tBOOH. Statistical significance is indicated by asterisks (***) and hash marks (##).

Fig. 6. Reversine sensitivity of cell shrinkage following oxidative stress. A. Original histograms of erythrocyte forward scatter following exposure for 15 minutes to Ringer solution (grey area), Ringer solution with 0.3 mM tert-butyl-hydroperoxide (t-BOOH) (black line) and Ringer solution with 0.3 mM tert-butyl-hydroperoxide (t-BOOH) in the presence of reversine (10 μ M) (red line). B. Arithmetic means $\pm$ SEM (n = 10) of erythrocyte forward scatter after a 15 minutes treatment with Ringer solution without (left bars) or with (right bars) 0.3 mM tert-butyl-hydroperoxide (t-BOOH) in the absence (white bars) and presence (black bars) of reversine (1-10 μ M). ***($p < 0.001$) indicates significant difference from the absence of t-BOOH (ANOVA).

Figure 6 consists of two panels. Panel A is a flow cytometry histogram showing cell counts (y-axis, 0 to 500) versus Forward Scatter (arb. units) on a linear x-axis (0 to 1000). It displays four curves: Ionomycin + Reversine (red line), Ionomycin (grey area), DMSO (black line), and Annexin V + (dotted line). Panel B is a bar graph showing Forward Scatter (FSC) (y-axis, 0 to 600) for different concentrations of Reversine (0, 1, 5, 10 μ M) under two conditions: -tBOOH and +tBOOH. The bars are white for -tBOOH and black for +tBOOH. Statistical significance is indicated by asterisks (***) and hash marks (##).

Similar to what has been observed following ionomycin treatment, exposure of the erythrocytes for 15 minutes to 0.3 mM tert-butyl-hydroperoxide was followed by a sharp increase of the percentage of annexin-V-binding erythrocytes (Fig. 5). The effect was slightly blunted in the presence of reversine, an effect reaching statistical significance at 10 μ M reversine. In the presence of 10 μ M reversine, tert-butyl-hydroperoxide still significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 5).

Exposure of the erythrocytes for 15 minutes to 0.3 mM tert-butyl-hydroperoxide was further followed by a decrease of forward scatter (Fig. 6). The effect was not significantly modified by reversine and in the presence of 1-10 μ M reversine, tert-butyl-hydroperoxide significantly increased the percentage of phosphatidylserine exposing erythrocytes (Fig. 6).

Discussion

The present observations reveal the somewhat unexpected inhibitory effect of reversine on eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface [33].

Reversine itself did not appreciably modify the percentage of phosphatidylserine exposing erythrocytes and cell volume, but significantly blunted the cell membrane scrambling following energy depletion, Ca²⁺ loading and oxidative stress. All three maneuvers are well known stimulators of eryptosis [33].

Reversine further blunted the cell shrinkage following ionomycin treatment. Ionomycin increases cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [33]. Reversine fails to significantly blunt the cell shrinkage following energy depletion or oxidative stress. The mechanisms accounting for the reversine resistance of cell shrinkage following energy depletion and oxidative stress remained elusive.

At least in theory, the inhibition of eryptosis by reversine could be therapeutically exploited in the treatment of anemia due to enhanced eryptosis. Enhanced eryptosis is observed in diverse clinical conditions including iron deficiency [33], dehydration [92], hyperphosphatemia [93], vitamin D excess [94], chronic kidney disease (CKD) [95-100], hemolytic-uremic syndrome [101], diabetes [102], hepatic failure [103, 104], malignancy [33, 105, 106], sepsis [107], sickle-cell disease [33], beta-thalassemia [33], Hb-C and G6PD-deficiency [33], as well as Wilson's disease [107]. The enhanced eryptosis leads to rapid clearance of the phosphatidylserine exposing erythrocytes from circulating blood [33]. Overt anemia is observed as soon as the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [33]. Excessive eryptosis may further interfere with microcirculation [89, 108-112], as phosphatidylserine exposing erythrocytes adhere to the vascular wall [113], stimulate blood clotting and trigger thrombosis [108, 114, 115]. Inhibitors of eryptosis may counteract anemia and impairment of microcirculation in the above clinical conditions. It must be kept in mind, however, that inhibition of eryptosis may prevent the clearance of defective erythrocytes from circulating blood. The defective erythrocytes may instead undergo hemolysis [33] with release of hemoglobin which may pass the renal glomerular filter, precipitate in the acidic lumen of renal tubules, occlude nephrons and thus lead to renal failure [116].

In contrast to its effect on eryptosis, reversine stimulates apoptosis [3, 5, 10, 23-27]. Mechanisms involved in the stimulation of apoptosis by reversine include inhibition of Akt/mTORC1 signaling [88], caspase activation [5, 10, 22, 88], mitochondrial depolarization [22], autophagy [3, 10, 88] and mitotic catastrophe [20, 25, 27].

In conclusion, reversine suppresses the stimulation of cell membrane scrambling following energy depletion, Ca^{2+} overload and oxidative stress. Reversine further interferes with the triggering of cell shrinkage following Ca^{2+} overload, but not following energy depletion and oxidative stress. The potential therapeutic implications of the present observations remain to be defined.

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Disclosure Statement

The authors have no competing interests to declare.

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