Original Paper

Suicidal Death of Human Erythrocytes Following Exposure to Pentostatin

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Key Words

Phosphatidylserine • Cell membrane scrambling • RBC • Cell volume • Calcium • ATP

Abstract

Background/Aims: Pentostatin (2′-deoxycoformycin), a purine analog, is used for the treatment of diverse B and T-cell malignancies as well as for immunosuppression. Pentostatin is at least in part effective by triggering apoptosis. Pentostatin sensitive mechanisms leading to apoptosis include accumulation of DNA strand breaks, altered transcription and mitochondrial depolarization. Erythrocytes lack nuclei and mitochondria but nevertheless may enter eryptosis, an apoptosis-like suicidal cell death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$), ceramide formation and energy depletion. The present study tested, whether and how pentostatin induces eryptosis. ***Methods:*** The phosphatidylserine exposure at the cell surface was estimated from annexin V binding, cell volume from forward scatter, hemolysis from hemoglobin release, $[Ca^{2+}]_i$ from Fluo3-fluorescence, ceramide abundance utilizing specific antibodies, and cytosolic ATP concentration utilizing a luciferin–luciferase assay kit. ***Results:*** A 48 hours exposure of human erythrocytes to pentostatin (≥5 µg/ml) significantly increased the percentage of annexin-V-binding cells and significantly decreased forward scatter. Pentostatin significantly increased $[Ca^{2+}]_i$, and significantly decreased cytosolic ATP, but did not significantly modify ceramide abundance. The effect of pentostatin on annexin-V-binding was significantly blunted, but not abolished by removal of extracellular Ca^{2+}. ***Conclusion:*** pentostatin triggers erythrocyte shrinkage and phospholipid scrambling of the erythrocyte cell membrane, effects paralleled by and at least partially due to entry of extracellular Ca^{2+} and cellular energy depletion.

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Introduction

Pentostatin (2'-deoxycoformycin), a purine analog and specific adenosine deaminase inhibitor [1-6], is effective against a variety of B- and T-cell malignancies [6-11] including hairy cell leukemia [1-3, 12-17], chronic lymphocytic leukemia [1, 4, 18, 19], lymphoplasmacytic lymphoma [8], cutaneous T-cell lymphoma [20], low grade non-Hodgkin's lymphoma [5], and autoimmune lymphoproliferative syndrome [21]. Moreover, the drug has been used as immunosuppressant [22, 23] and treatment of graft-versus-host disease [23, 24]. Pentostatin has further antiviral and antimalarial potency, and protects CNS cells against ischemic and hypoxic injury [5].

Pentostatin is at least in part effective by triggering apoptosis [3, 11, 24]. Pentostatin sensitive cellular mechanisms include accumulation of DNA strand breaks [3], activation of transcription factor p53 [3], release of cytochrome c from mitochondria, [3], activation of poly(ADP-ribose) polymerase (PARP) [3], and cellular depletion of nicotinamide adenine dinucleotide (NAD) and ATP [3].

Even though lacking nuclei and mitochondria, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage [25] and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface [26]. Cellular mechanisms involved in the stimulation of eryptosis include oxidative stress [26], Ca^{2+} entry with increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [26], ceramide [27], energy depletion [26], activated caspases [26, 28, 29], enhanced activity of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, p38 kinase and PAK2 kinase [26], as well as decreased activity of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [26].

The present study explored, whether and how pentostatin may stimulate eryptosis. To this end phosphatidylserine surface abundance and cell volume were determined by flow cytometry in human erythrocytes from healthy volunteers without and with prior exposure to pentostatin. Additional experiments addressed the influence of pentostation on $[Ca^{2+}]_i$, ceramide abundance ands cyotsolic ATP levels.

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) 5 glucose, 1 $CaCl_2$, 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to pentostatin (Sigma Aldrich, Hamburg, Germany) at the indicated concentrations.

Determination 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 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". The cells were analyzed by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

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.

Determination 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 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 and the threshold was set at the default value of "52".

Determination of ceramide formation

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. 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 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Determination of intracellular ATP concentration

For the determination of intracellular erythrocyte ATP, 90 µl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without pentostatin (10 µg/ml). 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 a 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.
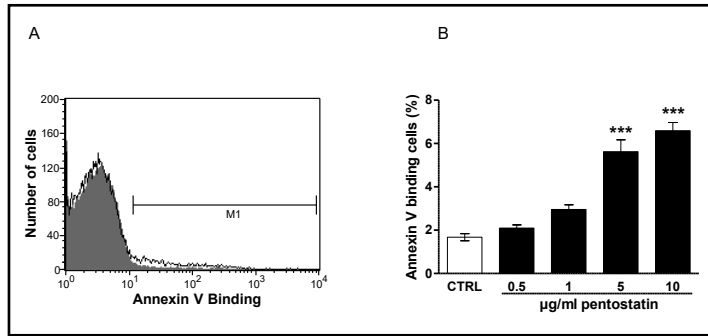
Statistics

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.
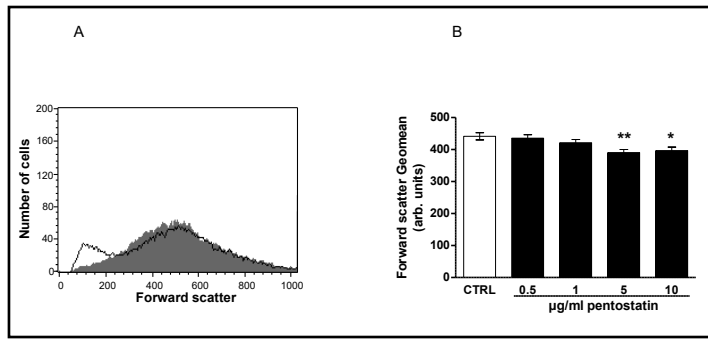
Results

The present study tested, whether pentostatin stimulates suicidal erythrocyte death or eryptosis. Hallmarks of eryptosis include cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 48 hours in Ringer solution without or with pentostatin (0.5 - 10 µg/ml). As illustrated in Fig. 1, a 48 hours exposure to pentostatin increased the percentage of annexin-V-binding, an effect reaching statistical significance at 5 µg/ml pentostatin concentration.

Fig. 1. Effect of pentostatin 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 µg/ml pentostatin. The marker M1 indicates the annexin-V positive erythrocytes. 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 pentostatin (0.5 - 10 µg/ml). ***(p<0.001) indicates significant difference from the absence of pentostatin (ANOVA).

Fig. 2. Effect of pentostatin 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 pentostatin. 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) pentostatin (0.5 - 10 µg/ml). *(p<0.05), **(p<0.01) indicate significant difference from the absence of pentostatin (ANOVA).
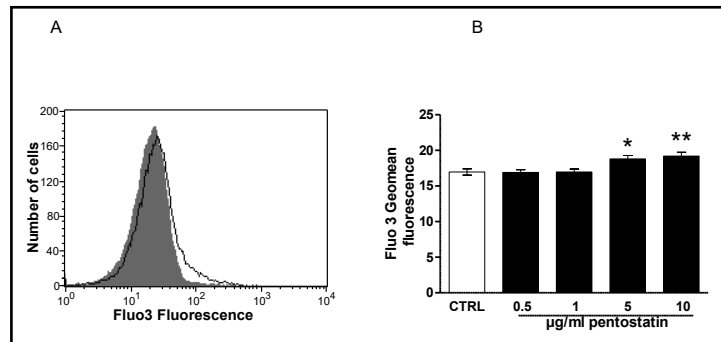

In order to quantify hemolysis, the hemoglobin concentration was determined in the supernatant by photometry. As a result, the percentage of hemolytic erythrocytes was after a 48 hours incubation similar in the absence of pentostatin (1.29 ± 0.42, n = 12) and in the presence of 0.5 µg/ml (1.24 ± 0.45, n = 12), 1 µg/ml (1.12 ± 0.29, n = 12), 5 µg/ml (1.07 ± 0.26, n = 12) and 10 µg/ml (1.36 ± 0.37, n = 12) pentostatin. Thus, pentostatin (0.5 - 10 µg/ml) did not significantly induce hemolysis.

The second hallmark of eryptosis is cell shrinkage. In order to estimate cell volume, forward scatter was determined utilizing flow cytometry following a 48 hours incubation in Ringer solution without or with pentostatin (0.5 – 10 µg/ml). As shown in Fig. 2, pentostatin decreased erythrocyte forward scatter, an effect reaching statistical significance at 5 µg/ml pentostatin concentration.
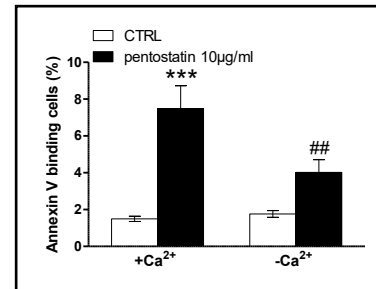
In order to determine, whether pentostatin modifies cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), Fluo3 fluorescence was taken as measure of $[Ca^{2+}]_i$. As shown in Fig. 3, a 48 hours exposure to pentostatin increased the Fluo3 fluorescence, an effect reaching statistical significance at 5 µg/ml pentostatin concentration.

A next series of experiments explored whether pentostatin-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca^{2+}. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 10 µg/ml pentostatin in the presence or nominal absence of extracellular Ca^{2+}. As illustrated in Fig. 4, removal of extracellular Ca^{2+} significantly blunted the effect of pentostatin on annexin-V-binding. Thus, pentostatin induced cell membrane scrambling was in large part due to entry of extracellular Ca^{2+}.
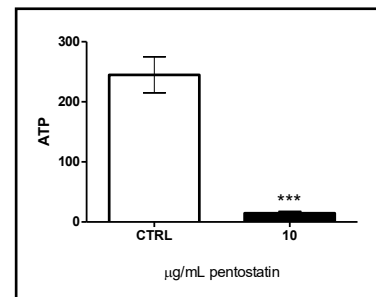
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Fig. 3. Effect of pentostatin on erythrocyte Ca^{2+} activity. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of pentostatin (10 μg/ml). B. Arithmetic means ± SEM (n = 20) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) pentostatin (0.5 – 10 μg/ml). *(p<0.05) **(p<0.01) indicate significant difference from the absence of pentostatin (ANOVA).

Fig. 4. Ca^{2+} sensitivity of pentostatin-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 pentostatin (10 μg/ml) in the presence (A) and absence (B) of extracellular Ca^{2+}. Arithmetic means ± SEM (n = 11) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) pentostatin (10 μg/ml) in the presence (left bars, $+Ca^{2+}$) and absence (right bars, $-Ca^{2+}$) of Ca^{2+}. ***(P<0.001) indicates significant difference from the absence of pentostatin, ##(p<0.01) indicates significant difference from the presence of Ca^{2+} (ANOVA).

Fig. 5. Effect of pentostatin on erythrocyte ATP levels. Arithmetic means ± SEM (n = 4) of cellular ATP (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) pentostatin (10 μg/ml). ***(p<0.001) indicates significant difference from the absence of pentostatin (ANOVA).

Stimulators of eryptosis in the absence of increased $[Ca^{2+}]_i$ include ceramide. Thus, specific antibodies were utilized to quantify ceramide abundance at the erythrocyte surface. As a result, the percentage of ceramide exposed erythrocytes was similar following a 48 hours incubation in the absence (12.76 ± 0.94, n = 11) and presence (13.65 ± 1.16, n = 11) of 10 μg/ml pentostatin.

Eryptosis is further stimulated by energy depletion. A luciferin–luciferase assay was employed to determine the cytosolic ATP level. As illustrated in Fig. 5, a 48 hours exposure to 10 μg/ml significantly decreased the cytosolic ATP concentration.

Discussion

The present observations reveal a novel effect of pentostatin, i.e. the triggering of erythrocyte death or eryptosis. Pentostatin treatment of human erythrocytes drawn from healthy individuals is followed by cell shrinkage and phosphatidylserine translocation to

the erythrocyte surface, the two hallmarks of eryptosis [26]. The pentostatin concentration (5 µg/ml) required for statistically significant stimulation of erythrocyte cell membrane scrambling was higher than the plasma concentrations of 0.5 µg/ml or 0.78 µg/ml [30] observed in patients. Moreover, *in vivo*, the pentostatin concentration may be lowered by plasma protein binding [30, 31]. Thus, pentostatin may induce eryptosis only after pentostatin intoxicaton rather than at therapeutic dosage. On the other hand, the possibility must be kept in mind that the susceptibility to triggers of eryptosis is enhanced by eryptosis-inducing clinical disorders. Eryptosis is induced by a wide variety of xenobiotics [26] and in several clinical conditions including dehydration [32], hyperphosphatemia [33] chronic kidney disease (CKD) [34-37], Hemolytic-uremic syndrome [38], diabetes [39], liver failure [40], malignancy [41], sepsis [42] Sickle-cell disease [40], beta-thalassemia [40], Hb-C [40], G6PD-deficiency [40] and Wilsons disease [43].

The effects of pentostatin on cell membrane scrambling and cell shrinkage was apparently in part due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), as they were significantly blunted by removal of extracellular Ca^{2+}. An increase of $[Ca^{2+}]_i$ triggers cell membrane scrambling by simulating an illdefined scramblase [26]. An increase of $[Ca^{2+}]_i$ further triggers erythrocyte shrinkage by activation of Ca^{2+} sensitive K^+ channels with subsequent K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [25]. As an increase of $[Ca^{2+}]_i$ triggers both, annnexin binding and cell shrinkage, the subpopulation of shrunken erythrocytes mainly includes erythrocytes undergoing eryptosis.

Removal of extraellular Ca^{2+} does not completely abrogate the effects of pentostatin on cell membrane scrambling and cell shrinkage. Thus, pentostatin may trigger cell membrane scrambling and cell shrinkage through some additional mechanism. Our observations did not reveal an effect of pentostatin on the abundance of ceramide, a well known stimulator of cell membrane scrambling not requiring increases of $[Ca^{2+}]_i$ [26]. Our observations disclosed, however, that pentostatin exposure leads to ATP depletion, which is a powerful stimulator of eryptosis [26]. An effect of pentostatin on ATP has been shown previously [44, 45].

Consequences of stimulated eryptosis include anemia due to rapid clearance of eryptotic erythrocytes from circulating blood [26]. As eryptosis triggers disposal of defective erythrocytes prior to hemolysis [26], it prevents release of hemoglobin, which would otherwise be filtered in renal glomerula, precipitate in the acidic lumen of renal tubules and thus occlude nephrons [46]. Eryptosis may be particularly important in malaria [47]. The malaria pathogen *Plasmodium* imposes oxidative stress on the infected host erythrocyte, which leads to activation of several host cell ion channels including Ca^{2+}-permeable erythrocyte cation channels [26, 48]. The subsequent Ca^{2+} entry triggers eryptosis with clearance of infected erythrocytes from circulating blood [47]. Sickle-cell trait, beta-thalassemia-trait, Hb-C and G6PD-deficiency erythrocytes are particularly sensitive to triggers of eryptosis and thus protect against a severe course of malaria by accelerating the removal of infected erythrocytes [26, 49-51]. Iron deficiency [52], and treatment with lead [52], chlorpromazine [53] or NO synthase inhibitors [53] similarly counteract parasitemia by accelerating eryptosis. It remains to be shown, whether pentostatin similarly fosters eryptosis in *plasmodium* infected erythrocytes.

Consequences of eryptosis may further include impairment of microcirculation [26]. Phosphatidylserine exposing erythrocytes may adhere to the vascular wall [54], trigger blood clotting and elicit thrombosis [55-57], events interering with microcirculation [27, 55, 58-61].

Conclusion

Pentostatin triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect paralleled by and in part due to Ca^{2+} entry and energy depeletion.

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