Stimulation of Eryptosis by Cadmium Ions

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Key Words

Cell volume • Annexin • Apoptosis • GARDOS channel • Calcium

Abstract

Cadmium ions are known to trigger apoptosis. Erythrocytes may similarly undergo suicidal death or eryptosis, which is characterized by exposure of phosphatidylserine at the erythrocyte surface. As macrophages are equipped with phosphatidylserine receptors, they bind, engulf and degrade phosphatidylserine exposing cells. Cellular mechanisms known to trigger cell membrane phospholipid scrambling include increased cytosolic Ca^{2+} activity and activation of a sphingomyelinase with formation of ceramide. The present experiments were performed to explore whether cadmium ions (Cd^{2+}) trigger phosphatidylserine exposure of erythrocytes and to possibly identify underlying mechanisms. Phosphatidylserine exposure was estimated from annexin V-binding as determined in fluorescence activated cell sorting (FACS) analysis. Exposure to Cd^{2+} ($\geq 5.5 \mu\text{M}$ Cd^{2+}) indeed significantly increased annexin V-binding. This effect was paralleled by erythrocyte shrinkage as apparent from the decrease of forward

scatter in FACS analysis. According to Fluo3 fluorescence, Cd^{2+} increased the entry of Ca^{2+} into erythrocytes. According to antibody binding, Cd^{2+} did not stimulate the formation of ceramide. In the nominal absence of extracellular Ca^{2+} and in the presence of cation channel inhibitor amiloride the effects of Cd^{2+} on erythrocyte phosphatidylserine exposure and forward scatter were blunted. In conclusion, in human erythrocytes Cd^{2+} stimulates entry of Ca^{2+} , which activates Ca^{2+} -sensitive K^+ channels leading to erythrocyte shrinkage and triggers Ca^{2+} -sensitive erythrocyte membrane scrambling leading to phosphatidylserine exposure.

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Introduction

Cadmium is a widespread environmental toxin [1, 2]. The pleiotropic effects of cadmium [3-8] include induction of suicidal death of nucleated cells or apoptosis [9-12].

Similar to apoptosis, the suicidal death of erythrocytes or eryptosis [13] is characterized by exposure of phosphatidylserine at their surface [14-16].

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As macrophages are equipped with receptors specific for phosphatidylserine [17-19], erythrocytes exposing phosphatidylserine at their surface will be rapidly recognized, engulfed and degraded [20, 21] and are thus expected to be rapidly eliminated from circulating blood.

Erythrocyte phosphatidylserine exposure is accomplished by cell membrane scrambling [22, 23] which is activated following increase in cytosolic Ca^{2+} activity [14-16]. Moreover, the scramblase is sensitized to Ca^{2+} by ceramide which is formed following activation of a sphingomyelinase [24]. Ca^{2+} activity may be increased by activation of a Ca^{2+} -permeable cation conductance which is activated by osmotic shock, oxidative stress and energy depletion [25-28]. Besides increase in scramblase activity, Ca^{2+} activates Ca^{2+} -sensitive K^{+} channels [29, 30], which similarly favours eryptosis [31].

Eryptosis has recently been shown to be triggered by lead [32], mercury [33], aluminium [34], copper [35], and gold ions [36]. Cd^{2+} has previously been shown to accelerate age-related changes in erythrocytes [37], i.e. to increase erythrocyte density and decrease filterability. The present experiments were performed to explore whether exposure to Cd^{2+} triggers phosphatidylserine exposure and to elucidate the underlying mechanisms.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers. Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 . Where indicated, 1 mM CaCl_2 was removed. Cd^{2+} ions (CdCl_2 from Sigma, Schnelldorf, Germany) were added to NaCl Ringer at final concentrations varying from 0.5 to 55 μM . Amiloride was purchased from Sigma and used at a concentration of 1 mM.

Measurement of hemolysis

After 48 hours of incubation at 37°C, the samples were centrifuged (3 min at 400 g, RT) and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Phosphatidylserine exposure and forward scatter

Fluorescence activating cell sorting (FACS) analysis was performed as described [38]. After incubation, cells were washed in Ringer solution + 4 mM CaCl_2 . Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500

dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 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 ceramide formation

To determine ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 $\mu\text{g/ml}$ anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, 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. Samples were then analysed by flow cytometric analysis in FL-1.

Determination of intracellular ATP concentration

90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without Cd^{2+} (final hematocrit 5%). All manipulations were then 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 concentration of the aliquots was determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics, Mannheim, Germany) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations refer to the cytosol of erythrocytes.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by unpaired t-test or ANOVA, $p < 0.05$ was considered as statistically significant.

Results

Incubation of freshly drawn erythrocytes in Ringer solution for 48 hours resulted in low phosphatidylserine exposure with subsequent annexin V-binding in $1.2 \pm 0.1\%$

Fig. 1. Stimulation of phosphatidylserine exposure at the erythrocyte surface by Cd²⁺ ions. A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (black line) or with (red line) 2 μg/ml (10.9 μM) CdCl₂. B. Arithmetic means ± SEM (n = 8-16) of the percentage of annexin V-binding erythrocytes after exposure for 48 hours to Ringer solution without (white bar) or with (black bars) CdCl₂ at the indicated concentrations. **, *** indicate significant difference from the respective values obtained in Ringer-treated controls (ANOVA; p < 0.01, p < 0.001). C. Arithmetic means ± SEM (n = 14) of the percentage of hemolyzed erythrocytes following a 48 hours incubation without (white bar) or with (black bars) CdCl₂ at the indicated concentrations. Hemolysis is expressed as the percentage of complete hemolysis in distilled water.

Fig. 2. Erythrocyte shrinkage following exposure to Cd²⁺ ions. A. Histogram of forward scatter in a representative experiment of erythrocytes incubated for 48 hours in Ringer solution (black line), or in Ringer solution containing 2 μg/ml (10.9 μM; red line) CdCl₂. B. Arithmetic means ± SEM (n = 8-16) of the normalized forward scatter of erythrocytes after a 48 hours treatment with Ringer solution as a function of the Cd²⁺ concentration. *** indicates significant difference (p < 0.001) from control (absence of Cd²⁺ ions).

(n = 16) of the cells. Addition of Cd²⁺ (Cd²⁺ concentrations varying from 0.5 to 55 μM) to the Ringer solution increased the percentage of annexin V-binding cells in a dose-dependent manner (Fig. 1A,B). Statistically significant increases were observed at concentrations of ≥ 5.5 μM Cd²⁺ (Fig. 1A,B). Cd²⁺ did not induce marked hemolysis (Fig. 1C).

Exposure to Cd²⁺ was further followed by a decrease of forward scatter reflecting erythrocyte shrinkage (Fig. 2A,B). A statistically significant decrease was observed at concentrations of ≥ 10 μM Cd²⁺ (Fig. 2B).

Further experiments were performed to explore the mechanisms underlying Cd²⁺-induced cell membrane phospholipids scrambling and decrease of forward scatter. To this end, Fluo3 fluorescence was utilized to meas-

ure changes of the intracellular Ca²⁺ concentration following incubation of erythrocytes in the absence and presence of Cd²⁺. As a result, even 0.5 μM Cd²⁺ significantly increased the intracellular Ca²⁺ concentrations of erythrocytes within 48 hours of incubation (Fig. 3).

As phosphatidylserine exposure is further stimulated by ceramide, additional experiments were performed to test for an effect of Cd²⁺ on ceramide formation. However, Cd²⁺ failed to induce an appreciable release of ceramide (data not shown).

To explore the significance of Cd²⁺-induced Ca²⁺ entry for PS-exposure and cell shrinkage, the effects of Cd²⁺ were tested in the nominal presence and absence of extracellular Ca²⁺. As shown in Fig. 4A,B, the stimulation of phosphatidylserine exposure by Cd²⁺ (5 μg/ml

Fig. 3. Increase in cytosolic Ca^{2+} activity in erythrocytes following exposure to Cd^{2+} . A. Histogram of forward scatter in a representative experiment of erythrocytes incubated for 48 hours in Ringer solution (black line), or in Ringer solution containing 2 $\mu\text{g}/\text{ml}$ (10.9 μM ; red line) CdCl_2 . B. Arithmetic means $\pm$ SEM ($n = 14$ -16) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (open bar) and with (closed bar) the indicated concentrations of CdCl_2 . *** indicates significant difference between presence and absence of Cd^{2+} (ANOVA; $p < 0.001$).

Fig. 4. Ca^{2+} dependence of the Cd^{2+} effect on erythrocyte annexin V-binding. A. Histogram of annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to 5 $\mu\text{g}/\text{ml}$ (37.4 μM) CdCl_2 either in the presence (black line) or absence (red line) of extracellular calcium. B. Arithmetic means $\pm$ SEM ($n = 12$) of the percentage of annexin V-binding erythrocytes following incubation for 48 hours in Ringer without (white bars) or with (black bars) CdCl_2 at the indicated concentration in the presence (left bars) or absence (right bars) of extracellular calcium. *** indicates significant difference from the respective values obtained in Ringer-treated controls (ANOVA; $p < 0.001$), ## indicates significant difference from respective values in the presence of calcium (ANOVA; $p < 0.01$). C. Arithmetic means $\pm$ SEM ($n = 4$ -12) of the percentage of annexin V-binding erythrocytes following incubation for 48 hours in Ringer without (white bars) or with (black bars) CdCl_2 at the indicated concentration in the absence (left bars) or presence (middle bars) of 1 mM amiloride, an inhibitor of the non-specific cation channel. Exposure of energy-depleted erythrocytes for 48 hours (incubation in glucose-free Ringer solution) to amiloride served as a positive control (right bars). *** indicates significant difference from the respective values obtained in Ringer-treated controls (ANOVA; $p < 0.001$), ### indicates significant difference from respective values in the absence of amiloride (ANOVA; $p < 0.001$).

CdCl_2 ($\approx 27.3 \mu\text{M}$) was slightly but significantly blunted in the absence of extracellular Ca^{2+} . Further experiments were performed to investigate the nature of Cd^{2+} -induced increase in the intracellular Ca^{2+} concentration. To this end, erythrocytes were treated with Cd^{2+} in the presence or absence of amiloride (1 mM), an inhibitor of the non-specific cation channel. As shown in Fig. 4C, amiloride significantly blunted but did not completely abrogate the eryptotic effect of Cd^{2+} . Exposure of energy-depleted

erythrocytes for 48 hours (incubation in glucose-free Ringer solution) to amiloride served as a positive control.

Triggers of eryptosis include energy depletion [39]. Therefore, the effect of Cd^{2+} on ATP concentration of erythrocytes was determined. As shown in Fig. 5, a 48 hours exposure to 55 μM Cd^{2+} significantly lowered the intracellular ATP concentration. As a positive control, erythrocytes were incubated for 48 hours in Ringer solution without glucose (Fig. 5).

Fig. 5. Effect of Cd^{2+} on erythrocyte ATP concentration. Arithmetic means $\pm$ SEM ($n = 4$) of the ATP concentration in erythrocytes from healthy volunteers incubated for 48 h in Ringer solution without (open bar) or with 10 $\mu\text{g/ml}$ (54.5 μM) CdCl_2 (closed bar) or in Ringer solution without glucose as a positive control. *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA).

Discussion

The present experiments disclose a novel action of Cd^{2+} ions on erythrocytes. The trace element stimulates erythrocyte cell membrane scrambling leading to phosphatidylserine exposure at the cell membrane surface. The concentrations needed, are in the range of those encountered in blood plasma [40, 41].

The effect of Cd^{2+} on cell membrane scrambling was at least in part due to increase in cytosolic Ca^{2+} activity, a known trigger of eryptosis [14-16, 28].

Ca^{2+} may have entered through the cation channels [28]. In addition, the increase of Ca^{2+} could have been due to impaired extrusion. Human erythrocytes do not possess a plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger [42] and the only active mechanism to extrude Ca^{2+} is the plasmalemmal Ca^{2+} ATPase [43, 44]. As Cd^{2+} reduces the levels of intracellular ATP, the increase in cytosolic Ca^{2+} activity could be secondary to decreased Ca^{2+} ATPase activity.

Ca^{2+} could further account for Cd^{2+} -induced erythrocyte shrinkage, another typical feature of apoptotic cell death [45]. Ca^{2+} is known to activate Ca^{2+} -sensitive K^+ channels [29, 30]. Activation of those channels leads to hyperpolarisation of the cell membrane driving Cl^- exit

in parallel to K^+ exit [31, 46]. The cellular loss of KCl and osmotically obliged water thus leads to cell shrinkage.

The stimulating effect of Cd^{2+} on cell membrane scrambling and Ca^{2+} -sensitive K^+ channels could have been at least in part due to more direct effects of Cd^{2+} . In Madin Darby canine kidney (MDCK) cells, the effect of Cd^{2+} was, however, similarly dependent on Ca^{2+} [47].

The activation of K^+ channels and subsequent cellular KCl loss could not only lead to Cd^{2+} -induced cell shrinkage but may contribute to Cd^{2+} -induced phosphatidylserine exposure. As reported recently [31], activation of the erythrocyte K^+ channels and subsequent cell shrinkage participate in the triggering of scramblase activation. Similarly, treatment of erythrocytes with the K^+ ionophore valinomycin leads to cell shrinkage and cell membrane scrambling [48]. The sensitivity of erythrocytes to cellular K^+ loss is similar to what is observed in other cell types. Cellular loss of K^+ has been demonstrated to parallel and to support apoptosis of a variety of nucleated cells [27, 49-54]. However, the stimulation of phosphatidylserine exposure may be augmented by but is not exclusively due to activation of the K^+ channels as neither increase in extracellular K^+ concentration nor pharmacological inhibition of the channels with clotrimazole or charybdotoxin fully reversed erythrocyte annexin V-binding following treatment of erythrocytes with ionomycin [31]. Rather, Ca^{2+} triggers cell membrane scrambling by mechanisms independent of cellular K^+ loss and shrinkage.

Cd^{2+} does not lead to appreciable ceramide formation, another important stimulator of cell membrane scrambling [24]. The proeryptotic effect of Cd^{2+} exposure also involves decrease of cytosolic ATP concentration, which has previously been shown to trigger eryptosis [28, 55].

According to the present observations, eryptosis may account for the anemia in Cd^{2+} intoxication [56-58]. Eryptosis has been observed in a wide variety of further anemic conditions, including sickle-cell anemia [59, 60], beta-thalassemia [55], glucose-6-phosphate dehydrogenase (G6PD)-deficiency [55], phosphate depletion [59], iron deficiency [61], Hemolytic Uremic Syndrome [62], sepsis [63], malaria [64] and Wilson disease [35]. Moreover, enhanced eryptosis could contribute to the anemia following excessive formation, treatment or intoxication with hemolysin Kanagawa [65], listeriolysin [66], PGE_2 [67], lipoxygenase inhibitor Bay-5884 [68], platelet activating factor [69], paclitaxel [70], methylglyoxal [71], chlorpromazine [72], amantadine [73], cyclosporine [34], curcumin [74], amyloid

peptides [75], aluminium [34], lead [32], mercury [33] and copper ions [35]. Thus, eryptosis participates in the pathophysiology of diverse diseases and contributes to side effects of a variety of drugs.

In conclusion, exposure to Cd^{2+} leads to Ca^{2+} entry with subsequent activation of K^+ channels, K^+ loss, erythrocyte shrinkage and scrambling of cell membrane phospholipids. Similar mechanisms may participate in the pleotropic effects of Cd^{2+} on nucleated cells.

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