Toxicology 249 (2008) 40–44

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Suicidal erythrocyte death triggered by cisplatin

Hasan Mahmud, Michael Föller, Florian Lang*

Department of Physiology, University of Tübingen, Germany

ARTICLE INFO

Article history:
Received 18 March 2008
Accepted 3 April 2008
Available online 15 April 2008

Keywords:
Cell volume
Annexin
Eryptosis
Calcium
Phosphatidylserine

ABSTRACT

Cisplatin, a cytotoxic drug for the treatment of cancer, induces suicidal death or apoptosis of nucleated cells. Side effects of cisplatin include anemia, which, at least in theory, could similarly result from suicidal cell death. Erythrocyte suicidal death or eryptosis is characterized by cell shrinkage and cell membrane scrambling, the latter leading to exposure of phosphatidylserine (PS) at the cell surface. PS-exposing cells are rapidly cleared from circulating blood. The present experiments explored whether cisplatin could trigger eryptosis. According to forward scatter in FACS analysis, a 48 h exposure to cisplatin (≥1 μM) indeed decreased cell volume and, according to annexin V-binding, cisplatin (≥1 μM, 48 h) indeed increased PS exposure at the cell surface. Cisplatin did not induce hemolysis. According to Fluo3 fluorescence, cisplatin increased cytosolic Ca^{2+} activity, a known stimulator of eryptosis. In the absence of extracellular Ca^{2+}, the effect of cisplatin on annexin V-binding was blunted. Cisplatin did not significantly modify the formation of ceramide, another stimulator of eryptosis. Cisplatin moderately decreased the cellular concentration of ATP, which is known to favour eryptosis. In conclusion, cisplatin triggers suicidal erythrocyte death at least partially by increasing cytosolic Ca^{2+} activity. The effect contributes to or even accounts for the development of anemia during cisplatin treatment.

1. Introduction

Cisplatin is a widely used drug for the treatment of cancer (Badary et al., 2000a,b; Glynne-Jones and Hoskin, 2007; Havelka et al., 2007; Saif and Kim, 2007). Cisplatin induces apoptosis (Havelka et al., 2007; Lock et al., 2007), an effect at least partially attributed to DNA damage (Havelka et al., 2007). Side effects of cisplatin include nephrotoxicity (Alfieri and Cubeddu, 2000; Chirino et al., 2008; Deegan et al., 1996; Francescato et al., 2007; Iseri et al., 2007; Naziroglu et al., 2004; Sato et al., 2002), neurotoxicity (Scott et al., 1995) and hemolytic uremic syndrome (Canpolat et al., 1994; Vandendries and Drews, 2006).

Hemolytic uremic syndrome has recently been shown to involve eryptosis, the suicidal death of erythrocytes (Lang et al., 2006a). Moreover, eryptosis is triggered by several anemia-inducing drugs or toxins, such as paclitaxel (Lang et al., 2006b), amantadine (Föller et al., 2008), chlorpromazine (Akel et al., 2006), cyclosporine (Niemoeller et al., 2006a), Bay-5884 (Shumilina et al., 2006), curcumin (Bentzen et al., 2007), valinomycin (Schneider et al., 2007), hemolysin (Lang et al., 2004b), listeriolysin (Foller et al., 2007), aluminium (Niemoeller et al., 2006b), lead (Kempe et al., 2005), mercury (Eisele et al., 2005), copper (Lang et al., 2007), gold (Sopjani et al., 2008), methylglyoxal (Nicolay et al., 2006) and amyloid peptides (Nicolay et al., 2007).

Eryptosis is characterized by exposure of phosphatidylserine (PS) at the erythrocyte surface (Berg et al., 2001; Brand et al., 2003; Bratosin et al., 2001; Daugas et al., 2001; Lang et al., 2003c). Phosphatidylserine-exposing erythrocytes are rapidly phagocytosed and thus eliminated from circulating blood (Kempe et al., 2006). Erythrocyte phosphatidylserine exposure results from phospholipid scrambling of the cell membrane (Dekkers et al., 2002; Woon et al., 1999). Triggers of cell membrane scrambling include increase in the cytosolic Ca^{2+} concentration (Berg et al., 2001; Bratosin et al., 2001; Daugas et al., 2001), which results from Ca^{2+} entry through Ca^{2+}-permeable cation channels (Duranton et al., 2002, 2003; Huber et al., 2001; Lang et al., 2003a). Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels (Bookchin et al., 1987; Brugnara et al., 1993) leading to exit of KCl and osmotically obliged water thus resulting in cell shrinkage (Lang et al., 2003b). Ca^{2+} sensitivity of phospholipid scrambling is enhanced by ceramide (Lang et al., 2004a).

The present study explored, whether cisplatin induces eryptosis, and if so, whether the effect involves an increase in the cytosolic Ca^{2+} concentration and/or ceramide formation.

* Corresponding author at: Physiologisches Institut, der Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen, Germany. Tel.: +49 7071 29 72194; fax: +49 7071 29 5618.
E-mail address: florian.lang@uni-tuebingen.de (F. Lang).

0300-483X/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2008.04.003

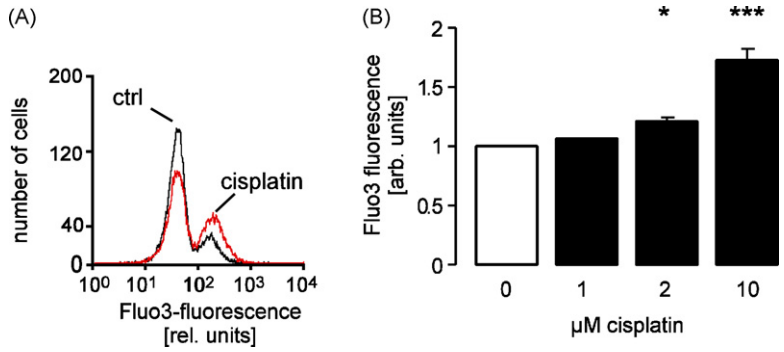

Fig. 1. Increase in cytosolic Ca^{2+} concentration in erythrocytes following exposure to cisplatin. (A) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 h to Ringer without (black line) and with (red line) 5 μM cisplatin. (B) Arithmetic means ± S.E.M. ($n = 20$) of the normalized Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer without (white bar) or with (black bars) cisplatin at the indicated concentrations. *, ***Indicate significant difference from the absence of cisplatin (ANOVA, $p < 0.05$, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

2. Materials and methods

2.1. Volunteers

Erythrocytes were drawn from healthy volunteers, who provided informed consent. The study has been approved by the Ethical commission of the University of Tübingen.

2.2. Solutions

The experiments with cisplatin 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, cisplatin (Sigma, Schnelldorf, Germany) was added to the NaCl Ringer at final concentrations ranging from 1 to 10 μM. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted for 1 mM glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA).

2.3. Measurement of hemolysis

After 48 h 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 supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

2.4. Phosphatidylserine exposure and forward scatter

Erythrocytes were washed once in Ringer solution +4 mM $CaCl_2$. The cells were then 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.

2.5. 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 containing 5 mM $CaCl_2$. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

2.6. Determination of ceramide formation

To determine ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h 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 1:5. After two washing steps with PBS-BSA, cells were stained for 30 min 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.

2.7. Determination of intracellular ATP concentration

For determination of erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37 °C in Ringer solution with or without cisplatin (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 $HClO_4$ (6%). 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 cystol of erythrocytes.

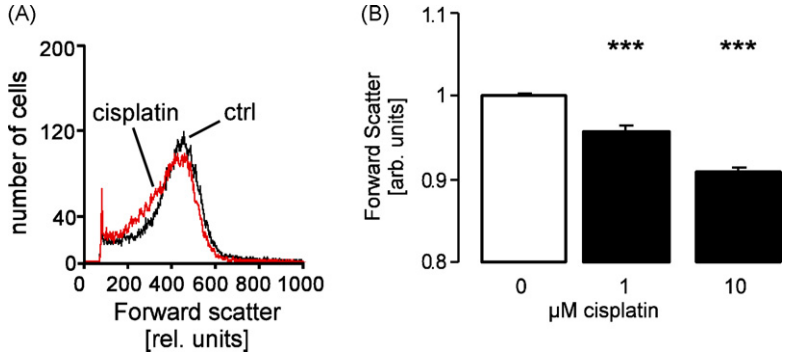

Fig. 2. Erythrocyte forward scatter following exposure to cisplatin. (A) Histogram of forward scatter in a representative experiment of erythrocytes incubated for 48 h in plain Ringer solution (black line) or in Ringer solution containing 10 μM cisplatin (red line). (B) Arithmetic means ± S.E.M. ($n = 20$) of the normalized forward scatter of erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) cisplatin at the indicated concentrations. ***Indicates significant difference ($p < 0.001$) from control (absence of cisplatin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)