Geldanamycin-Induced Phosphatidylserine Translocation in the Erythrocyte Membrane

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Key Words
Phosphatidylserine • Geldanamycin • Calcium • Ceramide • Cell volume • Eryptosis

Abstract

Background/aims: Geldanamycin, a benzoquinone ansamycin antibiotic, and its analogues induce apoptosis of tumor cells and are thus considered for the treatment of cancer. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine-exposure at the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca\textsuperscript{2+}-concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and formation of ceramide. The present study explored, whether geldanamycin modifies [Ca\textsuperscript{2+}], ceramide formation, cell volume and phosphatidylserine abundance at the erythrocyte surface. Methods: Erythrocyte volume was estimated from forward scatter, phosphatidylserine-abundance from annexin V binding, hemolysis from hemoglobin release, ceramide formation from binding of fluorescent antibodies and [Ca\textsuperscript{2+}] from Fluo3-fluorescence. Results: A 48 hours exposure to geldanamycin significantly decreased forward scatter (≥ 5 µM), significantly increased annexin-V-binding (≥ 25 µM), but did not significantly modify Fluo3-fluorescence (up to 50 µM). The annexin-V-binding following geldanamycin treatment was not significantly modified by removal of extracellular Ca\textsuperscript{2+} but was paralleled by significantly increased ceramide formation (50 µM). Conclusions: Geldanamycin stimulated eryptosis, an effect at least partially due to ceramide formation.

Introduction

The benzoquinone ansamycin antibiotic geldanamycin has been shown to inhibit heat shock protein Hsp90 [1-12], which prevents stress-induced cellular damage [2], stabilizes various oncogenic kinases [1, 7, 9, 10, 12] and influences gene expression e.g. by up-
regulating NF-κB [13, 14]. As Hsp90 expression is particularly high in cancer cells and is associated with tumor cell progression, invasion and formation of metastases, as well as development of drug resistance [2], geldanamycin and its analogues have been considered for treatment of cancer [2, 3, 12, 15-19]. Geldanamycin has been shown to induce apoptosis [1, 5, 6, 8-10, 15, 20-23], an effect paralleled by altered gene expression, downregulation of Akt, p38 MAPK activation, mitochondrial depolarization, reactive oxygen species formation, decline of reduced glutathion, lipid peroxidation and caspase activation [5, 9, 15, 20, 21]. On the other hand, geldanamycin may counteract neuronal injury, an effect attributed to destabilization of RIP1 protein [4, 7, 24].

Similar to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, the suicidal erythrocyte death characterized by cell membrane scrambling and cell shrinkage [25]. Eryptosis may be triggered by increased cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) resulting from Ca\(^{2+}\)-entry through Ca\(^{2+}\)-permeable cation channels [26, 27] or from permeabilization of the cell membrane e.g. by hemolysin [28]. Increased [Ca\(^{2+}\)]\(_i\) leads to cell shrinkage by activation of Ca\(^{2+}\)-sensitive K\(^+\) channels [29] with K\(^+\) exit, hyperpolarization, Cl\(^-\) exit and thus cellular loss of KCl and osmotically obliged water [30]. Increased [Ca\(^{2+}\)]\(_i\) further triggers phospholipid scrambling of the cell membrane with translocation of phosphatidylserine to the erythrocyte surface [31]. Ca\(^{2+}\) sensitivity of phospholipid scrambling is enhanced by ceramide [32]. Eryptosis may further be stimulated by energy depletion [33] and caspase activation [34-38]. Kinases participating in the regulation of eryptosis include AMP activated kinase AMPK [27], cGMP-dependent protein kinase [39], Janus-activated kinase JAK3 [40], casein kinase 1α [41, 42], p38 kinase [43], PAK2 kinase [44] as well as sorafenib [45] and sunitinib [46] sensitive kinases.

Eryptosis is a physiological mechanism preceding and actually preventing hemolysis of defective erythrocytes [32]. Excessive cell swelling may lead to rupture of the erythrocyte cell membrane, resulting in hemolysis with release of cellular hemoglobin, which is filtered in renal glomerula and subsequently occludes renal tubules [47]. The activation of K\(^+\) channels during eryptosis counteracts cell swelling and thus hemolysis [30].

The present study explored, whether geldanamycin modifies erythrocyte [Ca\(^{2+}\)]\(_i\), erythrocyte volume and/or phosphatidylserine abundance at the erythrocyte surface.

**Materials and Methods**

**Erythrocytes, solutions and chemicals**

Leukocyte-depleted erythrocytes 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 (184/2003V). 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), 5 glucose, 1 CaCl\(_2\); pH 7.4 at 37°C for 48 h. Where indicated, erythrocytes were exposed to geldanamycin (Enzo, Lörrach, Germany) at the indicated concentrations. In Ca\(^{2+}\)-free Ringer solution, 1 mM CaCl\(_2\) was substituted by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA).

**FACS analysis of annexin-V-binding and forward scatter**

After incubation under the respective experimental condition, 50 µl cell suspension was washed in Ringer solution containing 5 mM CaCl\(_2\) and then stained with Annexin-V-FTTC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin-V fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

**Measurement 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 2 µM Fluo-3/AM. The cells were
incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM CaCl₂. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca²⁺-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

**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 1:5. 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.

**Measurement of hemolysis**

For the determination of hemolysis the samples were centrifuged (3 min at 400 g, room temperature) after incubation, 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.

**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 addressed the effect of geldanamycin on eryptosis. Hallmarks of eryptosis include cell shrinkage. Thus, cell volume was estimated utilizing forward scatter. The forward scatter was determined by flow cytometry. As shown in Fig. 1A,B, a 48 hours
exposure to geldanamycin resulted in a decrease of forward scatter, an effect reaching statistical significance at 5 µM geldanamycin concentration.

The second hallmark of eryptosis is cell membrane scrambling with subsequent increase of phosphatidylserine abundance at the cell surface. Accordingly, phosphatidylserine exposing erythrocytes were identified by annexin-V-binding in flow cytometry. As illustrated in Fig. 2A,B, a 48 hours exposure to geldanamycin increased the percentage of annexin-V-binding erythrocytes, an effect reaching statistical significance at 25 µM geldanamycin concentration. Notably, the percentage of hemolytic erythrocytes remained almost one magnitude smaller than the percentage of phosphatidylserine exposing erythrocytes (Fig. 2C).

Fig. 2. Effect of geldanamycin on phosphatidylserine exposure and hemolysis. (A) Original histogram of annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 50 µM geldanamycin. (B) Arithmetic means ± SEM (n = 12) of erythrocyte annexin-V-binding (PS-(+) cells) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of geldanamycin (5-50 µM). (C) Arithmetic means ± SEM (n = 4) of the percentage of hemolysis following incubation for 48 hours to Ringer solution without (white bar) or with (grey bars) presence of geldanamycin (5-50 µM). *** (p<0.001) indicate significant differences from the absence of geldanamycin (ANOVA).

Fig. 3. Effect of geldanamycin on erythrocyte cytosolic Ca\(^{2+}\) concentration. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey shadow) and with (black line) presence of 50 µM geldanamycin. (B) Arithmetic means ± SEM (n = 12) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) geldanamycin (5-50 µM).
In an attempt to elucidate the mechanisms underlying the triggering of erythrocyte shrinkage and cell membrane scrambling following geldanamycin exposure, cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) was determined utilizing Fluo3 fluorescence. To this end, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis following incubation in Ringer solution without or with geldanamycin (1-50 µM). As illustrated in Fig. 3, a 48 hours exposure of human erythrocytes to geldanamycin up to 50 µM remained without significant effect on Fluo3 fluorescence.

To further elucidate the role of Ca\(^{2+}\), an additional series of experiments explored whether extracellular Ca\(^{2+}\) entry was required for the effect of geldanamycin on cell membrane scrambling. To this end, erythrocytes were exposed to 50 µM geldanamycin for 48 hours either in the presence of 1 mM Ca\(^{2+}\) or in the absence of Ca\(^{2+}\) and the presence of Ca\(^{2+}\) chelator EGTA (1 mM). As illustrated in Fig. 4, the effect of geldanamycin on annexin-V-binding was virtually the same in the presence and nominal absence of Ca\(^{2+}\).

Additional experiments explored, whether geldanamycin stimulates the formation of ceramide, which has previously been shown to trigger eryptosis even at constant [Ca\(^{2+}\)]\(_{i}\). Ceramide abundance at the erythrocyte surface was determined utilizing an anti-ceramide antibody. As shown in Fig. 5, a 48 hours exposure to geldanamycin increased the abundance of ceramide at the erythrocyte surface, an effect reaching statistical significance at 25 µM geldanamycin concentration.
Discussion

The present study uncovers a novel effect of geldanamycin, i.e. the stimulation of suicidal erythrocyte death. Exposure of human erythrocytes to geldanamycin is followed by erythrocyte shrinkage and erythrocyte membrane scrambling, both hallmarks of eryptosis. The concentrations required were similar to those previously observed in vivo [48].

Similar to geldanamycin, a wide variety of xenobiotics stimulate eryptosis [46, 49-75]. However, most xenobiotics triggering eryptosis do so by increasing cytosolic Ca\(^{2+}\) concentration [32]. They are effective by activation of the endogenous Ca\(^{2+}\)-permeable non-selective cation channels, which involve somehow the transient receptor potential channel TRPC6 [26] and are activated by oxidative stress [75]. Activation of those channels shrinks erythrocytes by stimulating entry of extracellular Ca\(^{2+}\) with subsequent increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), and activation of Ca\(^{2+}\)-sensitive K\(^{+}\) channels [29, 75]. The activation of the Ca\(^{2+}\)-sensitive K\(^{+}\) channels leads to K\(^{+}\) exit, cell membrane hyperpolarisation, Cl\(^{-}\) exit and thus cellular loss of KCl with osmotically obliged water [30]. Even though geldanamycin does not appreciably affect [Ca\(^{2+}\)]\(_{i}\), it still leads to a marked and robust decrease of forward scatter. The cell shrinkage is already maximal at geldanamycin concentrations, which have little effect on phosphatidylserine exposure. Possibly, geldanamycin shrinks erythrocytes by Ca\(^{2+}\)-independent activation of K\(^{+}\) channels, an effect already maximal at the lowest geldanamycin concentrations used and thus seemingly lacking dose-dependence.

The effect of geldanamycin on cell membrane scrambling is at least partially due to formation of ceramide, which sensitizes the cells to the eryptotic effects of [Ca\(^{2+}\)], and is thus capable to trigger eryptosis without increase of [Ca\(^{2+}\)] [32].

Excessive eryptosis contributes to several clinical disorders [25] including diabetes [38, 75, 76], renal insufficiency [75], hemolytic uremic syndrome [77], sepsis [78], malaria [79, 80], sickle cell disease [81], Wilson's disease [80], iron deficiency [82], malignancy [83], phosphate depletion [84], and metabolic syndrome [71]. Again, those disorders are mostly effective by increasing cytosolic Ca\(^{2+}\) concentration [32].

Eryptotic erythrocytes are cleared from circulating blood with subsequent development of anemia, if the accelerated loss of erythrocytes is not compensated by enhanced formation of new erythrocytes [25]. Phosphatidylserine exposing erythrocytes may further adhere to endothelial CXCL16/SR-PSO of the vascular wall [85], which may, at least in theory, interfere with blood flow [85-90]. Phosphatidylserine exposing erythrocytes may further stimulate blood clotting and thus favour the development of thrombosis [86, 91, 92]. In view of the present observations, those potential side effects must be considered during the use of geldanamycin.

Conclusions

Geldanamycin triggers eryptosis, an effect at least partially due to stimulation of ceramide formation. Geldanamycin stimulates cell membrane scrambling and cell shrinkage and thus suicidal death of human erythrocytes by mechanisms not requiring Ca\(^{2+}\) entry.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Ali Soleimanpour. The study was supported by the Deutsche Forschungsgemeinschaft and the Open Access Publishing Fund of Tuebingen University.
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DOI: 10.1159/000356596
Published online: December 03, 2013
© 2013 S. Karger AG, Basel
www.karger.com/cpb


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