Original Paper

Tannic Acid Induced Suicidal Erythrocyte Death

Majed Abed Tabea Herrmann Kousi Alzoubi Tatsiana Pakladok Florian Lang

Department of Physiology, University of Tübingen, Tübingen, Germany

Key Words

Phosphatidylserine • Tannic acid • Calcium • Cell volume • Eryptosis

Abstract

Background: The polyphenol tannic acid with antioxidant and antimicrobial potency may trigger suicidal death of nucleated cells or apoptosis and thus may counteract tumor growth. In analogy to apoptosis of nucleated cells, erythrocytes may undergo eryptosis, a suicidal death characterized by cell shrinkage and cell membrane scrambling with appearance of phosphatidylserine at the erythrocyte surface. A major trigger of eryptosis is increase of cytosolic Ca^{2+} -activity ($[\text{Ca}^{2+}]_i$). Erythrocytes could be sensitized to the eryptotic effect of cytosolic Ca^{2+} by ceramide. **Methods:** Cell volume has been estimated from forward scatter, phosphatidylserine abundance at the erythrocyte surface from annexin V binding, hemolysis from hemoglobin release, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence and ceramide utilizing fluorescent antibodies. **Results:** A 48 h treatment with tannic acid was followed by significant decrease of forward scatter ($\geq 1 \mu\text{g/ml}$) and significant increase of annexin-V-binding ($\geq 10 \mu\text{g/ml}$). Tannic acid did not significantly modify $[\text{Ca}^{2+}]_i$ (up to $50 \mu\text{M}$) but significantly increased ceramide formation ($50 \mu\text{M}$). The annexin-V-binding following tannic acid treatment ($50 \mu\text{M}$) was significantly blunted in the nominal absence of extracellular Ca^{2+} . **Conclusions:** Tannic acid stimulates eryptosis, an effect at least partially due to ceramide formation with subsequent sensitization of erythrocytes to cytosolic Ca^{2+} .

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Introduction

Tannins including tannic acid are water soluble complex polyphenols with antioxidant [1-5], antimicrobial [1, 5-7] antiviral [1, 5, 6], anti-inflammatory [1, 8] and anti-tumor [9-12] properties. Further effects attributed to tannins and/or their metabolites include stimulation of blood clotting [1], decrease of blood pressure [1], modification of glucose metabolism [1],

decrease of plasma lipids [1] and induction of liver necrosis [1]. Tannic acid may be useful in the treatment of malignancy [9-12]. The anticarcinogenic effect of tannic acid is at least partially due to stimulation of apoptosis [9-11, 13, 14]. Mechanisms invoked in tannic acid induced apoptosis include Fas-associated death domain (FADD) protein [15], proteasome activity [9, 14], inhibition of poly(ADP-ribose) glycohydrolase (PARG) expression [12], poly(ADP-ribose) (pADPr) [10, 12], cyclin-dependent kinase inhibitor p27(Kip1) [14], Bcl-2 homologous antagonist/killer (Bak) [15], proapoptotic protein Bax [14], mitochondrial depolarization [10], release of apoptosis-inducing factor [10, 12], activation of caspases [10, 12] and DNA fragmentation [10, 13]. Beyond that, tannic acid has both, antioxidant and oxidant potency [3].

Similar to nucleated cells, erythrocytes may enter suicidal death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage [16]. Eryptosis is triggered by increase of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) resulting mainly from Ca^{2+} entry through Ca^{2+} -permeable cation channels [17, 18]. The channels involve the transient receptor potential channel TRPC6 [17] and are activated by oxidative stress [19]. An increase of $[\text{Ca}^{2+}]_i$ leads to activation of Ca^{2+} -sensitive K^+ channels [20] with subsequent cell shrinkage due to K^+ exit, hyperpolarization, Cl^- exit and thus cellular loss of KCl and osmotically driven water [21]. Increased $[\text{Ca}^{2+}]_i$ further stimulates cell membrane scrambling with appearance of phosphatidylserine at the erythrocyte surface [22]. The cell membrane scrambling is sensitized for cytosolic Ca^{2+} by ceramide [23]. Further regulators of eryptosis include caspases [24-28], protein kinase C [29], AMP activated kinase AMPK [18], casein kinase [30, 31], cGMP-dependent protein kinase [32], Janus-activated kinase JAK3 [33], p38 kinase [34], PAK2 kinase [35] as well as sorafenib [36] and sunifinib [37] sensitive kinases.

Excessive eryptosis could result from exposure to xenobiotics [37-68] and complicates several diseases [16], such as diabetes [28, 69, 70], renal insufficiency [71], hemolytic uremic syndrome [72], sepsis [73], malaria [74], sickle cell disease [75], Wilson's disease [76], iron deficiency [77], malignancy [78], phosphate depletion [79] and metabolic syndrome [64].

Erythrocytes are devoid of mitochondria and nuclei, key elements in the machinery underlying apoptosis. Thus, analysis of eryptosis may uncover the significance of suicide mechanisms independent from those organelles. The present study thus explored, whether tannic acid triggers eryptosis. To this end, cell volume, phosphatidylserine abundance at the erythrocyte surface, $[\text{Ca}^{2+}]_i$, and ceramide abundance have been determined following exposure of human erythrocytes to Ringer solution without or with tannic acid.

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 tannic acid (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-FITC (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 in FL-1 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 μ M 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 μ l Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

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.

Confocal microscopy and immunofluorescence

For the detection of ceramide, erythrocytes were resuspended in PBS at 5×10^7 cells/ml and 10 μ l was smeared onto a glass slide. After being air dried for 30 min and fixed with 4% paraformaldehyde/PBS for 10 min, the slides were rinsed 3 times for 5 min with PBS. Then, the slides were blocked with 10% normal goat serum (Invitrogen, Paisley, UK) for 1 h and again rinsed 3 times with PBS for 5 min. The slides were incubated with mouse anti-ceramide antibody (diluted 1:100, Glycobiotech, Germany) at 4°C overnight and subsequently with fluorescence-labelled secondary Alexa Fluor 546 goat anti-mouse IgG (diluted 1:200, Invitrogen, UK) for 1 h at room temperature. The slides were mounted with Pro Long Gold antifade reagent (Invitrogen, UK). Images were taken on a Zeiss LSM5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Germany) with A-Plan 40x/1.2W.

Determination of ceramide formation

For the determination of ceramide abundance, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 h at 37°C with 1 μ 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. 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. Isotype control F(ab')₂/IgG1 (BD Biosciences, Heidelberg, Germany) was used as negative control according to the manufacturer instructions. The samples were then analyzed by flow cytometric analysis in FL-1.

Statistics

Following flow cytometry, the average of 10^4 cells has been calculated for each erythrocyte preparation. The arithmetic mean $\pm$ the standard error of the mean (SEM) has been calculated from the averaged values obtained from the different erythrocyte preparations. Data are provided as arithmetic means $\pm$ SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test or unpaired Student's t test, where 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 comparison of control and experimental conditions.

Results

The present study explored, whether treatment of human erythrocytes with tannic acid triggers the suicidal death of erythrocytes or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling. To estimate erythrocyte volume, forward scatter was determined in FACS analysis. As shown in Fig. 1, a 48 hours exposure to tannic acid resulted in a decrease of forward scatter, an effect reaching statistical significance at 1 μ M tannic acid concentration.

Fig. 1. Effect of tannic acid on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 50 µM tannic acid. B. Arithmetic means $\pm$ SEM ($n = 8$) of the normalized erythrocyte forward scatter (FSC) following incubation for 48 h to Ringer solution without (white bar) or with (black bars) tannic acid (0.1 – 50 µM). *** ($p < 0.001$) indicates significant difference from the absence of tannic acid (ANOVA).

Fig. 2. Effect of tannic acid on phosphatidylserine exposure and hemolysis. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 50 µM tannic acid. B. Arithmetic means $\pm$ SEM ($n = 8$) of erythrocyte annexin-V-binding following incubation for 48 h to Ringer solution without (white bar) or with (black bars) presence of tannic acid (0.1 – 50 µM). For comparison, arithmetic means $\pm$ SEM ($n = 6$) of the percentage of hemolysis is shown as grey bars. *** ($p < 0.001$) indicate significant differences from the absence of tannic acid (ANOVA), ## ($p < 0.01$) indicates significant difference from the respective values in the presence of Ca²⁺ (ANOVA).

In order to determine cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface, erythrocytes exposing phosphatidylserine at their surface were identified by annexin-V-binding in FACS analysis. As apparent from Fig. 2, a 48 h exposure to tannic acid was followed by an increase of annexin-V-binding. An arbitrary gate has been set to calculate the percentage of erythrocytes with excessive annexin V binding. As illustrated in Fig. 2B, the percentage of erythrocytes with excessive annexin V binding increased following exposure to tannic acid, an effect reaching statistical significance at ≥ 10 µM tannic acid concentrations.

A further series of experiments explored the effect of tannic acid on hemolysis, which was estimated by determination of hemoglobin in the supernatant. As a result, the

Fig. 3. Effect of tannic acid on erythrocyte cytosolic Ca^{2+} concentration. A. Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) and with (black line) presence of 50 μM tannic acid. B. Arithmetic means $\pm$ SEM ($n = 8$) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) tannic acid (0.1 – 50 μM).

Fig. 4. Effect of Ca^{2+} withdrawal on tannic acid induced annexin-V-binding. Arithmetic means $\pm$ SEM ($n = 5$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bar) or with (black bars) 50 μM tannic acid in the presence (left bars, + Calcium) and absence (right bars, - Calcium) of calcium. *** ($p < 0.001$) indicates significant difference from the absence of tannic acid (ANOVA), ## ($p < 0.01$) indicates significant difference from the respective values in the presence of Ca^{2+} (ANOVA).

percentage of hemolyzed erythrocytes increased slightly but significantly following exposure of erythrocytes for 48 h to tannic acid (Fig. 2).

An additional series of experiments was performed to elucidate the mechanisms underlying the stimulation of tannic acid induced cell membrane scrambling. In order to determine cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), the erythrocytes were incubated in Ringer solution in the absence or presence of tannic acid. The erythrocytes were subsequently loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis. As illustrated in Fig. 3, a 48 hours exposure of human erythrocytes to tannic acid concentrations did not significantly modify Fluo3 fluorescence.

In order to test, whether Ca^{2+} entry from the extracellular space was required for the effect of tannic acid on cell membrane scrambling, the erythrocytes were exposed to tannic acid for 48 hours in either the presence of 1 mM extracellular Ca^{2+} or in the absence of extracellular Ca^{2+} and presence of the Ca^{2+} chelator EGTA (1 mM). As shown in Fig. 4, the nominal absence of Ca^{2+} significantly blunted the effect of tannic acid on annexin-V-binding.

The observation that tannic acid induced cell membrane scrambling was dependent on the presence of Ca^{2+} but was not paralleled by an increase of $[\text{Ca}^{2+}]_i$ was suggestive for an increase of Ca^{2+} sensitivity of cell membrane scrambling by tannic acid. As sensitivity of cell membrane scrambling to $[\text{Ca}^{2+}]_i$ is enhanced by ceramide, additional experiments

Fig. 5. Effect of tannic acid on ceramide formation. A. Confocal images of ceramide abundance at the erythrocyte surface prior to (middle panel) and after (right panel) a 48 h exposure to tannic acid (50 μ M). The left panel shows erythrocytes stained only with secondary antibody as control. B. Original histogram of anti-ceramide FITC-fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (grey shadow) or with (black line) 50 μ M tannic acid. C. Arithmetic means $\pm$ SEM ($n = 4$) of ceramide abundance after a 48 h incubation in Ringer solution without (white bar) or with (black bars) tannic acid (50 μ M). Shown is the respective fluorescence intensity utilizing ceramide antibody (left bars) or isotype antibody (right bars). *** ($p < 0.001$) indicates significant difference from control (absence of tannic acid) (ANOVA).

were performed to determine the effect of tannic acid on ceramide formation. To this end, ceramide abundance at the erythrocyte surface was quantified utilizing FITC-labeled anti-ceramide antibodies. As illustrated in Fig. 5, tannic acid significantly increased the ceramide abundance at the erythrocyte surface by 385%. As a control, an isotype antibody has been employed. As illustrated in Fig. 5C, tannic acid application did not significantly modify the fluorescence upon use of an isotype antibody.

Discussion

The present observations reveal a completely novel effect of tannic acid, i.e. the triggering of erythrocyte shrinkage and erythrocyte cell membrane scrambling, both hallmarks of eryptosis, the suicidal death of erythrocytes. The concentrations of tannic acid required for the induction of eryptosis are well in the range of tannic acid concentrations triggering apoptosis, the suicidal death of nucleated cells [9, 10, 13, 14]. *In vivo*, reported dosages of tannic acid triggering methemoglobinemia include oral 2.0 to 4.6 g (1.1–2.7 mmol)/kg BW in mice [80], oral 8 g (4.7 mmol)/kg BW in sheep [80], and intraperitoneal 0.1 g (60 μ mol)/kg BW in sheep [80].

The erythrocyte shrinkage following tannic acid treatment may result from activation of Ca^{2+} sensitive K^+ channels [20, 81], leading to K^+ exit, cell membrane hyperpolarization, potential driven Cl^- exit and thus loss of cellular KCl along with the respective osmotically obliged water [21]. However, tannic acid did not significantly increase cytosolic Ca^{2+} activity. Thus, tannic

acid must have activated the K^+ channels by some other mechanism. Alternatively, tannic acid activated other transport systems leading to cellular KCl loss, such as KCl symport. Further experiments are required to fully elucidate the mechanisms underlying tannic acid induced erythrocyte shrinkage.

As apparent from Fig. 1A, erythrocytes do not uniformly shrink following treatment with tannic acid. Instead, a subpopulation of erythrocytes underwent dramatic cell shrinkage, whereas another subpopulation of erythrocytes did not appreciably alter cell volume. The sensitivity of individual erythrocytes to triggers of eryptosis has previously been shown to be a function of erythrocyte age [45].

The phosphatidylserine exposure at the erythrocyte surface due to cell membrane scrambling following tannic acid treatment was strongly decreased in the absence of extracellular Ca^{2+} and was thus in large part dependent on the presence of extracellular Ca^{2+} . However, tannic acid did not increase cytosolic Ca^{2+} activity. Thus, Ca^{2+} played a permissive role in the stimulation of cell membrane scrambling by tannic acid. The effect of tannic acid could thus have resulted from an increase of the Ca^{2+} sensitivity of cell membrane scrambling. The Ca^{2+} sensitivity could be enhanced by ceramide [23]. As a matter of fact, tannic acid treatment was followed by a marked increase of ceramide abundance at the erythrocyte surface. Ceramide production is presumably secondary to activation by acid sphingomyelinase [23]. Ceramide sensitizes the erythrocytes to the triggering of eryptosis by cytosolic Ca^{2+} . Thus, the eventual outcome for a given cell depends on ceramide abundance, cytosolic Ca^{2+} activity and other factors including erythrocyte age. The decrease of extracellular Ca^{2+} concentration is expected to decrease eryptosis by lowering cytosolic Ca^{2+} activity. Moreover, tannic acid may fail to trigger eryptosis despite triggering of ceramide formation, if e.g. the erythrocyte is young and thus relatively resistant to eryptosis.

Consequences of erythrocyte cell membrane scrambling include the clearance of the affected erythrocytes from circulating blood [16]. As long as the loss of erythrocytes is matched by increased formation of new erythrocytes, the stimulation of eryptosis may remain without appreciable effect on blood count. The increased formation of erythrocytes is apparent from increased reticulocyte numbers in blood, which may thus point to enhanced erythrocyte turnover [16]. Clinically overt anemia develops as soon as the loss of circulating erythrocytes by eryptosis exceeds the formation of new erythrocytes [16].

Besides its potential effect on blood count, excessive eryptosis could compromise microcirculation. Phosphatidylserine exposing erythrocytes have been shown to adhere to endothelial CXCL16/SR-PSO of the vascular wall [82], which is expected to impede blood flow [82-87]. In addition, phosphatidylserine fosters blood clotting thus potentially leading to thrombosis [83, 88, 89].

Eryptosis may, however, not only be harmful. Eryptosis may precede and thus protect against hemolysis, which may result from excessive swelling of defective erythrocytes and eventually lead to cell membrane rupture with release of cellular hemoglobin [68]. The hemoglobin may be filtered in renal glomerula, precipitate in renal tubules and thus occlude nephrons [90]. The activation of K^+ channels in the course of eryptosis counteracts cell swelling [21] and the exposure of phosphatidylserine at the surface of eryptotic cells fosters engulfment of the defective erythrocytes by phagocytosing cells prior to hemolysis [68].

In conclusion, tannic acid stimulates cell membrane scrambling and cell shrinkage, an effect partially due to ceramide formation with subsequent sensitization of erythrocytes to cytosolic Ca^{2+} .

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