Original Paper

Enhanced Suicidal Erythrocyte Death Contributing to Anemia in the Elderly

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Key Words

Phosphatidylserine • Ageing • Anemia • Oxidative stress • GSH • Ceramide • Eryptosis

Abstract

Background/Aims: Anemia, a common condition in the elderly, could result from impaired formation and/or from accelerated loss of circulating erythrocytes. The latter could result from premature suicidal erythrocyte death or eryptosis characterized by phosphatidylserine (PS) exposure at the erythrocyte surface. Triggers of eryptosis include increased cytosolic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$), oxidative stress and ceramide. The present study explored whether eryptosis is altered in elderly individuals and, if so, to identify underlying mechanisms.

Methods: Blood was drawn from healthy young ($n=11$, age 31.3 ± 1.7 years) and elderly ($n=16$, age 88.6 ± 0.9 years) individuals. PS exposure was estimated from annexin V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) from 2',7'-dichlorodihydrofluorescein fluorescence, reduced glutathione (GSH) from mercury orange fluorescence and ceramide from FITC-conjugated antibody binding in flow cytometry. Measurements were made in erythrocytes from freshly drawn blood and in erythrocytes exposed in vitro for 24 h to plasma from young or elderly individuals. **Results:** Elderly individuals suffered from severe anemia (hemoglobin 10.5 ± 0.3 g/100 ml) despite enhanced number of reticulocytes ($2.3 \pm 0.2\%$). The percentage of PS-exposing erythrocytes was significantly higher in the elderly ($2.5 \pm 0.2\%$) than in the young volunteers ($1.3 \pm 0.1\%$). The increase in PS exposure was paralleled by significant increase of ROS and significantly decreased levels of reduced GSH. Erythrocyte $[\text{Ca}^{2+}]_i$ and ceramide abundance tended to be higher in the elderly, differences, however, not reaching statistical significance. **Conclusions:** The anemia of elderly individuals is mainly if not exclusively due to enhanced eryptosis, resulting at least in part from GSH deficiency and increased oxidative stress.

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Introduction

Anemia is a common condition in the elderly and affects more than 50% individuals who are older than the age of 80 years [1]. The prevalence of anemia increases with age and sharply increases in the octogenarian and centenarian cohorts [2]. In the majority of the elderly population, the etiology of anemia remains elusive [3]. Anemia in the elderly is associated with decreased life quality, enhanced morbidity, and increased mortality [1, 4, 5]. In principle, anemia could result from impaired formation or from accelerated loss of erythrocytes.

Accelerated loss of erythrocytes could result from suicidal cell death or eryptosis, which is characterized by cell shrinkage [6] and cell membrane scrambling with exposure of phosphatidylserine at the cell surface [7].

Signaling involved in the stimulation of eryptosis includes increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [7], formation of ceramide [8], oxidative stress [7], energy depletion [7], caspase activation [9-13], activation of kinases such as casein kinase 1 α [14, 15], Janus-activated kinase JAK3 [16], protein kinase C [17], or p38 kinase [18], as well as inhibition or knockout of further kinases including AMP-activated kinase AMPK [19], cGMP-dependent protein kinase [10], PAK2 kinase [20], as well as sorafenib- and sunitinib-sensitive kinases [21, 22].

Eryptosis could be triggered by a wide variety of xenobiotics [7, 8, 21-68] and accelerated eryptosis is observed in several clinical pathologies including sepsis, malaria, sickle cell disease, Wilson's disease, malignancy, metabolic syndrome, diabetes, hepatic and renal insufficiency, hemolytic uremic syndrome, dehydration, hyperphosphatemia as well as phosphate depletion [7, 49, 69, 70].

The present study tested, whether eryptosis is altered in the elderly. To this end, phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, oxidative stress, GSH abundance and ceramide abundance were determined in erythrocytes drawn from elderly and young volunteers.

Materials and Methods

Patients, erythrocytes and treatments

Whole blood was drawn from 16 elderly (7 ♀, 9 ♂, age 83 - 96 years) and 11 young (6 ♀, 5 ♂, age 26 - 43 years) volunteers. The elderly were recruited from the patients at the Division of Internal Medicine and Geriatrics, Capio Franz von Prümmer Klinik. The clinical characteristics of the elderly are shown in table 1. The study was approved by the ethics committee of the University of Tübingen (184/2003V). Both, elderly and young volunteers provided written informed consent. In order to isolate erythrocytes, whole blood was centrifuged at 120 g for 20 min at 23 °C and the platelets and leukocytes-containing supernatant was disposed.

Measurements were made in isolated erythrocytes or in erythrocytes (O^- blood group) from healthy young individuals incubated *in vitro* with 500 μl plasma from young or elderly volunteers at a hematocrit of 0.4% for 24 h. For all measurements 50.000 cells were counted.

Analysis of annexin-V-binding and forward scatter

In order to determine Annexin-V-binding, 2 μl of freshly drawn blood was mixed in 500 μl Ringer solution containing 5 mM CaCl_2 and subsequently stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) and incubated at 37°C for 20 min under protection from light. Using Flow cytometry, the forward scatter (FSC) of the erythrocytes was determined and Annexin-V-FITC fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a FACS Calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

To quantify intracellular Ca^{2+} , 2 μl of freshly drawn blood was mixed in 500 μl Ringer solution containing 5 mM CaCl_2 , stained with Fluo-3/AM (5 μM ; Biotium, Hayward, USA) and incubated at 37°C for

30 min. 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.

Determination of ceramide formation

To determine ceramide abundance, a monoclonal antibody-based assay was used. Four μl erythrocytes were mixed in 1 ml Ringer. From the resulting cell suspension 100 μl was centrifuged (1600 rpm for 3 mins at RT) pelleting the erythrocytes. Subsequently, cells were stained for 1 h at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (1:10 dilution; clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA). After two washing steps with 100 μl PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (1:50 dilution; BD Pharmingen, Hamburg, Germany) in PBS-BSA. Unbound secondary antibody was removed by repeated washing with 50 μl PBS-BSA. The samples were resuspended in 200 μl PBS-BSA and then analyzed by flow cytometric analysis at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Quantification of reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). Four μl erythrocytes were mixed in 1 ml Ringer. From the resulting cell suspension 150 μl was centrifuged (1600 rpm for 3 mins at RT). Cells were stained with DCFDA (10 μM ; Sigma, Schnellendorf, Germany) in Ringer solution at 37°C for 30 min and then washed three times in 150 μl Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μl Ringer solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Determination of GSH abundance

The content of reduced glutathione was determined using mercury orange. Four μl erythrocytes were mixed in 1 ml Ringer. From the resulting cell suspension 150 μl was centrifuged (1600 rpm for 3 mins at RT). Cells were stained with mercury orange in PBS (40 μM , Sigma Aldrich, Germany) and incubated for 3 min at 37°C, washed once and resuspended in 200 μl PBS. The fluorescence intensity was measured with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 576 nm.

Microscopy

For the visualization of eryptotic erythrocytes, 20 μl erythrocytes (1×10^6 cells) were fixed in methanol/acetone and subsequently stained with Annexin V-FLUOS (1:100 dilution; Roche Diagnostics, Mannheim, Germany) in 200 μl Ringer solution containing 5 mM CaCl_2 . The erythrocytes were washed twice and finally resuspended in 200 μl Ringer solution containing 5 mM CaCl_2 . Forty μl were placed with PROlong Gold antifade reagent (Invitrogen, Darmstadt Germany) onto a glass slide, covered with a coverslip and images were taken on a Zeiss LSM 5 EXCITER confocal laser-scanning microscope or with the phase light (Carl Zeiss MicroImaging, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC. Scale bar 5 μm .

Statistics

Data are expressed as arithmetic means $\pm$ SEM. Mann-Whitney test, Spearman nonparametric analysis and Student's *t*-test were performed to determine statistical significance between the two groups using Graph Pad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA; *n* denotes the number of individuals. *P* < 0.05 was considered significant.

Results

From the 16 elderly participants, 10 individuals were anemic with a hemoglobin level lower than 11 g/dl. None of the young volunteers was found to be anemic. As evident from Table 1, the average erythrocyte number, hematocrit and hemoglobin concentration were all decreased in the elderly as compared to the young volunteers or to the respective control values. The anemia of the elderly was paralleled by significantly enhanced reticulocytosis, indicating enhanced erythrocyte turnover. As illustrated in Fig. 1A, the percentage of

Table 1. Characteristics of the volunteers. Values are given as arithmetic mean $\pm$ SEM. (* P <0.05, ** P <0.01, *** P <0.001, n.s.; non significant) indicates significant difference from healthy young volunteers using Student's *t*-test

	Elderly	Control
Age	88.6 $\pm$ 0.9 years (range 83-96)	31.3 $\pm$ 1.7 years (range 26-43)
Gender distribution	7 females, 9 males	6 females, 5 males
Plasma creatinine concentration	1.3 $\pm$ 0.1 mg/100 ml	1.0 $\pm$ 0.1 mg/100 ml
Plasma ferritin concentration	169.6 $\pm$ 47.5 μ g/100 ml *	48.9 $\pm$ 8.8 μ g/100 ml
Transferrin saturation	33.4 $\pm$ 7.5 %	31.8 $\pm$ 3.2 %
Erythrocyte number	3.5 $\pm$ 0.1 $\times 10^6$ / μ l ***	4.8 $\pm$ 0.1 $\times 10^6$ / μ l
Hematocrit	31.5 $\pm$ 0.8 % ***	42.2 $\pm$ 1.0 %
Hemoglobin	10.5 $\pm$ 0.3 g/100 ml ***	14.5 $\pm$ 0.4 g/100 ml
Reticulocytes (%)	2.3 $\pm$ 0.2 % ***	0.9 $\pm$ 0.1 %
Reticulocytes	77.5 $\pm$ 6.6 $\times 10^3$ / μ l ***	45.4 $\pm$ 3.6 $\times 10^3$ / μ l
Hemoglobin/ reticulocyte	0.9 $\pm$ 0.2 pg/cell **	1.7 $\pm$ 0.1 pg/cell
Diagnoses	Type 2 diabetes mellitus (n = 7), hypertension (n = 12), chronic kidney disease (CKD) stage 2 (n = 6), CKD stage 3 (n = 6), CKD stage 4 (n = 2), dialysis (n = 0), hepatic insufficiency (n = 4), heart failure (n = 4), myocardial infarction (n = 1), malignancy (n = 5), stroke (n = 5)	none

Fig. 1. Phosphatidylserine-exposure of erythrocytes from young and elderly volunteers. A. The percentage reticulocytes as a function of hemoglobin concentration [g/dl] in elderly individuals (P =****, R^2 =0.6022) using Spearman nonparametric analysis. B. Light Phase microscopy (upper panels, red arrows indicate abnormal erythrocytes) and confocal images of erythrocytes stained with Annexin V-FLUOS (green; lower panels, scale bar 5 μ m). The specimens were obtained from young healthy (left panels) and from elderly (right panels) individuals. C. Original representative histogram of Annexin V-binding of erythrocytes in freshly drawn blood from young healthy (grey) and from elderly (black) volunteers. M1 indicates the Annexin-V-fluorescence defining the percentage of Annexin-V-binding erythrocytes. D. Arithmetic means $\pm$ SEM (n = 11 - 16) of the percentage of Annexin V-binding erythrocytes in freshly drawn blood from healthy young (control, black circles) and elderly (black squares) individuals. *** (P <0.001) indicates significant difference from healthy young volunteers. (Mann-Whitney test).

and confocal images of erythrocytes stained with Annexin V-FLUOS (green; lower panels, scale bar 5 μ m). The specimens were obtained from young healthy (left panels) and from elderly (right panels) individuals. C. Original representative histogram of Annexin V-binding of erythrocytes in freshly drawn blood from young healthy (grey) and from elderly (black) volunteers. M1 indicates the Annexin-V-fluorescence defining the percentage of Annexin-V-binding erythrocytes. D. Arithmetic means $\pm$ SEM (n = 11 - 16) of the percentage of Annexin V-binding erythrocytes in freshly drawn blood from healthy young (control, black circles) and elderly (black squares) individuals. *** (P <0.001) indicates significant difference from healthy young volunteers. (Mann-Whitney test).

reticulocytes in the elderly individuals was inversely and significantly correlated with the hemoglobin concentration (R^2 =0.6022; P = 0.0004). Taken together, increased erythrocyte turnover rather than decreased erythrocyte formation accounts for anemia in the elderly.

Fig. 2. Forward scatter of erythrocytes from young and elderly volunteers. A. Original representative histogram of erythrocyte forward scatter in freshly drawn blood from young healthy (grey area) and from elderly (black line) volunteers. B. Arithmetic means $\pm$ SEM ($n = 11 - 16$) of the forward scatter of erythrocytes in freshly drawn blood from healthy young (control, white bar) and elderly (black bar) individuals (n.s. indicates non significant difference using Student's *t*-test).

The present study thus explored whether anemia of the elderly could in part be due to enhanced eryptosis. Eryptotic erythrocytes were identified by annexin-V-binding reflecting exposure of phosphatidylserine at the erythrocyte surface. As illustrated in Fig. 1B-D, the percentage of erythrocytes binding Annexin V was significantly higher in blood drawn from elderly individuals than in blood drawn from the control group. Confocal microscopy visualized the fluorescent labeled (green) Annexin V at the erythrocyte surface as shown in Fig. 1C. The percentage of Annexin V-binding erythrocytes was significantly higher in elderly patients than in young healthy volunteers (Fig. 1D).

In order to determine whether eryptosis is triggered by a plasma-borne component, erythrocytes from young healthy volunteers were exposed to plasma drawn from either, elderly patients or young healthy volunteers. As a result, the percentage of PS exposing erythrocytes drawn from young healthy individuals tended to be higher in plasma from elderly patients ($12.6 \pm 1.7\%$, $n = 16$) as compared to incubation in plasma from young healthy individuals ($8.8 \pm 1.4\%$, $n = 11$), an effect, however, not reaching statistical significance.

Forward scatter was determined in order to estimate cell volume of erythrocytes from the control group and from elderly individuals. As illustrated in Fig. 2, the average forward scatter was not significantly different between erythrocytes from elderly patients and erythrocytes from young healthy individuals.

Fluo3 fluorescence was employed to estimate $[Ca^{2+}]_i$ in freshly drawn blood from elderly patients and young healthy volunteers. The Fluo3 fluorescence tended to be higher in erythrocytes from elderly patients compared to young healthy individuals. The difference did, however, not reach statistical significance (Fig. 3).

A further series of experiments elucidated the role of oxidative stress. To this end, generation of reactive oxygen species (ROS) was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. As illustrated in Fig. 4, DCFDA-fluorescence was significantly higher in erythrocytes drawn from elderly patients than in erythrocytes drawn from young healthy volunteers.

To determine whether oxidative stress is triggered by a plasma-borne component, erythrocytes from young healthy volunteers (O- blood group) were exposed to plasma drawn from either, elderly patients or young healthy volunteers. As a result, the DCFDA fluorescence in erythrocytes drawn from young healthy individuals was similar following incubation in plasma from elderly patients (11.1 ± 0.5 a.u., $n = 16$) and following incubation in plasma from young healthy individuals (11.0 ± 0.1 a.u., $n = 11$).

The enhanced oxidative stress could have resulted from decreased GSH levels leading to impaired anti-oxidative defense. Thus, mercury orange staining was employed to quantify

Fig. 3. Intracellular Ca^{2+} activity of erythrocytes from young and elderly volunteers. A. Original histogram of Fluo3 fluorescence of erythrocytes in freshly drawn blood from young healthy (grey area) and from elderly (black line) volunteers. B. Arithmetic means $\pm$ SEM ($n = 11 - 16$) of the Fluo3 fluorescence of erythrocytes in freshly drawn blood from healthy young (control, white bar) and elderly (black bar) individuals. (n.s. indicates non significant difference using Student's t -test).

Fig. 4. Reactive oxygen species of erythrocytes from young and elderly volunteers. A. Original representative histogram of DCFDA fluorescence of erythrocytes in freshly drawn blood from young healthy (grey area) and from elderly (black line) volunteers. B. Arithmetic means $\pm$ SEM ($n = 11 - 16$) of the DCFDA fluorescence of erythrocytes in freshly drawn blood from healthy young (control, white bar) and elderly (black bar) individuals. ** ($P < 0.01$) indicates significant difference from healthy young volunteers using Student's t -test.

GSH abundance. As illustrated in Fig. 5, the mercury orange-dependent fluorescence was significantly lower in erythrocytes drawn from elderly patients than in erythrocytes drawn from young healthy volunteers.

In a next series of experiments, erythrocytes were stained with FITC-labeled antibodies to quantify ceramide using flow cytometry. As shown in Fig. 6, ceramide abundance tended to be higher in erythrocytes drawn from elderly patients than in erythrocytes drawn from young healthy volunteers, a difference, however, not reaching statistical significance.

Discussion

The present observations disclose accelerated suicidal erythrocyte death in elderly individuals. Phosphatidylserine exposure at the cell surface is the hallmark of eryptosis, the

Fig. 5. GSH levels of erythrocytes from young and elderly volunteers. A. Original representative histogram of mercury orange fluorescence of erythrocytes in freshly drawn blood from young healthy (grey area) and from elderly (black line) volunteers. B. Arithmetic means $\pm$ SEM ($n = 11 - 16$) of the mercury orange fluorescence of erythrocytes in freshly drawn blood from healthy young (control, white bar) and elderly (black bar) individuals. * ($P < 0.05$) indicates significant difference from healthy young volunteers using Student's *t*-test.

Fig. 6. Ceramide abundance of erythrocytes from young and elderly volunteers. A. Original representative histogram of ceramide-dependent FITC fluorescence of erythrocytes in freshly drawn blood from young healthy (grey area) and from elderly (black line) volunteers. B. Arithmetic means $\pm$ SEM ($n = 11 - 16$) of ceramide-dependent FITC fluorescence of erythrocytes in freshly drawn blood from healthy young (control, white bar) and elderly (black bar) individuals (n.s. indicates non significant difference using Student's *t*-test).

suicidal death of erythrocytes [7]. The percentage of erythrocytes with phosphatidylserine translocation to the erythrocyte surface was significantly enhanced in blood drawn from elderly individuals.

The enhanced cell membrane scrambling in erythrocytes drawn from elderly individuals was not paralleled by a significant decrease of cell volume. Erythrocyte $[Ca^{2+}]_i$ in elderly individuals tended to be higher as compared to erythrocytes drawn from younger individuals, a difference, however, not reaching statistical significance. Following various other triggers of eryptosis, erythrocytes decrease their volume at least in part by increase of $[Ca^{2+}]_i$ with subsequent activation of Ca^{2+} sensitive K^+ channels, K^+ exit, hyperpolarization of the cell membrane, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [6].

The enhanced eryptosis in elderly individuals was paralleled by increased oxidative stress paralleled by a decreased GSH abundance. Oxidative stress is a well-known stimulator of eryptosis [7]. The present observations do not allow safe conclusions as to the cause of enhanced oxidative stress.

In some elderly individuals accelerated eryptosis could have resulted from underlying disease rather than age *per se*. Eryptosis is fostered by several disorders encountered in elderly individuals, such as malignancy, metabolic syndrome, diabetes, renal insufficiency or dehydration [7, 49]. In the elderly individuals renal function was moderately decreased. However, the difference between plasma creatinine levels between the young volunteers and elderly patients was not significant and none of the elderly patients were on dialysis. According to Fig. 1D, the percentage of phosphatidylserine exposing erythrocytes was higher in each elderly individual than the average of the young individuals. Morbidity presumably contributed to but hardly accounted for the increased eryptosis in the elderly individuals.

Whatever the reason for the anemia, the enhanced eryptosis may well contribute to the ongoing anemia in elderly individuals, as eryptotic erythrocytes are rapidly removed by phagocytosis. Anemia develops as soon as the rate of eryptosis exceeds the formation of new erythrocytes [7]. Interestingly, according to the reticulocyte numbers, the formation of new erythrocytes appears to be accelerated in the elderly. Anemia in the elderly is thus not the result of impaired erythropoiesis but rather a consequence of enhanced suicidal erythrocyte death.

Eryptosis is similarly enhanced in the rapidly ageing Klotho-deficient mice [71]. Lack of Klotho is associated with increased oxidative stress [72]. It is thus tempting to speculate that accelerated eryptosis parallels oxidative stress and ageing in both, humans and mice. Additional studies are warranted to confirm this possibility.

Phosphatidylserine exposing erythrocytes could interfere with microcirculation [8, 73-77] due to binding of phosphatidylserine to endothelial CXCL16/SR-PSO and subsequent adherence of eryptotic erythrocytes to the vascular wall [28]. Moreover, phosphatidylserine exposing erythrocytes stimulate blood clotting and thrombosis [73, 78, 79]. The subsequently increased cardiovascular risk could contribute to the enhanced morbidity and mortality of anemic elderly individuals.

Taken together, our observations suggest that erythrocyte cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface is enhanced and may well contribute to the anemia in elderly individuals.

Disclosure Statement

No conflict of interest.

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