Original Paper

Stimulated Suicidal Erythrocyte Death in Arteritis

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Key Words

Reactive oxygen species • Phosphatidylserine • Calcium • Eryptosis • Anemia

Abstract

Background/Aims: Arteritis is an inflammatory disease of the vascular wall leading to ischemia and vascular occlusion. Complications of arteritis include anemia, which could, at least in theory, result from suicidal erythrocyte death or eryptosis, which is characterized by erythrocyte shrinkage and phosphatidylserine (PS) exposure at the erythrocyte surface. Cellular mechanisms involved in the stimulation of eryptosis include increased cytosolic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$), oxidative stress and ceramide formation. The present study explored whether and how arteritis influences eryptosis. **Methods:** Blood was drawn from patients suffering from arteritis ($n=17$) and from healthy volunteers ($n=21$). PS exposure was estimated from annexin V-binding, erythrocyte volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) from DCFDA fluorescence and ceramide abundance from FITC-conjugated antibody binding in flow cytometry. The patients suffered from anemia despite $2.8 \pm 0.4\%$ reticulocytes. **Results:** The percentage of PS-exposing erythrocytes was significantly higher in patients ($1.1 \pm 0.1\%$) than in healthy volunteers ($0.3 \pm 0.1\%$). The increase in PS exposure was paralleled by increase in oxidative stress and $[\text{Ca}^{2+}]_i$, but not by significant changes of ceramide abundance. Erythrocyte PS exposure and ROS production were significantly enhanced in erythrocytes exposed to patient plasma as compared to exposure to plasma from healthy volunteers. **Conclusion:** Arteritis is associated with enhanced eryptosis due to increased $[\text{Ca}^{2+}]_i$ and oxidative stress. The eryptosis contributes to or even accounts for the anemia in those patients. As eryptotic erythrocytes adhere to endothelial cells of the vascular wall, they could impede microcirculation and thus contribute to vascular occlusion.

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Introduction

Arteritis, an inflammatory disease of the vascular wall, may eventually lead to vascular occlusion with severe injury of the affected organs [1]. The vascular occlusion is considered

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to be, at least in part, due to activation of blood platelets [2]. Further complications of arteritis include anemia [1, 3-5]. At least in theory, anemia during arteritis may result from stimulation of eryptosis, the suicidal death of erythrocytes characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface [6]. Phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood and accelerated eryptosis is a common cause of anemia [6]. As phosphatidylserine-exposing erythrocytes bind to the surface of endothelial cells, they may impede microcirculation and thus contribute to vascular occlusion [6].

Cellular mechanisms involved in the stimulation of eryptosis include increased cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to Ca^{2+} entry through Ca^{2+} -permeable cation channels [6, 7], oxidative stress [6], ceramide formation [6], energy depletion [6], caspase activation [6] and deranged activity of a variety of kinases [6]. Enhanced eryptosis is common and contributes to the anemia of a variety of clinical conditions including chronic kidney disease (CKD), hemolytic-uremic syndrome, diabetes, hepatic failure, malignancy, sepsis, sickle-cell disease, and Wilsons disease [6, 8-10].

The present study explored, whether arteritis is associated with enhanced phosphatidylserine exposure of circulating erythrocytes and, if so, to identify the cellular mechanisms possibly involved.

Materials and Methods

Patients, erythrocytes and treatments

Seventeen patients (13 ♀, 4 ♂, age 65.1 ± 3.1 years) with arteritis were enrolled in the study. The clinical diagnosis and patient characteristics are shown in Table 1A. The patients suffered from anemia (Table 1B). Blood was drawn from patients and from age and sex matched healthy volunteers. The study was approved by the ethics committee of the University of Tübingen (184/2003V). Both, volunteers and patients provided written informed consent. In order to isolate the erythrocytes, whole blood was centrifuged at $120 \times g$ for 20 min at 23°C and the platelets and leukocytes-containing supernatant was disposed. Measurements were made in freshly isolated erythrocytes or in erythrocytes (O⁻ blood group) from healthy young individuals incubated *in vitro* with 500 μl plasma from patients or healthy volunteers at a hematocrit of 0.4% for 24 hours. For all measurements 50.000 cells were counted.

FACS analysis of annexin V-binding and forward scatter

In order to determine annexin-V-binding, 2 μl of freshly drawn blood were mixed in 500 μl Ringer solution containing 5 mM CaCl_2 , subsequently stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in Ringer solution containing 5 mM CaCl_2 at 37°C for 15 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for healthy erythrocytes and erythrocytes from arteritis patients. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters.

Reticulocyte count

For determination of the reticulocyte count, Lithium-Heparin-whole blood (2 μl) was added to 500 μl Retic-COUNT® (thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature in the dark and flow cytometry was performed according to the manufacturer's instructions. Forward scatter (FSC), side scatter (SSC) and thiazole orange-fluorescence intensity (in FL-1) of the blood cells were determined. The number of Retic-COUNT positive reticulocytes was expressed as the percentage of the total gated erythrocyte population. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using CellQuest software.

Table 1. Clinical diagnosis and characteristics of patients studied

A				
Number	Age	Sex	Clinical diagnosis	Medication
1	61	f	Takayasu's arteritis	Prednisolone
2	58	m	Inflammatory abdominal aortic aneurysm	Prednisolone
3	36	f	Polyarteritis nodosa	Prednisolone, Methotrexate
4	56	f	Giant cell arteritis	Azathioprine, Prednisolone
5	63	f	Takayasu's arteritis	Prednisolone, Mycophenolic acid
6	82	f	Large vessel vasculitis	Azathioprine, Prednisolone
7	47	m	Takayasu's arteritis	Azathioprine, Prednisolone
8	54	f	Takayasu's arteritis	Prednisolone, Methotrexate
9	75	f	Giant cell arteritis	Azathioprine, Prednisolone
10	72	f	Giant cell arteritis	Methotrexate
11	77	f	Giant cell arteritis	Azathioprine, Prednisolone
12	81	f	Temporal arteritis	Leflunomide, Prednisolone
13	72	m	Large vessel vasculitis	Prednisolone, Methotrexate
14	62	m	Granulomatosis with polyangiitis	Prednisolone
15	73	f	ANCA associated vasculitis	Azathioprine, Prednisolone
16	77	f	Giant cell arteritis	Prednisolone
17	60	f	Takayasu's arteritis	Prednisolone, Mycophenolic acid
B			aQ	
Characteristics	Patient values*		Control values	
Age	65.1 ± 3.1		51.4 ± 1.7	
Plasma creatinine concentration, (mg/100 ml)	0.87 ± 0.06		0.5-0.8 (♀), 0.6-1.1 (♂)	
Plasma uric acid, (mg/100 ml)	5.39 ± 0.33		2.4-5.7 (♀), 3.4-7.0 (♂)	
C-reactive protein, (mg/100 ml)	1.12 ± 0.28		< 0.5	
Erythrocyte sedimentation rate, (mm/h)	23.31 ± 3.60		< 30 (♀), < 20 (♂)	
Erythrocyte number, (x10 ⁶ /μl)	4.14 ± 0.12		4.94 ± 0.11	
Hemoglobin, (g/dl)	12.49 ± 0.51		14.65 ± 0.22	
Hematocrit, (%)	37.12 ± 1.37		41.94 ± 0.70	
Reticulocytes, (%)	2.78 ± 0.40		1.98 ± 0.15	
Plasma ferritin concentration, (μg/100 ml)	11.28 ± 4.48		1.0-20 (♀), 3.0-30 (♂)	
RBC distribution width, (%)	15.08 ± 0.78		< 14	
MCH, (pg/erythrocyte)	30.28 ± 1.06		27.0-34.0	
MCHC, (g/100 ml)	33.57 ± 0.30		32.0-36.0	
MCV, (fl)	89.96 ± 2.57		85.32 ± 1.51	
Plasma total protein, (g/100 ml)	7.0 ± 0.20		6.5-8.5	
Leucocytes, (/μl)	9269 ± 949		4100-11800 (♀), 3800-10300 (♂)	
Lymphocytes, (%)	15.95 ± 2.44		20-45	
Monocytes, (%)	6.27 ± 0.66		2-8	
Thrombocytes, (x10 ³ /μl)	267.7 ± 14.45		150-450	

* Values are given as arithmetic mean ± SEM.

Measurement of intracellular Ca²⁺

In order to quantify intracellular Ca²⁺ concentration, 2 μl of freshly drawn blood were mixed in 500 μl Ringer solution containing 5 mM CaCl₂, stained with Fluo-3/AM (5 μM; Biotium, Hayward, USA) and incubated at 37°C for 30 min. Ca²⁺-dependent 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. Afterwards, the geomean of the Ca²⁺ dependent fluorescence was determined.

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) and the erythrocytes were pelleted. Subsequently, cells were stained for 1 h at 37°C with 1 μg/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 μ l PBS-BSA. The samples were resuspended in 200 μ l PBS-BSA and then analyzed in FL-1 at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. Finally, the geomean of the ceramide dependent fluorescence was determined.

Quantification of reactive oxygen species (ROS)

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

Confocal microscopy and immunofluorescence

For the visualization of eryptotic erythrocytes, 20 μ l erythrocytes (1×10^6 cells) were stained with Annexin-V-FLUOS (1:100 dilution; Roche Diagnostics, Mannheim, Germany) in 200 μ l Ringer solution containing 5 mM CaCl_2 . The erythrocytes were washed twice and finally resuspended in 200 μ l Ringer solution containing 5 mM CaCl_2 . Forty μ l were spread onto a glass slide and dried for 15 mins on RT. The slides were covered with PROlong Gold antifade reagent (Invitrogen, Darmstadt Germany). 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 20 μ m.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. Mann-Whitney test 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

The present study explored the putative impact of arteritis on suicidal erythrocyte death. In a first step, we analyzed the differences in blood parameters of arteritis patients and healthy volunteers. As a result, erythrocyte count, hemoglobin concentration and hematocrit were significantly lower in arteritis patients as compared to healthy patients, suggesting that arteritis patients suffered from anemia (Fig. 1A,B,C). The percentage of reticulocytes was significantly higher in the arteritis patients in comparison to healthy volunteers suggesting increased erythropoiesis in arteritis patients (Fig. 1D).

In a next step, we explored the extent of eryptosis in patients suffering from arteritis. The key hallmark of eryptosis is phosphatidylserine translocation to the erythrocyte surface. In order to identify phosphatidylserine exposing erythrocytes, annexin V positive cells were detected by confocal microscopy. As shown in Fig. 2A, the number of phosphatidylserine exposing erythrocytes was higher in fresh blood drawn from arteritis patients compared to the healthy control group. The percentage of annexin V binding cells was in addition determined by FACS analysis. As illustrated in Fig. 2C, the percentage of annexin V positive cells was significantly higher (about three times) in patients suffering from arteritis than in healthy volunteers. Eryptosis is typically paralleled by cell shrinkage. Accordingly, forward scatter was determined in flow cytometry as a measure of erythrocyte volume. The average forward scatter geomean was, however, not significantly different between erythrocytes from patients (516 ± 12 a.u., *n* = 15) and erythrocytes drawn from healthy volunteers (498 ± 5 a.u., *n* = 18).

Phosphatidylserine translocation may be triggered by an increase in erythrocyte Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Fluo3 fluorescence was thus employed to estimate $[\text{Ca}^{2+}]_i$ in

Fig. 1. Blood parameters in freshly drawn blood drawn from arteritis patients and healthy volunteers. Means $\pm$ SEM of erythrocyte count (A), hemoglobin concentration (B), hematocrit (C) and percentage of reticulocytes (D) in freshly drawn blood from healthy volunteers (n=18) (white bars) and from arteritis patients (n=15, black bars). * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$) indicate significant difference from healthy volunteers (unpaired *t* test).

Fig. 2. Phosphatidylserine-exposing erythrocytes in freshly drawn blood from arteritis patients and healthy volunteers. A. Confocal images of FITC-dependent fluorescence and light microscopy images of human erythrocytes stained with FLUOS-conjugated annexin V. The specimens were obtained from healthy volunteers (left panels) and from arteritis patients (right panels). B. Original histogram of annexin V-binding of erythrocytes in freshly drawn blood from healthy volunteers (red area) and from arteritis patients (black line). C. Arithmetic means $\pm$ SEM of the percentage of annexin V-binding erythrocytes in freshly drawn blood from healthy volunteers (n=18, black circles) and from arteritis patients (n=15, black squares). *** ($P<0.001$) indicates significant difference from healthy volunteers (Mann Whitney test).

freshly drawn blood from arteritis patients and healthy volunteers. As shown in Fig. 3A,B, Fluo3 fluorescence in erythrocytes was significantly higher in erythrocytes from freshly drawn blood of arteritis patients than in erythrocytes from freshly drawn blood of healthy volunteers.

Fig. 3. Erythrocyte Ca^{2+} concentration and reactive oxygen species production in freshly drawn blood from arteritis patients and healthy volunteers. A. Original histogram of Fluo3 fluorescence in erythrocytes from freshly drawn blood of healthy volunteers (red area) and from arteritis patients (black line). B. Arithmetic means $\pm$ SEM of Fluo3 fluorescence in erythrocytes from freshly drawn blood of healthy volunteers ($n=21$, white bar) and from arteritis patients ($n=17$, black bar). C. Original histogram of DCFDA fluorescence in erythrocytes from freshly drawn blood of healthy volunteers (red area) and from arteritis patients (black line). D. Arithmetic means $\pm$ SEM of DCFDA fluorescence in erythrocytes from freshly drawn blood of healthy volunteers ($n=18$, white bar) and from arteritis patients ($n=13$, black bar). * ($P<0.05$) and ** ($P<0.01$) indicate significant difference from healthy volunteers (unpaired t test).

Fig. 4. Effect of patient plasma on phosphatidylserine exposure and reactive oxygen species in erythrocytes from healthy volunteers. A. Original histogram of annexin V-binding of erythrocytes from healthy volunteers following a 24 hours exposure to plasma from healthy volunteers (red area) and to plasma from arteritis patients (black line). B. Arithmetic means $\pm$ SEM of the percentage of annexin V-binding erythrocytes from healthy volunteers exposed for 24 hours to plasma from healthy volunteers ($n=18$, white bar) and to plasma from arteritis patients ($n=13$, black bar). C. Original histograms of DCFDA fluorescence of erythrocytes from healthy volunteers following a 24 hours exposure to plasma from healthy volunteers (red area) and to plasma from arteritis patients (black line). D. Arithmetic means $\pm$ SEM of DCFDA fluorescence in erythrocytes from healthy volunteers exposed for 24 hours to plasma from healthy volunteers ($n=18$, white bar) and from arteritis patients ($n=13$, black bar). * ($P<0.05$) indicates significant difference from healthy volunteers (unpaired t test).

As phosphatidylserine translocation may further result from oxidative stress, reactive oxygen species in erythrocytes was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. As illustrated in Fig. 3C,D, DCFDA-fluorescence was significantly higher in erythrocytes from freshly drawn blood of arteritis patients than in erythrocytes from freshly drawn blood of healthy volunteers.

As ceramide similarly stimulates phosphatidylserine translocation in erythrocytes, ceramide abundance was determined by FITC-labeled anti-ceramide antibodies. As a result, the ceramide abundance tended to be higher in erythrocytes from freshly drawn blood of arteritis patients (13.1 ± 0.8 a.u., $n = 13$) than in erythrocytes from freshly drawn blood of

healthy volunteers (11.9 ± 0.4 a.u., $n = 18$), a difference, however, not reaching statistical significance.

The present study further elucidated whether eryptosis is triggered by a blood-borne component. To this end, erythrocytes from healthy volunteers were exposed for 24 hours to plasma from either healthy volunteers or plasma from arteritis patients. As illustrated in Fig. 4A,B, the percentage of annexin-V-binding erythrocytes from healthy volunteers was significantly higher following a 24 hours exposure to plasma from arteritis patients than following a 24 hours exposure to plasma from healthy volunteers. The average forward scatter of erythrocytes following exposure to plasma from arteritis patients tended to be lower (460 ± 14 a.u., $n = 13$) than following exposure to plasma from healthy volunteers (473 ± 12 a.u., $n = 18$), a difference, however, not reaching statistical significance.

The Fluo3 fluorescence and ceramide abundance in erythrocytes from healthy individuals were not significantly different between erythrocytes exposed to patient plasma and erythrocytes exposed to plasma from healthy volunteers (data not shown).

According to DCFDA fluorescence, ROS was significantly higher following exposure to plasma from arteritis patients than following exposure to plasma from healthy volunteers (Fig. 4C,D). Thus, a plasma component in arteritis patients imposes oxidative stress on erythrocytes.

Discussion

The present study reveals that arteritis is associated with enhanced appearance of phosphatidylserine-exposing erythrocytes in circulating blood. Accordingly, arteritis leads to accelerated suicidal erythrocyte death or eryptosis.

The percentage of phosphatidylserine-exposing erythrocytes was about three times higher in freshly drawn blood from the patients as in blood drawn from healthy volunteers. As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood [6], a doubling of phosphatidylserine-exposing erythrocytes in circulating blood should decrease erythrocyte life span to half. If erythrocyte formation would not be enhanced, the reduction of erythrocyte life span to half would be expected to reduce the number of circulating erythrocytes by 50%. However, the anemia is followed by enhanced release of erythropoietin, which stimulates the formation of new erythrocytes. The enhanced erythropoiesis is apparent from the enhanced percentage of reticulocytes in circulating blood. Besides stimulating erythropoiesis, erythropoietin may enhance the susceptibility of erythrocytes for triggers of eryptosis, an effect, at least in theory, contributing to enhanced eryptosis in arteritis patients [11].

The observed eryptosis in arteritis patients is, at least in part, the result of stimulation by a component in plasma, as treatment of erythrocytes from healthy individuals with plasma from arteritis patients triggered cell membrane scrambling with phosphatidylserine translocation. Possibly, eryptosis does not result from a single component, but may result from altered concentration of several blood components. Eryptosis is stimulated by a wide variety of small molecules [12-37] including the inflammatory mediators prostaglandin E2 [38] and leukotriene [39] and enhanced eryptosis is observed in several clinical disorders [6, 8, 40]. Thus, stimulation of eryptosis in arteritis may result from systemic inflammation rather than from specific injury of the vascular wall. Future experiments may uncover some of the components contributing to the stimulation of eryptosis in arteritis.

The present observations further shed some light on the underlying cellular mechanisms participating in the stimulation of eryptosis. According to our observations, the stimulation of eryptosis is paralleled by increases in cytosolic Ca^{2+} activity and involves oxidative stress but is not paralleled by increased ceramide abundance. Both, oxidative stress and ceramide are well known stimulators of eryptosis [6]. The present observations do, however, not rule out additional mechanisms participating in the triggering of eryptosis. Future experiments will be required to fully define the mechanisms underlying eryptosis in arteritis.

In addition to its effect on erythrocyte life span, the stimulation of phosphatidylserine translocation to the erythrocyte surface is expected to foster thrombosis and thus to interfere with microcirculation [6]. Phosphatidylserine-exposing erythrocytes adhere to the vascular wall due to interaction of erythrocytic phosphatidylserine with endothelial CXCL16/SR-PSO [41]. Erythrocyte adherence to the vascular wall is expected to interfere with blood flow [6, 41]. Moreover, phosphatidylserine-exposing erythrocytes trigger blood clotting [6, 42]. Phosphatidylserine exposing erythrocytes could activate the complement system [42, 43] leading to further stimulation of eryptosis [44] and aggravation of vascular inflammation [43].

Conclusion

Arteritis is paralleled by stimulation of eryptosis, an effect at least partially due to increased cytosolic Ca^{2+} activity and oxidative stress. Eryptosis could in turn lead to anemia, to further vascular inflammation and thus to further impairment of microcirculation. Thus, therapeutic considerations in the treatment of arteritis may include strategies to counteract eryptosis.

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Disclosure Statement

All authors of this manuscript declare that they have no competing interests.

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