Eryptosis as an Underlying Mechanism in Systemic Lupus Erythematosus-Related Anemia

Peipei Jiang a  Maohong Bian a  Wenjuan Ma b  Chunqiu Liu a  Peng Yang a  Bangqiang Zhu a  Yuanhong Xu c  Meijuan Zheng c  Jinpin Qiao c  Zongwen Shuai d  Xueyong Zhou e  Dake Huang f

a Department of Blood Transfusion, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China; b Department of Laboratory Medicine, Hangzhou Elderly hospital, Hangzhou, Zhejiang, China; c Department of Laboratory Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China; d Department of Rheumatism and Immunology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China; e Blood Center of Anhui Province, Hefei, Anhui, China; f Integrated Laboratory of Anhui Medical University, Hefei, Anhui, China.

Key Words
Anemia • Eryptosis • Phosphatidylserine • Calcium • Reactive oxygen species • Systemic lupus erythematosus

Abstract

Background: The progression of systemic lupus erythematosus (SLE) leads to anemia in patients, adversely affecting prognosis. The diverse causes of anemia may include excessive eryptosis or premature suicidal erythrocyte death characterized by cell shrinkage and phosphatidylserine (PS) exposure on the cell surface. The present study explored if SLE enhances eryptosis and the underlying mechanisms. Materials and Methods: Eryptosis was assessed using flow cytometry in healthy volunteers (n = 20) and anemic patients hospitalized for SLE (n = 22), for parameters including PS exposure, cell volume, cytosolic calcium ion (Ca^{2+}) levels and reactive oxygen species (ROS) and ceramide abundance. These indicators were measured in erythrocytes of experimental subjects and erythrocytes treated with plasma from healthy volunteers or SLE patients. Results: The hemoglobin and hematocrit levels were significantly lower in anemic SLE patients than in healthy volunteers (**p<0.001, *p<0.001, respectively). The percentage of PS-exposing erythrocytes was significantly higher in SLE patients than in healthy volunteers (p<0.001), accompanied by an increase in cytosolic Ca^{2+} levels, oxidative stress. The measurements of PS and Ca^{2+} levels were significantly higher in the erythrocytes of healthy volunteers following incubation in plasma of SLE patients than in plasma of healthy volunteers for 24h (**p<0.001, *p<0.05 respectively). Conclusion: Eryptosis is enhanced in SLE and may contribute to anemia. The probable underlying mechanisms may be an excessive formation of ROS in erythrocytes. Also, some plasma components may trigger eryptosis by increasing the cytosolic Ca^{2+} concentration.
Introduction

The majority of patients with systemic lupus erythematosus (SLE) suffer from anemia in the later stages, which mainly originates from chronic disease, iron deficiency, and autoimmune hemolytic anemia [1-6]. In addition, an accelerated eryptosis could potentially result in anemia in SLE. Indeed, some studies have reported that diseases associated with anemia including end-stage renal disease, heart failure, hemolytic uremic syndrome, sepsis, malaria, and iron deficiency can enhance eryptosis [7-12]. Eryptotic erythrocytes are recognized by phagocytosed and engulfed cells, and thus, cleared from the circulating blood. The accelerated eryptosis can be attributed to premature suicidal erythrocyte death, resulting in the reduction of circulating erythrocytes, and thereby, leading to the development of anemia.

Eryptosis, also known as suicidal erythrocyte death, is characterized by the erythrocyte shrinkage, phosphatidylserine exposure at the cell surface [13, 14]. Eryptosis is triggered by increased cytosolic Ca\(^{2+}\) concentration, which may due to activated unselective cation channels facilitate calcium ion (Ca\(^{2+}\)) entry into the cell. An increased cytosolic Ca\(^{2+}\) concentration stimulates phospholipids scrambling by phospholipid translocator along with subsequent phosphatidylserine exposure at the cell surface and triggers the cell shrinkage by the activation of Ca\(^{2+}\)-sensitive K\(^{+}\) channels [15-17]. Signaling involved in the triggering of eryptosis further includes oxidative stress [18], ceramide [19], hyperosmotic shock [20], energy deficiency [21], caspase activation [22] and deranged activity of several kinases such as CK1\(\alpha\), PKC, AMPK, GSK, PAK2, JAK3, p38-MAPK [14, 17, 19, 23-25].

We hypothesized that eryptosis might be one of the causes of anemia in SLE, and the plasma component of the patients may shorten the lifespan of erythrocytes. In the present study, we substantiated our hypothesis by measuring the indexes of eryptosis in erythrocytes of SLE patients and healthy volunteers exposed to plasma from SLE patients.

Materials and Methods

Screening for patients and treatments of erythrocytes

Blood was withdrawn from SLE patients (21 females, 1 males, age 18–75 years) recruited from the Department of Rheumatism, First Affiliated Hospital of Anhui Medical University and healthy volunteers (17 females, 3 males, age 21–58 years). Exclusion criteria: those patients who were transfused blood products and were accompanied by autoimmune hemolytic anemia, aplastic anemia, and thrombotic microangiopathic hemolytic anemia. The hematological parameters of the patients and healthy volunteers

| Table 1. Hematological parameters of study subjects. Values are given as arithmetic mean ± SEM |
|---------------------------------------------|---------------------------------------------|
| Gender distribution                        | SLE (n=22)                                  |
| Age (years)                                | 31.8±2.2 years (range16-46)                 |
| Erythrocytes (+10\(^9\)/ul)                | 3.2±1.08                                   |
| Hemoglobin (g/dl)                          | 8.82±0.29                                  |
| Hematocrit (%)                             | 27.4±0.8                                   |
| Reticulocytes (+10\(^9\)/ul)               | 86.6±6.1                                   |
| Reticulocytes (%)                          | 2.9±0.21                                   |
| MCV (fl)                                   | 84.6±1.5                                   |
| Autoantibodies                             | anti-dsDNA antibody (n=17), anti-nDNA antibody (n=9), anti-Sm antibody (n=10), anti-Ro antibody (n=9), Anti-ribosomal P protein antibodies (n=5), Anti-C1q antibody (n=9) |

| Control (n=29)                             |
|---------------------------------------------|---------------------------------------------|
| Gender distribution                        | 17 females, 3 males                         |
| Age (years)                                | 29.6±1.9 years (range22-43)                 |
| Erythrocytes (+10\(^9\)/ul)                | 4.2±0.03                                   |
| Hemoglobin (g/dl)                          | 12.6±0.09                                  |
| Hematocrit (%)                             | 39.7±0.3                                   |
| Reticulocytes (+10\(^9\)/ul)               | 57.3±2.4                                   |
| Reticulocytes (%)                          | 1.2±0.15                                   |
| MCV (fl)                                   | 91.6±0.6                                   |

Only weakly positive results in individual.
are shown in Table 1. The study was approved by the Ethics Committee of the Anhui Medical University, and written informed consents were obtained from all the study subjects.

Whole blood was centrifuged at 1900 x g for 3 min at 25°C and compacted erythrocytes were conserved. Estimations were made in freshly isolated erythrocytes or erythrocytes (O+ blood group) from healthy individuals exposed in vitro to plasma from patients or healthy volunteers at a hematocrit of 0.4% for 24 h.

PS exposure and forward scatter
50 μL of erythrocyte suspension at a hematocrit of 0.4% was washed in Ringer’s solution containing 5 mM CaCl$_2$. Subsequently, the mixture was exposed to phosphatidylserine reacted with 5 μL annexin V-fluorescein isothiocyanate (FITC) (BestBio, Shanghai, China) in the 400 μL binding buffer at 37°C for 20 min in the dark. The annexin V staining and forward scatter were measured by FACS Calibur (BD Biosciences, CA, USA) using an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Detection of intracellular calcium
100 μL of erythrocyte suspension at a hematocrit of 0.4% was washed in Ringer’s solution and loaded with Fluo-3/AM (Dojindo, Japan) at a final concentration of 2 μM in Ringer’s solution containing 5 mM of CaCl$_2$. The cells were incubated at 37°C for 30 min and washed twice in CaCl$_2$-Ringer’s. The Fluo-3/AM-loaded erythrocytes were resuspended in 400 μL of Ringer’s. The measurement conditions of Ca$^{2+}$-dependent fluorescence intensity was similar to that of annexin V.

Measurement of ceramide abundance
Anti-ceramide antibody and species-specific secondary antibodies labeled with FITC were used to estimate the ceramide abundance. 100 μL of erythrocyte suspension at a hematocrit of 0.4%, was washed in Ringer’s solution. Subsequently, the cells were incubated with the anti-ceramide antibody (1:100; clone MID 15B4; Sigma, USA) for 1 h in phosphate-buffered saline (PBS, pH=7.40) containing 0.1% bovine serum albumin (BSA). Then, the cells were washed and stained for 30 min at 37°C with the polyclonal FITC-conjugated goat anti-mouse IgM-specific antibody (1:50; Bioss, Beijing, China) in PBS-BSA. After repeated washings, the samples were resuspended in 400 μL PBS-BSA and analyzed as that for annexin V.

Quantification of reactive oxygen species (ROS)
2, 7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect the level of oxidative stress. In the presence of ROS, DCFH converts into DCF through a biochemical reaction and exhibits fluorescence. 100 μL of erythrocyte suspension at a hematocrit of 0.4% was washed in Ringer’s solution and stained with DCFH-DA (10 μM; Beyotime, Shanghai, China). The erythrocytes were incubated at 37°C for 25 min and then washed in Ringer’s. The DCF-loaded erythrocytes were resuspended in 400 μL Ringer’s, and ROS-dependent fluorescence intensity was measured at the same condition used for annexin V.

Confocal laser scanning microscopy
To visualize the eryptotic erythrocytes, 20 μL of the suspension at a hematocrit of 0.4% was stained with 2 μL FITC-conjugated annexin V (BestBio) in the 200 μL binding buffer for 15 min. The erythrocytes were washed and resuspended in 100 μL binding buffer. Subsequently, 50 μL were placed with Prolong Gold antifade reagent (Beyotime) in the confocal dish, and images captured on a Leica TCS SP5 confocal laser scanning microscope (Leica, Germany).

Plasma total antioxidant capacity
2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) was used to detect the total antioxidant capacity in blood plasma. Trolox was used to prepare different concentrations of the standard solution. The working solution was prepared by mixing ABTS and oxidant solutions in equal volumes. After 16 h of chemical reaction at room temperature in the dark, the working solution was diluted with PBS in order to obtain an absorbance of 0.7 ± 0.05 at 734 nm. A new working solution was prepared for each assay. The standards and samples (10 μL) were mixed with 200 μL of working solutions, respectively, and the absorbance was measured after 6 min. The results of plasma total antioxidant capacity were expressed as Trolox equivalent antioxidant capacity (TEAC).
Statistics

Data were expressed as mean ± SEM. Student’s t-test compared the differences between the two groups using GraphPad Prism version 5.00 for Windows, (GraphPad Software, La Jolla CA, USA). p<0.05 was considered as statistically significant.

Results

The SLE patients suffering from anemia revealed a hemoglobin concentration (8.8 ± 1.3 g/dL), and hematocrit (27.4 ± 3.5%), which were significantly lower than the hemoglobin concentration (12.6 ± 0.5 g/dL) and haematocrit (38.2 ± 1.2%) in healthy volunteers (**p<0.001, p<0.001 respectively), although the reticulocyte number in patients was higher than that in the healthy volunteers. The present study aimed to explore whether anemia of SLE patients could partially be due to enhanced eryptosis and to assess the influence of plasma component on eryptosis. The flow cytometry experiments analyzed the erythrocytes from fresh blood. The confocal microscopy visualized the fluorescent-labeled annexin V (green) on the erythrocyte surface (Fig. 1A). The percentage of PS-exposing erythrocytes, reflecting the percentage of eryptosis, was significantly higher in SLE patients (4.1 ± 0.24%) than in the healthy volunteers (2.5 ± 0.19%), (**p<0.001), which was paralleled by an increase in cytosolic Ca²⁺ levels and oxidative stress (Fig. 1B, 1C). Forward scatter was measured to estimate the cell volume. The average forward scatter was significantly higher (**p<0.01) in erythrocytes from healthy volunteers than from the SLE patients (Fig. 1D, 1E).
Jiang et al.: Eryptosis in Systemic Lupus Erythematosus

Fig. 2. Ca2+ levels, ceramide, and ROS in erythrocyte from fresh blood of patients and healthy volunteers. A, C, E. Representative histograms of Fluo3 fluorescence (A), ceramide-dependent fluorescence (C), and DCFDA-fluorescence (E), in fresh erythrocytes from healthy volunteers (black line) and SLE patients (red line). B, D, F. Mean ± SEM (n=20–22) of Fluo3 fluorescence (B), ceramide-dependent fluorescence (D), and DCF-fluorescence (F) in fresh erythrocytes from healthy volunteers (white bars) and SLE patients (black bar). ***p<0.001, **p<0.01 indicates significant difference to healthy volunteers, and n.s indicates no significant difference to healthy volunteers (Student’s t-test).

Fig. 3. Influence of patient plasma on PS-exposure and volume in erythrocytes from healthy volunteers. A. Light microscopy (upper panels) and confocal laser scanning images (middle and lower panels) of erythrocytes from healthy volunteers dyed with FITC-labeled annexin V the following exposure to plasma from healthy volunteers (left panels) and SLE patients (right panels) for 24h. The length of line in photographs represents 25μm. B. A representative histogram of annexin V-binding illustrating the PS-exposure of erythrocytes of healthy volunteers following a 24h exposure to plasma from healthy volunteers (black line) and SLE patients (red line). C. Individual percentages of annexin V-binding erythrocytes following erythrocytes of healthy volunteers exposed to plasma from healthy volunteers and SLE patients for 24h and mean ± SEM (n=20–22) of the two groups. ***p<0.001 indicates statistically significant difference between the two groups (Student’s t-test). D. A representative histogram of forward scatter demonstrating the cell volume of erythrocytes from healthy volunteers following exposure to plasma from healthy volunteers (black line) and SLE patients (red line) for 24 hours. E. Mean ± SEM (n=20–22) of the forward scatter of erythrocytes from healthy volunteers following exposure to plasma from healthy volunteers (white bar) and SLE patients (black bar) for 24 hours. n.s indicates no significant difference to healthy volunteers (Student’s t-test).
An increased cytosolic Ca$^{2+}$ concentration is a primary trigger of eryptosis. Fluo3 fluorescence was used to detect the cytosolic Ca$^{2+}$ concentration in fresh erythrocytes from SLE patients and healthy volunteers, and was found to be significantly higher (**p<0.001) in erythrocytes from SLE patients than from the healthy volunteers (Fig. 2A, 2B).

Since ceramide can activate the PS translocator that promotes PS exposure, we sought to determine the ceramide abundance in erythrocytes. The ceramide abundance tended to be higher in the erythrocytes drawn from SLE patients than the healthy volunteers; however, the difference did not reach statistical significance (Fig. 2C, 2D).

Another stimulus of eryptosis is oxidative stress, which was quantified employing DCFH-DA fluorescent dye. The DCF fluorescence was significantly higher (**)p<0.01) in erythrocytes from fresh blood of SLE patients than that of healthy volunteers (Fig. 2E, 2F).

Whether eryptosis was stimulated by the plasma component was assessed by the flow cytometry experiments. Herein, the erythrocytes from healthy volunteers were incubated in the plasma from patients and healthy volunteers for 24h. As illustrated in Fig. 3A-3C, the percentage of PS-exposing erythrocytes from healthy individuals was significantly higher (**p<0.01) following incubation in plasma of SLE patients in comparison to that from healthy volunteers. However, a significant difference was not observed in the average forward scatter between erythrocytes incubated in the plasma from the patients and incubated in the plasma from healthy volunteers (Fig. 3D, 3E).

The Fluo3 fluorescence intensity demonstrated that the cytosolic Ca$^{2+}$ levels were significantly higher (*)p<0.05) in the erythrocytes exposed to plasma of SLE patients than that exposed to plasma of healthy volunteers (Fig. 4A, 4B).
Neither the ceramide nor the ROS abundance showed significant differences between the erythrocytes exposed to plasma of SLE patients and healthy volunteers (Fig. 4C-4F). Similarly, the plasma total antioxidant capacity did not differ significantly between the patients (0.88mM) and healthy volunteers (0.92mM) (Fig. 5).

Discussion

We hypothesized that suicidal erythrocyte death might contribute towards anemia in SLE patients attributed to the following reasons. First, according to the previous studies [26, 27], the prevalence of anti-RNP, anti-dsDNA antibody, and anti-Sm in SLE patients is positively correlated to the impairment of hematological system, in which the erythrocyte series damage is the most common; anti-RNP is closely associated with the erythrocyte series damage. Furthermore, the percentage of apoptosis of the peripheral blood mononuclear cells in the presence of nuclear targeting auto-antibodies is higher than in their absence [28]. In addition, the oxidative stress presented in SLE patients [29-31] is the main trigger of eryptosis [32, 33].

The current study demonstrates that the percentage of PS-exposed erythrocytes in the blood from SLE patients is significantly higher than in the blood from healthy volunteers. Therefore, eryptosis is enhanced in the blood of SLE patients suffering from anemia. Given that eryptotic erythrocytes are rapidly cleared from circulating blood, anemia develops as soon as the accelerated loss of erythrocytes is not compensated by the formation of new erythrocytes. In SLE patients, however, the anemia prevails despite the increased reticulocyte count, implying that anemia is not the result of the reduced formation of erythrocytes but rather a consequence of enhanced eryptosis. Simultaneously, a significant decrease in average cell volume in fresh erythrocyte from SLE patients could be observed, which is another characteristic of eryptosis [14].

Herein, several key established triggers for eryptosis were studied. We found an increased cytosolic Ca$^{2+}$ concentration in both fresh erythrocytes from patients and healthy volunteers after incubation in plasma from patients. This phenomenon implies that the enhanced eryptosis of SLE patients at least partially could be attributed to increased cytosolic Ca$^{2+}$ levels. Furthermore, eryptosis has been strengthened by incubation of erythrocytes from healthy volunteers in plasma obtained from patients. Thus, the components of the patient plasma may at least partly be responsible for Ca$^{2+}$ influx and PS exposure. The current study reveals the connection between the increased cytosolic Ca$^{2+}$ concentration and enhanced eryptosis in SLE, conforming to the previous study that underlined the vital function of Ca$^{2+}$ influx in eryptosis [15-17].

In patients with SLE, ROS that are primarily derived from the mitochondria of lymphocytes, vascular endothelial cells, and erythrocytes have been involved in the apoptosis of T cell and the pathogenesis of SLE [29, 34-36]. Similarly, the rate of eryptosis was positively correlated
with the ROS abundance as shown in this study. The increased percentage of eryptosis in fresh blood from SLE patients was equivalent to an increased ROS abundance, which may be attributed to the excessive production of ROS and antibodies against anti-oxidant enzymes [37-39]. Notably, in the present study, neither of the plasma total antioxidant capacity of patients and ROS in erythrocytes incubated in plasma from patients exhibited a significant change as compared to the control groups. Thus, it can be speculated that the amount of reducing substance in plasma from the patients is normal and these factors that drive eryptosis do not include oxidative stress.

Similarly, the increased percentage of PS-exposure in fresh erythrocytes from SLE patients or in erythrocytes distributed with plasma from patients was not paralleled by increased ceramide abundance, another trigger of eryptosis.

In our study, three patients suffered from lupus nephritis and 31% of patients presented iron depletion. Considering the effect of other various clinical manifestations and drugs that accelerate eryptosis, such as iron deficiency, renal failure, fever, cyclophosphamide, azathioprine, saponin and thioridazine, enhanced eryptosis may probably be multifactorial [7, 12, 40-44].

In conclusion, altogether the data suggests that SLE is indeed accompanied by enhanced eryptosis and shrunken erythrocytes, which are at least in part ascribed to Ca$^{2+}$ influx and increased ROS abundance in erythrocytes, thereby contributing to anemia in SLE patients.

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Disclosure Statement

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

References

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