Cytoskeleton alterations of erythrocytes from patients with Fanconi's anemia

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Received 7 December 1999; received in revised form 27 December 1999

Edited by Vladimir Skulachev

Abstract Fanconi's anemia (FA) is a very rare genetically heterogeneous disease which has been hypothesized to be defective in the detoxification of reactive oxygen species. In this work we report the results obtained by morphometric and biochemical analyses on the red blood cells (RBCs) from FA patients. With respect to RBCs from healthy donors the following changes have been detected: (i) a variety of ultrastructural alterations, mainly surface blebbing typical of acanthocytes and stomatocytes; (ii) a significant quantitative increase of these altered forms; (iii) modifications of spectrin cytoskeleton network; (iv) an altered redox balance, e.g. a decreased catalase activity and significant variations in the GSSG/GSH ratio. We hypothesize that remodeling of the redox state occurring in FA patients results in cytoskeleton-associated alterations of red blood cell integrity and function.

Key words: Fanconi's anemia; Erythrocyte; Cytoskeleton; Reactive oxygen species

1. Introduction

Fanconi's anemia (FA) is a rare (about 1:400 000) autosomal recessive disease characterized by somatic anomalies, pancytopenia, retarded development, hyperpigmentation of the skin, propensity to developing acute leukemia and solid tumors [1]. The disease is usually fatal, with a life expectancy of approximately 16 years.

In FA patients, typical signs of anemia were found and studied since 1927 [2]. Considering that the oxidative imbalance is involved in determining cell aging and cell death [3] and that oxidative stress could be considered as a phenotypic hallmark of Fanconi's anaemia (reviewed by Pagano et al. [4]), the possible role of a redox alteration, resulting in red blood cell loss, can be taken into account. In particular, as a general rule, an excess formation of reactive oxygen species (ROS) and/or an impairment in the activity of antioxidant enzymes causing severe subcellular oxidative lesions was hypothesized [5]. Thus, in order to investigate red blood cell (RBC) loss detected in Fanconi's congenital disorder, erythrocytes from FA patients with cytogenetically confirmed diagnosis were isolated and examined by means of morphometric and biochemical analyses. Particular emphasis was given to the redox alterations and to oxidative changes occurring in RBC cytoskeletal components.

2. Materials and methods

2.1. Patients and blood drawing

FA patients have been recruited from different parts of Italy and examined at the Hematological Department of the St. Eugenio Hospital, Tor Vergata University (Rome, Italy) after their informed consent. The group of FA patients studied (12 patients for biochemical analyses and, among them, seven for morphometric analyses, aged 3–15 years) exhibited symptoms of prolonged anemia. The FA diagnosis (complementation group A) was confirmed clinically and cytogenetically [6]. As control, six healthy members of our laboratory staff and 14 healthy children (aged 8–15 years) were used after their or their relatives' informed consent. Venous blood was obtained by venipuncture using Becton-Dickinson (Rutherford, USA) heparinized vacutainers (10 ml) in the morning before any food.

2.2. Erythrocyte isolation

Human erythrocyte suspensions were prepared from fresh venous blood collected into heparinized tubes and centrifuged for 10 min at $1500 \times g$. The plasma and buffy coat were then removed. The red cell preparations were washed two times in Buffer G (50 mM Tris, 50 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM D-glucose, 10 mM $CaCl_2$, 50 mM NaCl, 5 mM KCl and 0.1% bovine serum) and resuspended in the same buffer to the initial hematocrit concentration. No appreciable cell lysis was observed during the preparation procedure.

2.3. Scanning electron microscopy (SEM) and morphometry

Control erythrocytes and those from FA patients were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min, seeded on glass coverslips coated with polylysine and, after adhesion to the glass surface (about 10 min), postfixed in 1% osmium tetroxide for 30 min. These blood cells were then dehydrated through graded ethanols, critical point dried in CO_2 and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope. Altered erythrocyte shape was evaluated by counting at least 500 cells (50 erythrocytes for each SEM field at a magnification of 3000×) of healthy individuals and FA patients in triplicate.

2.4. Cytoskeletal analyses

Erythrocytes from healthy donors and those from FA patients were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature and after washing in the same buffer, were permeabilized

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PII: S0014-5793(00)01187-X

with 0.5% Triton X-100 (Sigma) in PBS for 5 min also at room temperature. The erythrocytes were then stained with anti-spectrin (Sigma) at 37°C for 30 min and subsequently incubated with anti-rabbit IgG-fluorescein linked whole antibody (Sigma) at 37°C for 30 min. Finally, after washing, all the samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope or by a confocal microscope.

2.5. Redox analyses

To determine reduced and oxidized glutathione (GSH/GSSG) content and activity of enzyme with antioxidant properties as CuZnSOD and catalase, the washed erythrocytes were lysed by hypotonic shock with distilled water and 0.1 ml of hemolysate was mixed with 0.7 ml cold water, 0.2 ml ethanol and 0.1 ml chloroform. The mixture was shaken vigorously for 3–5 min and centrifuged at $3000 \times g$ for 10 min. The clear top layer possessing SOD and catalase activities was frozen immediately at −20°C until analyses.

The CuZnSOD activity was determined by the inhibition of superoxide-mediated lucigenin-amplified chemiluminescence produced during the xanthine oxidation by xanthine oxidase [7]. Catalase activity was determined by Aebi method [8]. Reduced glutathione (GSH) concentration was measured in the erythrocyte sediment according to Hughes by evaluating the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by sulfhydryl compounds at 412 nm. The levels of oxidized glutathione (GSSG) in the cell extracts were determined as stated elsewhere [9] in the presence of glutathione reductase to prevent additional GSH oxidation. The activity of erythrocyte antioxidant enzymes were expressed as units of enzyme per gram of protein. The protein content in the ethanol–chloroform extract was determined by the Lowry method [10] using a protein kit. The zinc content in erythrocytes was determined by atomic absorption spectroscopy [11].

Superoxide generation by erythrocytes was measured using SOD-sensitive cytochrome *c* reduction. Erythrocyte samples were incubated at 37°C for 1 h with 50 mM cytochrome *c* with or without 10 mg/ml of bovine SOD. Then, erythrocytes were sedimented by centrifugation at $1500 \times g$ for 5 min and light absorbance of clear supernatant was measured at 550 nm (Beckman DU 600 spectrophotometer). Each sample was incubated and measured in duplicate, the variation coefficient between duplicates did not exceed 10%. Reagent mixture without red cells was used as blank. The spontaneous superoxide release from erythrocytes was calculated as the difference between absorbances in the absence and presence of exogenous SOD using the $\varepsilon_M = 21\text{p}000$ for reduced cytochrome *c*. The results were expressed in nmol/60 min/ml 10^{10} RBC.

2.6. Lipid analyses

The extracts for lipid analyses were prepared with 0.5 ml of packed erythrocytes. These were hemolyzed for 15 min with an equal volume of water, then 11 ml of isopropanol was added slowly while stirring, as well as 7 ml of chloroform. The lipid extracts, filtered on anhydrous sodium sulfate and dried under nitrogen, were used for the analytical determination. The fatty acid pattern of erythrocyte membrane lipids was determined by gas-chromatographic analysis of fatty acid methyl esters as described previously [12].

2.7. Chemicals

The following reagents were purchased from Sigma (St. Louis, MO, USA): lucigenin, diepoxybutane, xanthine, xanthine oxidase, potassium phosphate buffer (PBS), cytochrome *c* (type VIII), DTNB, metrizoate, reduced and oxidized glutathione, glutathione reductase, bovine erythrocyte SOD (EC 1.15.1.1) and catalase (EC 1.11.1.6). Hydrogen peroxide was from Merck (Darmstadt, Germany). It was titrated with $KMnO_4$.

2.8. Statistical analysis

Data are reported as means ± S.D. for each group examined. The comparison between variables was analyzed by Student's *t*-test. Significance was accepted at the $P < 0.05$ level.

3. Results

3.1. Analytical cytology. Qualitative studies

In order to determine whether erythrocytes from FA patients differ in surface morphology from the RBC of healthy donors, qualitative SEM analyses were first conducted. As shown in Fig. 1, the surface of the red blood cells isolated from the patients was greatly altered with respect to control erythrocytes. In particular, surface blebbing typical of acanthocytes (Fig. 1b) and rare stomatocytes (Fig. 1c,d) were observed. Moreover, other shrunk forms, e.g. of leptocytic morphology, were also found (Fig. 1d).

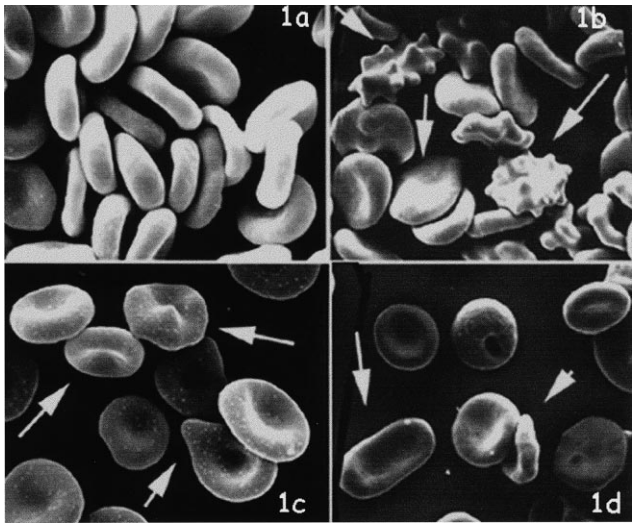

Fig. 1. Scanning electron microscopy. In panel (a) erythrocytes from healthy donors can be observed. By using this technical approach these cells appear as typical biconcave discs. By contrast, erythrocytes from FA patients show different types of alterations: surface blebbing typical of acanthocytes (b); rare swollen RBCs and stomatocytes (c and d) and shrunk forms of leptocytic morphology (d). Magnification 1680×.

3.2. Quantitative analyses

Studies were then carried out in order to evaluate statistical significance of these findings. Morphometric analyses were thus performed considering the alterations observed in the erythrocytes of each FA patient as specified in Section 2. RBCs from healthy donors were considered as control. Different values found in each FA patient are reported in Fig. 2. Morphometric analyses clearly indicate a variable and remarkable percentage of altered RBCs from seven different FA patients with respect to that found in control samples. In particular, the mean percentage of altered red blood cells in the FA patient group was of 42.8 ± 4.9% while that found in the control group was 15.0 ± 4.7% ($P < 0.01$).

3.3. Cytoskeletal analyses

Because of the importance of cytoskeleton in cell shape maintenance and in surface bleb formation [13], experiments were also conducted in order to learn whether the microfilament network could be altered. The cytoskeleton network formed by spectrin molecules appeared peculiarly modified in the erythrocytes from FA patients (Fig. 3b) with respect to the RBC of healthy donors (Fig. 3a). A subtle spectrin boundary network was visible in RBCs from control subjects (Fig. 3a). By contrast, significant changes were found in RBCs from FA patients: a patching, or a side by side bundling and cross linking of spectrin filaments, which appeared as thick positive fibers, was detected (Fig. 3b).

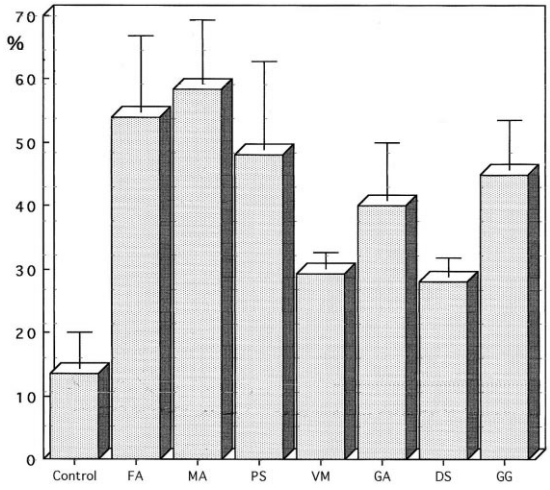

Fig. 2. Percentages of altered erythrocytes evaluated in seven different subjects with FA (FA, MA, PS, VM, GA, DS, GG) compared to healthy donors. For each patient the values are expressed as mean ± S.D. of different samples evaluated as stated in Section 2. Healthy donors are pooled together and only the mean value ± S.D. is shown.

3.4. Biochemical analyses

It was observed that the mean value of SOD activity in FA patients was significantly lower (−44%, $P < 0.05$) than in healthy donors. No difference in zinc content and superoxide release was detected between the studied groups. The mean value of catalase activity was also significantly decreased (−77%, $P < 0.05$) in the RBCs of patients compared to control values. Evaluation of intracellular thiol pools indicated significant variations. In fact, the GSH level in erythrocytes from FA patients was increased and, more important, the GSSG/GSH ratio was twice as high in FA patients as compared to the control RBCs ($P < 0.01$). The polyunsaturated fatty acid (PUFA) composition of erythrocyte membrane lipids, considered as a substrate of oxygen radical attack, was also studied by gas-chromatographic analysis. However, no significant difference both in fatty acid composition and in PUFA content was found in control samples (29.2 ± 2.0) and in RBCs from FA patients (33.0 ± 2.5) (Table 1).

4. Discussion

It has been suggested that disorders of the erythrocyte membrane, such as hereditary spherocytosis, hereditary elliptocytosis and hereditary poikilocytosis, are characterized by heterogeneity in their clinical and laboratory manifestations but they share, as common feature, alterations of RBC deformability and plasticity, usually associated with cytoskeleton changes [14,15]. These changes are strictly related to those

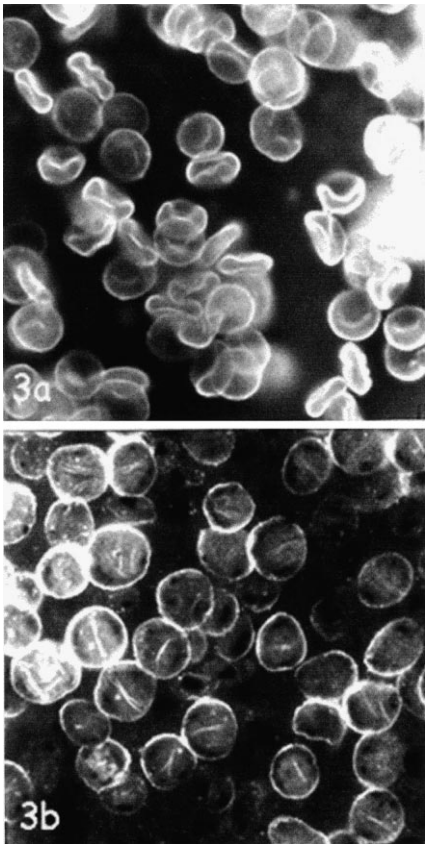

Fig. 3. Immunofluorescence microscopy. Spectrin labeling. a: Erythrocytes from healthy donors. A thin boundary spectrin positive filament network is visible. b: Erythrocytes from FA patients. Spectrin cytoskeleton appears rearranged forming patches and cross-links. In particular, a side by side bundling of spectrin filaments is evident. Magnification 1400×.

alterations of morphological and rheological properties of circulating erythrocytes which can be detected either in the aging process or in some pathologic conditions including FA [17–19]. A possible general role exerted by redox alterations in these modifications has also been proposed [4,16]. In fact, in the present work we show that RBCs from FA patients are significantly modified by a mechanism involving cytoskeleton integrity and we also hypothesize a role for redox imbalance in these changes. An extensive body of evidence has associated the FA phenotype with the occurrence of oxidative stress [4,20]. For instance, it has been shown that FA fibroblasts and leukocytes exhibited lower than normal levels of SOD and/or catalase activities [21]. Our results support these findings indicating that erythrocytes from FA homozygotes possess a significantly decreased activity of above enzymes. At the same time, the ratio GSSG/GSH was significantly higher in

Table 1

	Controls	FA patients
Superoxide release (nmol/60 min × ml 10^{10} RBC)	76 ± 31	146 ± 135
SOD* (mU/mg protein)	24.8 ± 5.3	14.1 ± 3.5
Catalase* (mU/mg protein)	362 ± 42	83.6 ± 23.5
Zinc (ppm)	33.7 ± 17.2	35.4 ± 7.8
GSH* (μmol/g Hb)	6.6 ± 1.0	7.8 ± 0.2
GSSG/GSH**	1.8 ± 0.6	3.6 ± 1.7

*$P < 0.05$; **$P < 0.01$.

the FA patients in comparison with that found in the control group. This fact points to the excess amount of oxidized glutathione in FA erythrocytes that could be a consequence of hyperoxidation of GSH.

The cytoskeleton was largely described as a subcellular structure involved in oxidative modifications leading to cell injury [13,15]. In this context, the results reported in this work represent the first evidence for cytoskeleton changes occurring in RBCs from FA patients and suggest a possible role for actin binding proteins, e.g. spectrin, in altered RBC function in this disease. In fact, fluorescent labeling of spectrin molecules with specific antibodies employed in order to assess the integrity of spectrin–actin junction sites, known to be responsible for the red cell deformability and shape maintenance [22,23], clearly indicated a profound alteration of the microfilament system network. Thus, significant alterations of RBC shape, mainly represented by cell swelling, blebbing and/or shrinking, have been detected. These data are suggestive for a specific 'chronic' oxidative modification, denaturation and crosslinking (or defective bundling) of cytoskeletal components in RBCs from these subjects. This should result in a decreased deformation ability (or an increased rigidity).

In conclusion, the present study corroborates the evidence for the occurrence of oxidative damage in FA (or at least FAA) phenotype. This evidence is both substantiated by the observed alterations in RBC cytoskeleton and by the reduced antioxidant, e.g. catalase, activity. Thus, it can be suggested that the FA phenotype can be included among those congenital conditions sharing oxidative stress as a phenotypic hallmark [4,24]. In particular, the multifaceted damage which is detectable in FA patients seem to indicate the erythrocyte integrity, its cytoskeleton and the redox state of these cells as an important tool in the diagnostic characterization of this rare disease. These features may also contribute to the understanding of the functional defects observed in FA disease progression.

Altogether the data reported here partially address the mechanisms underlying the pathogenesis of the anemia at least in terms of RBC aging process, add some new insight in the field of FA as a cytoskeleton-associated pathology and suggest, according to literature data [25], the possible use of specific antioxidants and/or radical scavengers in the management of Fanconi's anemia.

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