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Iron Release in Erythrocytes from Patients with β-Thalassemia

LUCIA CICCOLI[a], CINZIA SIGNORINI[a], CLELIA SCARANO[b], VIVIANA ROSSI[a], SABRINA BAMBAGIONI[a], MARCO FERRALI[a] and MARIO COMPORTI[a,*]

[a]*Istituto di Patologia Generale, Università di Siena, Via Aldo Moro, I-5300 Siena, Italy;*
[b]*Centro per lo Studio della Microcitemia, Ospedali Riuniti, Foggia, Italy*

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Our previous studies have shown that iron is released in a free (desferrioxamine-chelatable) form when erythrocytes undergo oxidative stress (incubation with oxidizing agents or aerobic incubation in buffer for 24–60 h (a model of rapid *in vitro* ageing)). The release is accompanied by oxidative alterations of membrane proteins as well as by the appearance of senescent antigen, a signal for termination of old erythrocytes. In hemolytic anemias by hereditary hemoglobin alterations an accelerated removal of erythrocytes occurs. An increased susceptibility to oxidative damage has been reported in β-thalassemic erythrocytes. Therefore we have investigated whether an increased iron level and an increased susceptibility to iron release could be observed in the erythrocytes from patients with β-thalassemia. Erythrocytes from subjects with thalassemia intermedia showed an extremely higher content (0 time value) of free iron and methemoglobin as compared to controls. An increase, although non-statistically-significant,was seen in erythrocytes from subjects with thalassemia major. Upon aerobic incubation for 24 h the release of iron in β-thalassemic erythrocytes was by far greater than in controls, with the exception of thalassemia minor. When the individual values for free iron content (0 time) seen in thalassemia major and intermedia were plotted against the corresponding values for HbF, a positive correlation ($P < 0.001$) was observed. Also, a positive correlation ($P < 0.01$) was seen between the values for free iron release (24 h incubation) and the values for HbF. These results suggest that the presence of HbF is a condition favourable to iron release. Since in β-thalassemia the persistance of HbF is related to the lack or deficiency of β chains and therefore to the excess of α chains, the observed correlation between free iron and HbF, is consistent with the hypothesis by others that excess of α chains represents a prooxidant factor.

Keywords: Erythrocytes, β-thalassemia, oxidative stress, iron release, foetal hemoglobin (HbF)

INTRODUCTION

As is known, erythrocytes undergo oxidative modifications during their ageing in blood.[1] Also it is known that a number of oxidative alterations occur in erythrocytes incubated with oxidants *in vitro*.[1–8] Similar modifications have been found in erythrocytes with hereditary abnormal hemoglobins. For instance fluorescent phospholipids,[9] oxidized cysteinyl residues and

* Corresponding author. Tel.: +39-577-227003. Fax: +39-577-227009. E-mail: comporti@unisi.it.

high-molecular-weight aggregates in band 4.1 membrane protein[10] have been found in sickle erythrocytes. Analogous alterations as well as increased susceptibility to oxidative damage have been reported in erythrocytes from patients with β-thalassemia,[11] hemoglobin Köln[12] and glucose-6-phosphate dehydrogenase deficiency.[13] What remains to be elucidated is the source of oxidative stress in these erythrocytes.

As for β-thalassemic erythrocytes, recent studies seem to demonstrate that unbalance between α and β chains plays a crucial role in producing such an oxidative stress.[14–16] As is known, persistence of fetal hemoglobin (HbF, $\alpha_2\gamma_2$), owing to absent or reduced synthesis of β chains (and consequently of HbA_1), is a feature of β-thalassemia. Because of the impossibility of producing β chains at normal rate, staminal cells substitute them with γ chains (persistance of HbF) and with δ chains (slight increase in HbA_2). Therefore in β-thalassemia a marked excess of α chains occurs and such excess probably represents the source of oxidative stress. In fact it has been observed that isolated α chains directly generate oxygen species.[17,18] In addition, because of inherent instability of a chains, damage to red cells by oxidative means may be further potentiated by heme, or heme derived iron, released from the excess of α chains.[19–21] Finally, *in vitro* sensitivity of α chains loaded[22] erythrocytes to exogenous oxidants correlates with the amount of membrane bound hemoglobin or heme iron. Also β-thalassemic red cells show evidence of elevated oxidative damage[11,23,24] and produce hydroxyl radicals when stimulated with ascorbate.[25]

Our previous studies[26,27] have shown that iron is released in a free form when mouse erythrocytes are incubated with a number of oxidizing agents, such as phenylhydrazine and others. Iron is released from hemoglobin;[28] the release is accompanied by methemoglobin (Met-Hb) formation and, under conditions of glutathione (GSH) depletion, by lipid peroxidation and hemolysis, too. A similar release of iron also occurs[29] during erythrocyte ageing, experimentally induced by aerobic incubation of calf or human erythrocytes in buffer for 24–60 h (a model of rapid *in vitro* ageing). The release is accompanied[29] by oxidative alterations of membrane proteins as well as by the appearance of senescent antigen, as measured by autologous IgG binding. Intracellular chelation of the released iron by chelators which enter erythrocytes, prevents both membrane protein alterations and senescent antigen formation. Increased amount of IgG with antiband 3 specificity has been found in sickle and hemoglobin Köln erythrocyte[30] and probably even in β-thalassemic erythrocyte.[31] We therefore suggested the possibility that the release of iron in a reactive form is a relevant factor in membrane protein oxidation and consequent generation of senescent antigen.

Since senescent antigen acts as a specific signal for termination of old cells, and since in hemolytic anemias by hereditary hemoglobin alterations an accelerated removal of erythrocytes occurs, we investigated whether an increased free iron level and an increased susceptibility to iron release could be observed in erythrocytes from patients with these hemolytic diseases. In this note we present the results obtained in β-thalassemia. The release of iron in possible experimental models [32] of thalassemia is also presented.

MATERIALS AND METHODS

Patients studied Informed consent was obtained from control subjects and patients. Patients were from the Center for the Study of Microcitemias, Ospedali Riuniti, Foggia, Italy. Five patients with β-thalassemia major (all splenectomized), five patients with β-thalassemia intermedia (one, R.A. – see Table I – splenectomized) and five patients with β-thalassemia minor (all non-splenectomized) were studied. All the patients were adult and the diagnosis of the various forms of thalassemia had been performed in childhood and based on

TABLE I HbF, HbA_2, HbA, total Hb content, MCV (mean cell volume), RBC (red blood cell) and hematocrit in β-thalassemic patients and controls

	Patients	HbF ($\alpha_2\gamma_2$) (%)	HbA_2 ($\alpha_2\delta_2$) (%)	HbA (%)	Total Hb (g/dL)	MCV (fl)	RBC ($10^6/\mu l$)	Hematocrit (%)
Thalassemia major	C.A.	5.4	2.0	92.6	8.9	85.7	3.22	27.6
	C.M.	25.3	1.8	72.9	9.8	90.0	3.40	30.6
	S.G.	3.9	2.5	93.6	9.7	85.9	3.44	29.5
	V.V.	10.9	1.8	87.3	9.2	82.0	3.62	29.7
	B.P.	7.1	2.1	90.8	7.6	80.2	2.86	23.5
	Mean ± SEM	10.5 ± 3.9	2.0 ± 0.1	87.4 ± 4.7	9.0 ± 0.4	84.8 ± 1.7	3.30 ± 0.1	28.2 ± 1.3
Thalessemia intermedia	B.P.	92.5	2.9	4.6	7.1	75.7	3.22	24.4
	C.R.	20.6	5.3	74.1	7.8	79.5	3.47	27.6
	F.P.*	75.0	3.2	13.2	9.5	74.7	4.10	31.2
	R.A.	11.9	4.6	83.5	7.2	72.7	3.52	25.6
	C.G.	17.0	6.2	76.8	6.3	69.5	3.28	22.8
	Mean ± SEM	43.4 ± 16.7	4.4 ± 0.6	50.4 ± 17.1	7.6 ± 0.5	74.4 ± 1.7	3.52 ± 0.15	26.3 ± 1.4
Thalessemia minor	P.F.	1.7	4.7	93.6	11.1	64.9	5.73	37.2
	A.L.	1.6	4.2	94.2	11.1	61.8	5.51	34.0
	B.A.	n.d.	4.6	95.4	9.9	58.4	5.39	31.4
	M.V.	n.d.	4.4	95.6	9.5	56.5	5.45	30.8
	B.A.	n.d.	5.7	94.3	10.6	56.1	6.09	34.2
	Mean ± SEM		4.7 ± 0.3	94.6 ± 0.4	10.4 ± 0.4	59.5 ± 1.7	5.63 ± 0.12	33.5 ± 1.1
Controls	C.R.	n.d.	—	—	11.3	81.2	4.50	36.6
	L.M.	n.d.	—	—	14.2	78.9	5.59	44.1
	D.M.P.	n.d.	2.8	97.2	12.5	88.0	4.43	39.0
	C.A.	0.6	2.3	97.1	12.8	85.1	4.59	39.0
	T.D.	0.5	2.6	96.9	12.9	89.1	4.39	39.1
	Mean ± SEM		2.6 ± 0.1	97.1 ± 0.1	12.7 ± 0.5	84.5 ± 1.9	4.70 ± 0.2	39.6 ± 1.2

The thalassemia major samples (from splenectomized patients) were obtained prior to the next transfusion, typically 4 weeks after the last transfusion. Out of the thalassemia intermedia patients, two (including the splenectomized one, R.A.) required transfusion at irregular intervals and none had been transfused 4–5 months prior to blood withdrawal.
*In this patient Hb Lepore (8.6%) was also present.
n.d.: not detectable.

electrophoretic analysis of hemoglobin, presence of erythroblasts in peripheral blood and clinical data. At the time of withdrawal the various types of hemoglobin as well as the other hematological parameters were as reported in Table I.

The determination of the various types of hemoglobin was performed using an automated cation-exchange HPLC system (Diamat, Bio-Rad) and three BIS-TRIS/phosphate buffers of increasing ionic strength (Hemoglobin Dual Kit, Bio-Rad).

Erythrocyte incubation The erythrocytes were prepared by centrifugation from heparinized blood and washed three times with 0.123 M NaCl, 28 mM sodium phosphate/potassium phosphate buffer, pH 7.4, and resuspended in the same buffer as a 50% (v/v) suspension. Iron contamination was removed from the buffer as previously described.[26] The incubation was carried out aerobically for 24 h in the presence of antibiotics (20 units penicillin and 20 μg streptomycin/ml of buffer). At 0 time and at the end of the incubation, samples were withdrawn for the determination of free (desferrioxamine (DFO)-chelatable) iron, Met-Hb,[33] GSH[34] and hemolysis.[26] Free iron was determined as a DFO-iron complex (ferrioxamine) as previously reported.[26]

In separate experiments, erythrocytes from normal subjects were incubated as above in the presence of phenylhydrazine (5 mM) or methylhydrazine (5.7 mM) for 2 h, and free iron, Met-Hb, GSH and hemolysis were determined as above.

α and β chains were isolated from hemoglobin of normal subjects according to Bucci and Fronticelli.[35]

RESULTS AND DISCUSSION

As shown in Table II, erythrocytes from subjects with thalassemia intermedia showed an extremely higher content (0 time value) of free iron as compared to controls. An increase (+92%), although non-statistically-significant, was also seen in erythrocytes from subjects with thalassemia major (who were transfused monthly), while essentially no increase was found in thalassemia minor. Upon aerobic incubation for 24 h, a release of iron was seen in all the erythrocytes, including control (this latter result confirms our previous data[28] on iron release in *in vitro* ageing of normal erythrocytes). However, iron release in erythrocytes with abnormal hemoglobins was by far greater than in controls, with the exception of thalassemia minor. The highest values were found in thalassemia intermedia, even if in the latter, because of the high 0 time value, the difference between 24 h and 0 time values was similar to that found in thalassemia major. Methemoglobin content (0 time) and formation (after 24 h incubation) were markedly increased in erythrocytes with thalassemia intermedia.

No significant differences were found in the GSH content (0 time) or decrease (after incubation) in abnormal erythrocytes with respect to controls, even if a tendency to a lower GSH content (0 time) was observed in thalassemia intermedia and minor. The hemolysis observed after the aerobic incubation was higher in thalassemia intermedia, while in the other forms of thalassemia was similar to that occurring in controls.

One patient (RA) of the thalassemia intermedia group showed free iron values (0 time, 3.9; incubation, 8.3) similar to those of controls, when trasfused relatively recently (1.5 month). She showed higher free iron levels (0 time 5.7; incubation, 18.5) when transfused 2.5 months in advance; and highest free iron levels (0 time, 10.7; incubation, 12.4 nmol/ml) when transfused 4.5 months in advance. In the latter case it can be imagined that the erythrocytes are not mixed with normal (transfused) cells and that the free iron content is proper of the thalassemia intermedia group.

As shown in Table I, the percentage of HbF was highest in erythrocytes from subjects with thalassemia intermedia; it was increased, as compared to controls, in erythrocytes with thalassemia major, and to a much lesser extent (at least when detectable) in thalassemia minor. This is in agreement with the current knowledge. The higher amount of HbF in thalassemia intermedia as

TABLE II Free iron (DFO-chelatable), methemoglobin (Met-Hb), glutathione (GSH) and hemolysis in β-thalassemic erythrocytes at 0 time (real content) and after 24 h of aerobic incubation

	Incubation time (h)	Free iron (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	Hemolysis (%)
Controls	0	1.2 ± 0.3	82 ± 7	1388 ± 213	1.5 ± 0.1
	24	6.2 ± 0.6	402 ± 59	155 ± 42	5.4 ± 0.8
Thalassemia major	0	2.3 ± 0.7*	113 ± 28	1372 ± 120	1.3 ± 0.5
	24	13.0 ± 2.0	599 ± 54	147 ± 44	3.1 ± 0.5
Thalassemia intermedia	0	16.0 ± 4.8	788 ± 235	855 ± 111	1.5 ± 0.6
	24	24.2 ± 5.1**	1376 ± 88	320 ± 88	9.2 ± 1.2***
Thalassemia minor	0	1.7 ± 0.1	97 ± 25	967 ± 59	1.1 ± 0.1
	24	5.7 ± 1.2	486 ± 99	188 ± 15	3.9 ± 1.1

The results are the means ± SEM of 5 samples. Results are expressed as nmol/ml of incubation mixture, with the exception of hemolysis.
*Not significantly different ($0.10 > P > 0.05$) from the respective control, 0 time value.
**Significantly different ($P < 0.05$) from the respective 0 time value, as measured with t-test for paired samples.
***Significantly different ($P < 0.05$) from the respective control, 24 h value.

compared to thalassemia major was obviously due to the fact that in the latter all the subjects were continously transfused. In spite of this, however, in the latter subjects thalassemic erythrocytes must have been present at the time of withdrawal, as shown by the large increase in HbF as compared to controls. This is the reason why the thalassemia major samples were considered in the following plots.

As shown in Figure 1, when the individual values for free iron content (0 time) seen in thalassemia major and intermedia were plotted against the corresponding values for HbF, a positive correlation ($P < 0.001$) was observed (Figure 1(A)). Also a positive correlation ($P < 0.01$) was seen between the values for free iron release (24 h incubation) and the values for HbF (Figure 1(B)). On the contrary, no correlation was found between iron content or release on one hand and Met-Hb content or formation on the other (data not shown).

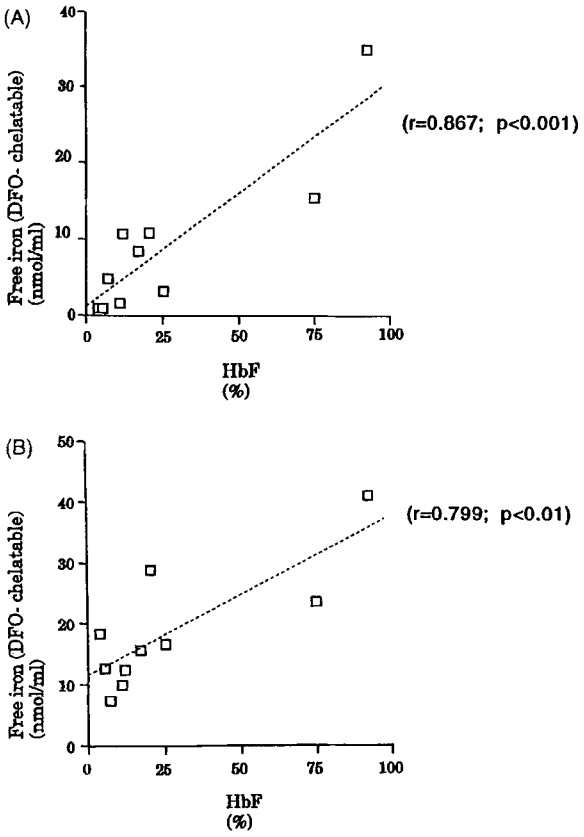

FIGURE 1 (A) Correlation between free iron and HbF content in erythrocytes of β thalessemic patients (major and intermedia). (B) Correlation between free iron release (24 h incubation) and HbF content in erythrocytes of β-thalassemic patients (major and intermedia).

Since, as stated above, in β-thalassemia the persistance of HbF is related to the lack or deficiency of β chains and therefore to the excess of α chains, the observed correlation between free iron and HbF, is in agreement with the hypothesis that excess of α chains represents a prooxidant factor. This is probably due to the fact that α chains are more prone to release iron in a free form. In considering this, we carried out studies in which isolated α and β chains were incubated separately under aerobic conditions with the aim of demonstrating an increased iron release from α chains as compared to β chains. However, no clear results were obtained because both isolated chains, and especially α chain, precipitated to some extent during the incubation, thus rendering difficult or impossible free iron determination. Nevertheless HbF is considered an unstable hemoglobin[36] and therefore its persistance is consistent with an increased iron release.

It has been shown that excess of unpaired α- and β-globin chains interact with the membrane skeleton of erythrocytes in β- and α-thalassemia, respectively.[37,38] Also it has been shown[32,39] that incubation of normal erythrocytes with phenylhydrazine or methylhydrazine induces association of oxidized α- or β-globin chains with membrane skeleton, thus mimicking, at least in part, the cytoskeletal alterations seen in β- and α-thalassemia, respectively. Table III shows that incubation of normal erythrocytes with phenylhydrazine or methylhydrazine also induces a considerable release of iron and Met-Hb formation. Therefore iron release can be observed, after aerobic incubation, in both models of thalassemia, which reinforces the view that the susceptibility to release iron in a free form is a feature of erythrocytes in these hereditary hemoglobin alterations. This is also true for the only case of sickle cell anemia (HbSS) examined in

TABLE III Free iron (DFO-chelatable), methemoglobin (Met-Hb), glutathione (GSH) and hemolysis in human erythrocytes incubated with phenylhydrazine or with methylhydrazine

	Incubation time (h)	"Free" iron (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	Hemolysis (%)
Control	0	1.4 ± 0.3	77 ± 11	896 ± 58	1.5 ± 0.5
	2	1.5 ± 0.5	90 ± 14	839 ± 50	1.9 ± 0.5
Phenylhydrazine	2	39.9 ± 5.9	4380 ± 40	312 ± 59	1.0 ± 0.1
Methylhydrazine	2	31.1 ± 5.1	2862 ± 215	329 ± 78	1.2 ± 0.3

Erythrocytes are incubated with phenylhydrazine (5 mM) or with methylhydrazine (5.7 mM) for 2 h at 37°C. The results are the means ± SEM of 4–8 experiments.

which the erythrocyte content of free iron was 16 nmol/ml and the release after incubation was 38 nmol/ml.

Heme iron, non-heme iron and free iron were found to be increased in inside-out membranes from erythrocytes of HbSS, HbSC and splenectomized β-thalassemic patients.[20,40,41] In α-hemoglobin chain loaded-erythrocytes, considered a very interesting model of β-thalassemic cells,[16] membrane bound heme and iron were significantly elevated and the cells were more susceptible to lipid peroxidation, which could be inhibited by entrapment of an iron chelator.

Our results confirm and extend these observations in that they demonstrate and offer the direct measurement of iron in a free form. The markedly increased level of free iron both in native cells and after the oxidative stress imposed by the prolonged aerobic incubation may represent the trigger for the oxidative damage seen in β-thalassemic erythrocytes. It may also represent the mechanism of formation of senescent cell antigen, or any other membrane event responsible for the early removal of erythrocytes from the blood stream.

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