J Membrane Biol (2009) 228:43–49
DOI 10.1007/s00232-009-9157-5

Derangement of Erythrocytic AE1 in Beta-Thalassemia by Caspase 3: Pathogenic Mechanisms and Implications in Red Blood Cell Senescence

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Received: 15 October 2008 / Accepted: 27 January 2009 / Published online: 24 February 2009

Abstract Considering its complex molecular pathophysiology, beta-thalassemia could be a good in vivo model to study some aspects related to erythrocyte functions with potential therapeutic implications not only within the frame of this particular hemoglobinopathy but also with respect to conditions in which the cellular milieu, altered by a deranged anion exchanger, could display a significant pathogenetic role (i.e., erythrocyte senescence, complications of red cell storage, renal tubular acidosis and some abnormal protein thesaurismosis). This work evaluates the anionic influx across band 3 protein in normal and beta-thalassemic red blood cells (RBCs) and ghosts. Since redox-mediated injury is an important pathway in the destruction of beta-thalassemic RBCs, we studied the anion transport and the activity of caspase 3 in the absence and presence of *t*-butylhydroperoxide in order to evaluate the effect of an increase of cellular oxidative stress. Interestingly, beta-thalassemic erythrocytes show a faster rate of anion exchange than normal RBCs and absence of any modulation mechanism of anion influx. These findings led us to formulate a hypothesis about the metabolic characteristics of beta-thalassemic erythrocytes, outlining that one of the main targets of caspase 3 in RBCs is the cytoplasmic domain of band 3 protein.

Keywords Hemoglobinopathy · Beta-thalassemia · Anion exchanger 1 · Red blood cell · Oxygen transport · Glucose-6-phosphate metabolism

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Introduction

The red blood cell (RBC) is particularly vulnerable as it is exposed to important oxidative insults during its life span, being confronted, in a unique fashion among cells, to very large concentrations of both iron and oxygen. This potentially dangerous combination (of iron and O_2) within the RBC is normally kept in check by a sophisticated reducing system (Scott et al. 1989, 1991; Chiu et al. 1982).

However, redox balance is greatly altered in some pathological conditions like hemoglobinopathies characterized by severe hemoglobin instability (Carrel et al. 1975; Kahane et al. 1978). In particular, in beta-thalassemic syndromes, decreased or impaired biosynthesis of beta-globin leads to accumulation of unpaired alpha-globin chains, giving rise to formation of hemichromes (HCRs), on the inner leaflet of the RBC membrane (Scott et al. 1993). Above all, considering the higher binding to the cytoskeleton of alpha-chains with respect to beta-chains (Giardina et al. 1995), autoxidation of the unpaired alpha-hemoglobin chains and the large concentrations of free iron and prosthetic heme group, which lead to the generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), correlate to massive damage of the cellular membrane (Kuross and

Hebbel 1988; Repka et al. 1993; Browne et al. 1998; Cappellini et al. 1999; Droge 2002).

Cross-linked aggregates of spectrin and hemoglobin are the major components of the erythroid skeleton (Sauberman et al. 1983; Leb et al. 1983; Snyder et al. 1985); moreover, various observations support the idea that partly oxidized globin chains cross-link with adjacent cytoskeleton proteins (Advani et al. 1992). The presence of such aggregates has been implicated in pathophysiological effects such as reduced deformability and enhanced rigidity of erythrocytes and their enhanced phagocytosis by macrophages (Schrier et al. 1989; Schrier 1994; Knyszynski et al. 1979; Wiener and Wickramasinghe 1985).

Some recent genetic and clinical evidence confirms and expands these pathogenetic mechanisms. In fact, molecular analysis of the so-called dominantly inherited beta-thalassemia showed a peculiar group of beta-globin chains associated with a thalassemic phenotype in the heterozygous state. The molecular lesions include mutations and deletions at the level of exon 3, which give rise to elongated or truncated beta-globin chains. Interestingly, mutations that produce truncated beta-chains, up to 72 residues in length, are usually associated with a mild phenotype in heterozygotes. Of note, the truncated globins should still be able to bind heme since only helix H is missing. These are hyperunstable chains that could enhance the oxidative stress linked to the excess of alpha-chains. Moreover, these heme-containing products seem to possess a secondary structure which renders them less susceptible to proteolytic degradation. Furthermore, the lack of helix H, which would expose one of the hydrophobic patches of helix G and the hydrophobic patches of helices E and F, would induce a polyaggregation of these truncated products.

Since the band 3 anion exchanger (AE1) is the major integral protein of the RBC membrane (about 10^6 copies per RBC), its interaction with the unpaired alpha-hemoglobin chains is highly probable. In this respect, we have to recall that the monomer of this multifunctional protein is constituted by three domains, a membrane-spanning domain that carries out chloride–bicarbonate exchange, a short C-terminal cytoplasmic domain and a large N-terminal cytoplasmic domain (Tanner 1997, 2002).

The C-terminal cytoplasmic domain binds carbonic anhydrase II (CAII), while the N-terminal cytoplasmic domain binds proteins of the cytoskeleton, glycolytic enzymes, hemoglobin (Hb), HCRs and some drugs (Zhang et al. 2000; Tellone et al. 2008); such interactions contribute to regulate the anion exchange, metabolism and therefore the structural and functional integrity of the RBC (Galtieri et al. 2002; Giardina et al. 1995; Russo et al. 2008). In particular, intracellular binding of denatured hemoglobin (i.e., HCRs) to the membrane causes a clustering of band 3 protein and erythrophagocytosis (Low et al. 1985; Low 1991; Brovelli et al. 1991; Castellana et al. 1992; Giuliani et al. 1993; Cappellini et al. 1999). Studies on RBCs in vivo and vitro showed that oxidation plays a relevant role in this process since oxidative modifications of band 3 were found in senescent cells and in many pathological cells with decreased life span.

Recently, it has been demonstrated that human mature erythrocytes contain functional procaspase, such as caspase 3, and that its activation by oxidative stress leads to proteolytic cleavage of the N-terminal cytoplasmic domain of band 3 (Mandal et al. 2002, 2003; Radi et al. 1991; Matarrese et al. 2005; Clementi et al. 2007). This cleavage, altering the modulating function of cdb3, could well represent a significant event with respect to the erythrophagocytic process.

These premises have led us to study the kinetic characteristics of the anionic influx through AE1 in normal and heterozygous beta-thalassemic erythrocytes.

Materials and Methods

Material

All reagents were from Sigma-Aldrich (St. Louis, MO). Citrate fresh human blood was obtained from informed heterozygous beta-thalassemic and healthy donors aged 30–50 years under the declaration that they had avoided any drug treatment for at least 1 week before sample collection, in accordance with principles outlined in the Declaration of Helsinki.

Preparation of RBCs

Citrate blood samples were washed three times with an isoosmotic NaCl solution. During washings white blood cells were discarded from the pellet. After washing, RBCs were resuspended (hematocrit 3%) in the incubation buffer (35 mM Na_2SO_4, 90 mM NaCl, 25 mM *N*-(2-hydroxyethyl)-piperazine-N^1-2-ethanesulfonic acid [HEPES], 1.5 mM $MgCl_2$), adjusted to pH 7.4 or 7.3 and 310 ± 20 mOsmol/kg, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco, Kyoto, Japan).

Preparation of RBC Ghosts

RBC membranes were isolated by hypotonic lysis of buffy coat free packed erythrocytes in 10 volumes of ice-cold 5 mM sodium phosphate, pH 8, with the addition of 1 mM EDTA and protease inhibitors (2 mM). The resulting membranes were centrifuged at 20,000 × *g* for 20 min at 4°C. They were then washed three to five times in 10 mM

Tris-HCl (pH 7.5). The hemoglobin-free membrane suspension (ghosts) was finally resuspended in the same buffer.

Kinetic Measurements

Cells were incubated in the above incubation buffer at 25°C, under different experimental conditions. At several time intervals, 10 μM of the stopping medium 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) were added to each test tube containing RBC suspension. Cells were then separated from the incubation medium by centrifugation (J2-HS Centrifuge; Beckman, Palo Alto, CA) and washed three times at 4°C with a sulfate-free medium to remove the sulfate trapped outside. After the last washing the packed cells were lysed with perchloric acid (4%) and distilled water. Lysates were centrifuged for 10 min at 4,000 × *g* (4°C), and membranes were separated from the supernatant. Sulfate ions were precipitated from the supernatant by adding glycerol/distilled water mixture (1:1, V/V), 4 M NaCl, 1 M HCl and 1.23 M $BaCl_2 \cdot 2H_2O$ in order to obtain a homogeneous barium sulfate precipitate. The absorbance of this suspension was measured at 350–425 nm.

The sulfate concentration was determined using a calibrated standard curve, obtained by measuring the absorbance of suspensions with known sulfate amounts (Romano et al., 1998). Experimental data of sulfate concentration as a function of incubation time were analyzed by best-fitting procedures according to the following equation:

$$c(t) = c_\infty\left(1 - e^{-kt}\right)$$

where $c(t)$ represents sulfate concentration at time t, c_∞ intracellular sulfate concentration at equilibrium and k the rate constant of sulfate influx.

Analysis of Caspase 3 Activity

Caspase activity was measured using a Sigma (St. Louis, MO) assay kit, following the manufacturer's instructions. Acetyl-DEVD-*p*-nitroanilide (Ac-DEVD-pNA) was used as a colorimetric substrate.

Erythrocytes were incubated for 2 h at 25°C in HEPES 25 mM, NaH_2PO_4 1 mM, NaCl 110 mM, KCl 5 mM, $MgCl_2$ 2 mM, at pH 7.4, and 290 ± 5 $mOsm/kg^{-1}$ measured by the Osmostat OM-6020. Cell suspensions were then separated from the incubation medium by centrifugation (J2-HS Centrifuge) and washed three times at 4°C with HEPES washing buffer. After the last washing, packed cells were lysed with cycles of freezing and defrosting. Lysate was centrifuged at 10,000 × *g* for 1 min at 4°C, and supernatant was used for the caspase 3 activity. Protein concentration was determined by the BCA assay. Protease activity was determined by spectrophotometric detection at 405 nm of the chromophore pNA after its cleavage by caspase 3 from the labeled caspase-3-specific substrate (DEVD-pNA). Acquired data were fitted to a polynomial 2° grade equation. Before performing spectrophotometric determinations, hemolysates were filtered (50,000 cut-off) in order to eliminate interferences by Hb spectra.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Treated and untreated erythrocytes were osmotically lysed in hypotonic solution, and the lysate was incubated overnight with rabbit anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody at 4°C. Protein A/G agarose (Santa Cruz Biotechnology) was added and incubated for 1 h at room temperature, and the immunoprecipitate was washed, denatured and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, as previously described (Castellana et al. 1992). Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-caspase 3 antibody, followed by alkaline phosphatase–linked goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) and finally visualized using Bio-Rad (Hercules, CA) color developing reagent. Images of nitrocellulose membranes were acquired (Bio-Rad Gel Doc 2000) and scanned (Bio-Rad GS800) using Bio-Rad Quantity One software.

Statistical Analysis

Group means were compared by analysis of variance (ANOVA), followed by a multiple comparison of means by Tutteg's test. Upon occurrence the group means were analyzed with a two-tailed Student's *t*-test. The results are expressed as means ± SD of at least three different experiments performed in triplicate; unless otherwise specified, $P < 0.05$ was considered significant.

Results

As shown in Figures 1 and 2 the sulfate flux measured in RBCs and ghosts of normal and heterozygous for beta-thalassemia adults revealed a sensible difference between the two cellular types. Specifically, the rate of sulfate influx in thalassemic erythrocytes was about 75% ($P < 0.001$) higher with respect to normal erythrocytes. However, the situation was wholly inverted in the case of ghosts. Thus, in this latter experimental setting, the sulfate flux rate was about 23% ($P < 0.001$) lower in thalassemic ghosts with respect to normal membranes (Table 1). The wide difference in the rate

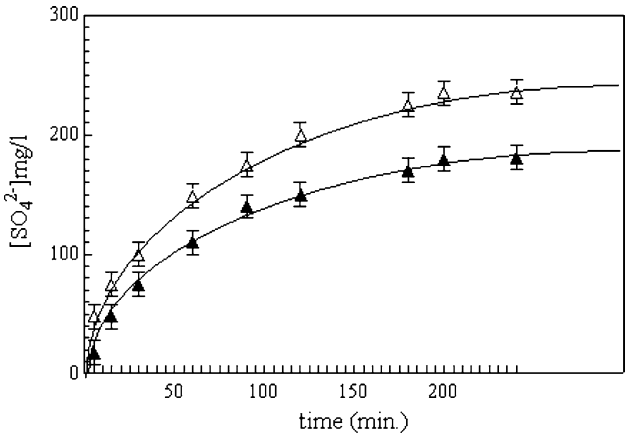

Fig. 1 Sulfate concentration (mg/l) determined in RBC lysates at different incubation times. Results of a typical experiment performed by incubating normal (*closed symbols*) and pathological (*open symbols*) erythrocytes in a medium containing sulfate. Curves were obtained by fitting experimental data with the equation $C(t) = C_\infty$ (1 – e^{-kt}). See "Materials and Methods" for further experimental details. $P < 0.0005$ with respect to control

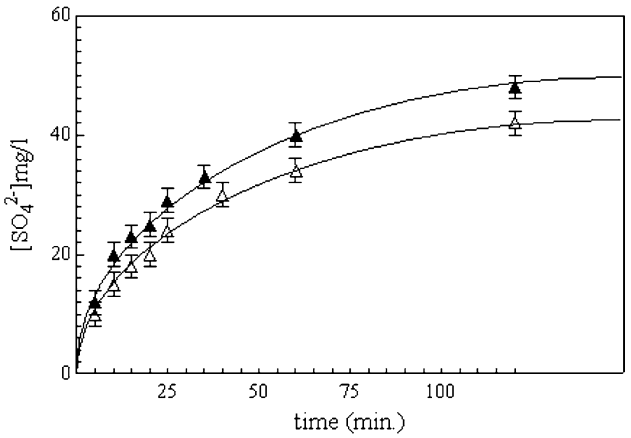

Fig. 2 Sulfate concentration (mg/l) determined in ghost lysates at different incubation times. Results of a typical experiment performed by incubating normal (*closed symbols*) and pathological (*open symbols*) ghosts in a medium containing sulfate. Curves were obtained by fitting experimental data with the equation $C(t) = C_\infty$ (1 – e^{-kt}). See "Materials and Methods" for further experimental details. $P < 0.005$ with respect to control

Table 1 Rates of sulfate transport measured in normal and beta-thalassemic adult human RBCs and ghosts

	Rate constant (min^{-1})		*P*
	Normal	Beta-thalassemic	
RBC	0.012 ± 0.0025 ($n = 6$)	0.021 ± 0.0024 ($n = 5$)	0.0002
Ghost	0.034 ± 0.0025 ($n = 5$)	0.026 ± 0.0019 ($n = 4$)	0.0015

$P < 0.05$ was considered significant

of anion exchange found between normal red cells and their ghosts (0.012 ± 0.0025 vs. 0.034 ± 0.0025, respectively; an increase of about + 180%, $P < 0.001$) clearly indicates the presence of an important flux modulation in the integral cell. This modulation was strongly reduced in thalassemic samples, even if the sulfate flux remained significantly upregulated in ghosts with respect to erythrocytes (0.026 ± 0.0019 vs. 0.021 ± 0.0024, respectively; an increase of about + 24%, $P < 0.05$). The higher sulfate influx of beta-thalassemic samples compared to controls should be considered in the light of recent studies which reported significant changes in the relative content of AE1 in membranes from normal (20.6% ± 0.7), beta-thalassemic erythrocytes (15.6% ± 0.9) and heterozygous for beta-thalassemia erythrocytes (14.3% ± 0.9) (Alekperova et al. 2004). Hence, the AE1 deficit in thalassemia did not slow the rate of sulfate influx; on the contrary, the cell showed high exchange activity through the anion channel. This could be explained on the basis of the loss, in thalassemic samples, of the modulation mechanism of the anion influx, which characterizes normal erythrocytes in which the presence of normal Hb optimizes the functional behavior of band 3 protein (Giardina et al. 1995).

Furthermore, we cannot disregard the different caspase 3 activity observed in the two cellular types. Thus, Western blot analysis (Fig. 3) indicated the presence of a higher concentration of the active form of caspase 3 in beta-thalassemic vs. normal RBCs. The appearance of the active form of caspase 3 is represented by the band at 20 kDa, while the procaspase (inactive form) is represented by the band at 32 kDa, which is clearly more present in the normal RBCs.

Figure 4 shows the different caspase 3 activity determined in normal and beta-thalassemic RBCs. In particular, in heterozygous beta-thalassemic erythrocytes the increase of caspase 3 activity leads to the proteolytic cleavage of cdb3 (Mandal et al. 2002, 2003) and could be the cause of the measured anionic flux variation. In fact, cleavage of cdb3 prevents the AE1 physiological modulation because the protein lacks its hemoglobin-binding site. This is further supported by the fact that the difference in the beta-thalassemic RBC rate of anion flux was similar to that observed in the beta-thalassemic ghost, where normal protein–protein interactions are not present.

On the whole, the emerging scheme points to an excess of oxidative stress that in beta-thalassemic RBCs would stimulate caspase 3 activation. In this regard, we evaluated the effect of *t*-butylhydroperoxide (tBHP), a well-known oxidant agent that strongly affects caspase 3 activity.

Figure 4 shows the effect of tBHP in both beta-thalassemic and normal RBCs. In both cases a significant increase of enzymatic activity in the presence of the oxidant agent is observed. This effect in turn clearly induces an increase of the anion flux, as reported in Table 2.

The pronounced cleavage of cdb3 in beta-thalassemic erythrocytes together with the reported deficit in band 3

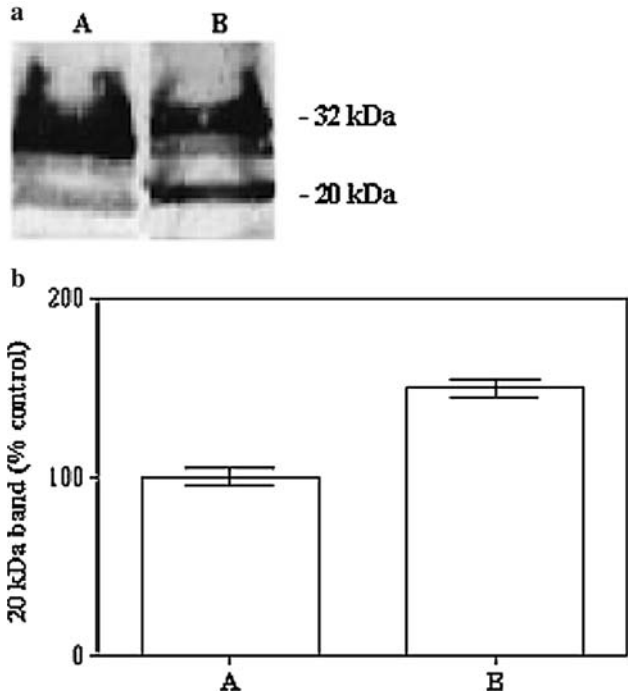

Fig. 3 Caspase 3 in normal and beta-thalassemic RBCs. Lysates were immunoprecipitated with anti-caspase 3 antibody and analyzed for caspase 3 by Western blotting as described in "Materials and Methods." **a** Lanes A and B represent, respectively, normal and beta-thalassemic erythrocytes. The 20- and 32-kDa bands represent, respectively, the activated and inactivated forms of caspase 3. **b** Quantification of intensities of the 20-kDa band (activated caspase 3) determined by densitometric scanning of nitrocellulose membrane. Results are from four different independent experiments ± SD. $P < 0.005$ with respect to control

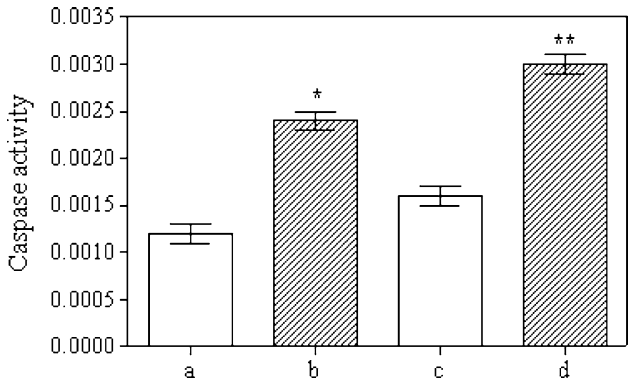

Fig. 4 Effect of tBHP concentration on caspase activity. Results of a series of experiments performed by incubating normal (a, b) and beta-thalassemic (c, d) adult human erythrocytes with (*shaded bars*) and without (*white bars*) 3 mmol/l of tBHP. See "Materials and Methods" for further experimental details. Results are from four different independent experiments ± SD. $*P < 0.005$ with respect to the corresponding control, $**P < 0.005$ with respect to the beta-thalassemic control

protein (Alekperova et al. 2004) could represent, according to recent studies (Miki et al. 2007; Mandal et al. 2003; Suzuki et al. 2007), a mechanism that signals to

Table 2 Effect of tBHP concentration on rates of sulfate transport in normal (RBC_N) and beta-thalassemic (RBC_β) human RBCs

	Rate constant (min^{-1})		*P*
	Control	Plus 50 μM tBHP	
RBC_N	0.012 ± 0.0025 ($n = 6$)	0.042 ± 0.0017 ($n = 5$)	<0.0001
RBC_β	0.021 ± 0.0024 ($n = 5$)	0.047 ± 0.0021 ($n = 4$)	0.0003

$P < 0.05$ was considered significant

macrophages the senescence and/or dysfunction of the erythrocyte. However, in our opinion, in particular clinical settings (i.e., thalassemic heterozygotes and other hemoglobinopathies with a pathogenetic oxidative component) this could also represent a sort of anemia-induced molecular adaptation to compensate for the serious derangement of band 3.

Discussion

Our results show that AE1 in heterozygous thalassemic patients presents a peculiar derangement in its anion exchange function which is significantly increased with respect to normal erythrocytes. Interestingly, in normal ghosts the anion flux was strongly increased. On the contrary, thalassemic ghosts showed a significant reduction of sulfate flux with respect to normal ghosts. On the whole, data obtained on ghosts are in strong support of the modulator function of hemoglobin on the anion flux through AE1. Moreover, incubation of normal and thalassemic erythrocytes with tBHP induced a significant further stimulation of the anion flux in both cell samples. These data not only confirm the peculiar derangement of band 3 but outline the modulating role of its NH_2-cytoplasmic domain.

Finally, exposure of human erythrocytes to redox-mediated injury could be the primary event that induces activation of caspase 3 and consequently a structural modification of the anion exchanger.

In fact, caspase 3 action, once primed, mediates cleavage of the band 3 cytoplasmic domain at positions 45 and 205 (Mandal et al. 2003), leading AE1 to lose that part of the molecule which is involved in the metabolic control of the erythrocyte. In particular, cleavage of cdb3 abolishes functional interactions (protein–protein) with deoxyhemoglobin and glycolytic enzymes and partially alters the intrinsic functionality of the anionic exchanger itself.

The band 3 cytoplasmic domain moreover plays a crucial structural role in the phospholipidic bilayer through its linkage with spectrin and ankyrin, which are fixed to the cytoskeletal net (Bennett and Stenbuck 1980; Korsgren and

Cohen 1986; Han et al. 2000). In this way, oxidative stress–mediated caspase 3 activation may also perturb the structural features of human erythrocytes. These processes gradually would lead the RBC toward phagocytosis by exposure of unusual epitopes (Kay et al. 1984; Kay 1984). Thus, one of the proposed processes for the antigenicity of band 3 involves the intracellular binding of denatured hemoglobin to the membrane, which causes aggregation and clustering of band 3 protein, thereby providing antigenic sites for the binding of anti-band 3 autologous antibodies (Low et al. 1985; Low 1991). In all these processes oxidation plays a relevant role since oxidative modification of AE1 was found in senescent cells (Brovelli et al. 1991; Castellana et al. 1992) and in many pathological cells with decreased life span (Giuliani et al. 1993; Santos Silva et al. 1995).

As far as the biochemical integrity of the cell is concerned, we have to remember that glycolysis Embden-Meyerhof pathway (EMP) is modulated via competition between glycolytic enzymes and deoxyhemoglobin for the N-terminal domain of band 3 (Giardina et al. 1991). Alterations at the level of this complex network of interactions would, very likely, imply a further increase of oxidative stress. Thus, more glucose-6-phosphate would be metabolized through EMP because of the loss of cdb3 binding sites for glycolytic enzymes. As a consequence, since glucose consumption is constant, less glucose would be metabolized by the pentose phosphate pathway (PPP) and the small amount of NADPH produced would be unable to perform normal RBC protection (De Rosa et al. 2007). All these phenomena contribute to an increased production of oxygen free radicals and, above all, of hydrogen peroxide, which stimulates more caspase 3 activation and increases phosphorylation (Mandal et al. 2002; Radi et al. 1991; Matarrese et al. 2005). In this respect, the case of beta-thalassemic erythrocytes is of particular interest. Thus, in beta-thalassemic erythrocytes, the deficit in band 3 protein (about 30% less) and the pronounced cleavage of cdb3 by caspase 3 action would lead the cell unequivocally toward excessive consumption of glucose-6-phosphate through the glycolytic pathway at the expense of NADPH production by the PPP. Hence, we observed a further increase of the oxidative free radicals that stimulate more caspase 3 activation, which would quickly lead beta-thalassemic RBCs toward phagocytosis. However, beta-thalassemic erythrocytes seem to be characterized by a sort of metabolic strategy adopted to limit the consequences of oxidative stress and to extend the life span of the abnormal cell. This strategy may be summarized as follows:

1. An increased rate of anion exchange that, even if not regulated, could compensate, perhaps more than necessary, for the deficit of the anionic protein.
2. The absence (due to loss of cdb3) of anchoring sites for hemoglobin aggregates, which would lead to the anticipated thalassemic erythrocyte elimination.
3. A great decrease ($\sim$30%) of glyceraldehyde-3-phosphate dehydrogenase (Alekperova et al. 2004), a key enzyme of the EMP; this allows redirection of more glucose-6-phosphate to the PPP with a more adequate NADPH production, indispensable to counteracting oxidative stress and redox damage.

These results not only give new information on some molecular pathogenetic events in beta-thalassemia but are important for some possible cellular alterations linked to red cell senescence, with implications for red cell storage.

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