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Original Contribution

Oxidative stress and autologous immunoglobulin G binding to band 3 dimers in newborn erythrocytes

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Abstract

Since birth-induced oxidative stress (OS) results in the removal of erythrocytes from the blood stream, we studied the binding of autologous IgG to erythrocyte band 3 dimers (the 170-kDa band, which marks the erythrocytes for removal) in preterm and term newborns and in adults. The 170-kDa band was present in as much as 74% of preterm, in 21% of term newborns, and in 10% of adults. During erythrocyte ageing "in vitro" (0, 24, and 48 h aerobic incubation), the appearance of the band occurred much faster with erythrocytes from newborns (particularly preterm) than with those from adults. When the blots for the 170-kDa band were quantified by scanning densitometry, it was seen that the 0 time values were significantly higher in preterm compared to term and adult values. After aerobic incubation a progressive increase in the optical density was observed in each group and the densities were higher in preterm than in the other groups. The course of iron release during the various incubations was analogous to that of the 170-kDa band blots, and significant correlations were found at 0 and 48 h. Methemoglobin formation roughly paralleled iron release. Esterified F_2-isoprostanes (markers of OS) and $O_2^{\bullet -}$ production in the nonincubated (0 time) erythrocytes were much higher in newborn (preterm and term) than in adult erythrocytes. Plasma free F_2-isoprostanes were significantly higher in preterms than in terms and in terms than in adults. Plasma non-protein-bound iron (NPBI) was higher in preterm than in term newborns and not detectable in adults. In conclusion dimers of band 3 with autologous IgG are found under conditions in which OS can be detected in erythrocytes or in plasma: namely in newborns or in aged erythrocytes.
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Keywords: Oxidative stress; Newborns; Band 3 dimers; Iron release; Membrane esterified F_2-isoprostanes; Superoxide anion; Plasma F_2-isoprostanes; Non-protein-bound iron

Introduction

Erythrocyte ageing is associated with a decrease in the activity of several enzymes [1] and with modifications in membrane proteins [2]. In particular modifications in membrane band 3 protein, by proteolytic cleavage, clustering or exposure of unusual epitopes, trigger the binding of specific anti-band 3 autoantibodies, marking the cell for removal [3–7].

Our previous studies have shown that iron is released from hemoglobin [8] in a desferrioxamine-chelatable (DCI) form when erythrocytes are exposed to oxidative stress, such as incubation with oxidizing agents [9–11], or prolonged aerobic incubation in physiological buffer (a model of rapid in vitro ageing) [12]. Iron release is accompanied by methemoglobin (MetHb) formation [10–12] and by oxidative alterations of membrane proteins, in particular band 3 protein [12]. Iron release seems to be the cause of oxidation of membrane proteins which promotes the autologous immunoglobulin G (IgG) binding [12,13]. In fact, cell-permeable iron chelators (such as ferrozine, quercetin, and fluor-benzoil-pyridoxal hydrazone) prevent both membrane protein oxidation and autologous IgG

Abbreviations: DCI, desferrioxamine chelatable iron; MetHb, methemoglobin; IgG, immunoglobulin G; NPBI, non-protein-bound iron; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; DFO, desferrioxamine; PBS, phosphate-buffered saline; MCLA, methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one; BHT, butylated hydroxytoluene; PGF2α, prostaglandin F2α; GC/NICI-MS/MS, gas chromatography/negative-ion chemical ionization tandem mass spectrometry; C_{18}, octadecylsilane; NH_2, aminopropyl; NTA, nitrilotriacetic acid; CP22, 3-hydroxy-1propyl-2-methyl-pyridin-4-one.

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binding, suggesting the possibility that a metal-catalyzed oxidation of membrane protein underlies erythrocyte ageing [12–15]. Furthermore, in two conditions in which an accelerated removal of erythrocytes occurs, namely β-thalassemia (major and intermedia) [16] and perinatal period [17], the erythrocyte DCI content and even more the release of iron after 24 h of aerobic incubation are greatly increased as compared to normal adult erythrocytes [18,19], suggesting that β-thalassemic and newborn erythrocytes are subjected to an oxidative stress more than normal adult erythrocytes. β-Thalassemic and newborn erythrocytes show a high content in fetal hemoglobin (HbF) (even higher in preterm infants). HbF, at least under many experimental conditions, has greater oxygen affinity than adult hemoglobin and is more subjected to denaturation and oxidation [20–23]. A positive correlation between HbF and DCI content (and release) in thalassemia major and intermedia has also been observed [18], suggesting that the presence of HbF is a condition favourable to iron release. The erythrocyte DCI content and release after incubation are higher in preterm than in term newborns [19]. Birth is an oxidative challenge for the newborn, due to the sharp postnatal transition from the relatively low oxygen intrauterine pressure (pO_2 20–25 Torr) to the significantly higher oxygen extrauterine environment (pO_2 100 Torr) [24–26]. Such oxidative challenge is exacerbated by the low efficiency of natural antioxidant systems in the newborns, particularly in preterm babies [26,27]. In addition in most newborns [28–30] non-protein-bound iron (NPBI), a form of non-transferrin-bound iron, is detectable in plasma, a condition similar to that occurring in plasma of patients with iron overload such as primary or secondary hemocromatosis [31,32]. Plasma NPBI is higher in preterm than term newborns and is not detectable in healthy adults [19]. Furthermore, F_2-isoprostanes, prostaglandin F_2-like compounds formed by free radical-catalyzed lipid peroxidation of phospholipid-bound arachidonic acid and considered the most reliable marker of oxidative stress [33–35], are significantly higher in plasma of newborns than of healthy adults and are higher in preterm than in term newborns [36].

Since an accelerated removal of erythrocytes from the blood stream occurs in preterm and term newborns, we investigated whether neonatal erythrocyte exposure to oxidative stress results in the binding of autologous IgG to band 3 dimers in a higher percentage of newborns as compared to adults and of preterm as compared to term babies. The susceptibility of neonatal and adult erythrocytes exposed to in vitro ageing to bind autologous IgG was also investigated. The release of iron (DCI) and the formation of esterified F_2-isoprostanes in erythrocytes and the occurrence of free F_2-isoprostanes and of NPBI, as markers of oxidative stress in the erythrocytes and plasma, respectively, were also studied.

Materials and methods

Materials

Desferrioxamine (DFO) was supplied by Ciba-Geigy. Centrifugal filter devices (Centriplus®, YM-30) were from Amicon. The reservoirs for silicic acid column chromatography were from Varian. The solvents used for HPLC were of HPLC grade. The methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-α]pyrazin-3-one (MCLA) was from Molecular Probes. The nitrocellulose Hybond-C extra was supplied by Amersham Life Science. The secondary antibody was goat anti-human IgG (Fc-specific) alkaline phosphatase conjugate from Sigma Immunochemicals. 5-Bromo-4-cloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system (BCIP/NBT) was from Sigma-Aldrich. Tetradeuterated 8-epi-PGF2α were obtained from Cayman (Ann Arbor, MI), Sep-Pak Vac® C_{18} (500 mg) and Sep-Pak Vac® NH_2 (500 mg) cartridges were purchased from Waters (Milford, MA). Nitrilotriacetic acid (NTA) was from Sigma.

Subjects studied

One hundred thirty subjects were randomly selected from 9 am to 2 pm to allow preliminary blood processing on the same day, from January to October 2004. Thirty-five were excluded due to the lack of parental consent, 23 were excluded for suspected sepsis, thalassemias or other abnormal hemoglobins (screened by HPLC) or glucose-6-phosphate dehydrogenase deficiency (estimated by a Biotech kit), 12 were not enrolled due to the impossibility to draw cord blood. Sixty newborn infants (37 term and 23 preterm) were examined at birth. Seventy-five percent of preterm newborns received antenatal corticosteroids. Four milliliters of heparinized blood was collected from the umbilicain vein immediately after cord clamping. All tests were performed in cord blood immediately after cord clamping in healthy term and in preterm babies without any severe illness at birth (for more detailed information see Table 1). All postnatal procedures cannot influence obviously cord blood data.

Venous blood was also drawn from 50 healthy adult subjects.

Informed consent was obtained from the parents of the newborn infants and from the adults. The study was approved by the Human Ethics-Deontology Committee of the Medical Faculty of the University of Siena.

Table 1
Clinical characteristics of babies

	Preterms	Terms
Number	23	37
Gestational age (weeks)	34.7 ± 0.47 (28–36)	38.70 ± 0.22 (37–41)
Male/Female	12/11	19/18
Birth weight (kg)	2.45 ± 0.12 *	3.04 ± 0.08
Vaginal delivery (*n*)	–	16
Elective cesarean section (*n*)	17	20
Emergency cesarean section (*n*)	6	1
Apgar-1 min score	6.74 ± 0.46 **	8.32 ± 0.27
Apgar-5 min score	8.78 ± 0.39 **	9.72 ± 0.10
pH	7.31 ± 0.01	7.30 ± 0.01
Peack of bilirubin (mg/dl)[a]	11.3 ± 0.68	11.2 ± 0.74

The data are the means ± SE.
[a] As measured by Bilicheck (from Burke and Burke).
* $P < 0.001$ preterms vs terms.
** $P < 0.01$ preterms vs terms.

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