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Original Contribution

Oxidative damage and genotoxicity biomarkers in transfused and untransfused thalassemic subjects

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ABSTRACT

Chronic anemia and tissue hypoxia increase intestinal iron absorption and mitochondrial impairment in thalassemic patients. Regular blood transfusions improve hemoglobin levels but determine an iron overload that induces reactive oxygen species (ROS) overproduction. The aim of this study was to assess cellular oxidative damage by detection of ROS, lipid peroxidation, 8-oxo-dG, and mitochondrial transmembrane potential ($\Delta\psi_m$) in transfused and untransfused thalassemic patients. We have also evaluated genotoxicity by CBMN and comet assay. Our data show that ROS and lipid hydroperoxides are significantly higher in thalassemic patients than in controls, especially in untransfused thalassemia intermedia patients. Moreover, the latter have a significant decrease in $\Delta\psi_m$ that highlights the energetic failure in hypoxic state and the ROS overproduction in the respiratory chain. 8-OHdG levels are higher in thalassemics than in controls, but do not differ significantly between the two patient groups. Both genotoxicity biomarkers highlight the mutagenic potential of hydroxyl radicals released by iron in the Fenton reaction. Values for percentage of DNA in the comet tail and micronuclei frequency, significantly higher in transfused patients, could also be due to active hepatitis C virus infection and to the many drug treatments. Our biomonitoring study confirms the oxidative damage in patients with thalassemia major and shows an unexpected cellular oxidative damage in untransfused thalassemic patients. In addition to iron overload, the results highlight the important role played by hypoxia-driven mitochondrial ROS overproduction in determining oxidative damage in β-thalassemias.

The β-thalassemias are autosomal recessive disorders characterized by absent or reduced synthesis of β-globin chains. The imbalance of α- and β-globin chains causes a premature death of erythroid progenitors in the bone marrow and in the spleen for the excess of α-globin chains, which precipitate as tetramers in the red blood cells. This leads to severe anemia, tissue hypoxia, marked hemocatheresis, hepatosplenomegaly, and ineffective erythropoiesis [1].

Hypoxia downregulates the expression of hepcidin, a key regulator of iron homeostasis, and increases intestinal iron absorption [2,3].

Chronic anemia and iron overload are the main complications of thalassemic patients. Thalassemia intermedia has a clinical heterogeneity and, generally, the milder anemia does not require a steady transfusion treatment [1]. Thalassemia major patients are regularly transfused to improve hemoglobin levels and to decrease the ineffective erythropoiesis, but they show an increase in non-transferrin-bound iron (NTBI)[1]. Iron overload, due to the higher enteric iron absorption and, mostly, to transfusions, induces reactive oxygen species (ROS) overproduction [4]. Clinical effects of the abnormal iron stores are liver disease, cardiac dysfunction, arthropathy, gonadal insufficiency, and other endocrinal disorders. Iron-chelating compounds, desferrioxamine,

[1] *Abbreviations used:* NTBI, non-transferrin-bound iron; ROS, reactive oxygen species; CBMN, cytokinesis-block micronucleus; MNi, micronuclei; NPB, nucleoplasmic bridge; NBUD, nuclear bud; %TDNA, percentage of DNA in the tail; %TSAT, percentage of transferrin saturation; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; DCF-DA, 2′,7′-dichlorofluorescein diacetate; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; $\Delta\psi_m$, mitochondrial transmembrane potential; R123, rhodamine 123; DPPP, diphenyl-1-pyrenylphosphine; FU, fluorescence units.

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deferiprone, and deferasirox, are used to counteract iron overload and improve life expectancy [1].

Iron is required in biochemical processes such as metabolism, oxygen delivery, and DNA synthesis. Its biological functions are based on its ability to participate in one-electron reduction–oxidation reactions between the ferric (Fe^{3+}) and the ferrous (Fe^{2+}) states. However, iron is a leader in free radical production, because of the ability of Fe^{2+} to catalyze the production of hydroxyl radicals by Fenton reaction [5]. Redox imbalance determines the oxidation of membrane phospholipids, glycoxidation, and DNA damage that trigger apoptosis or carcinogenesis [6,7].

Tissue hypoxia, in addition to enteric iron absorption increases [2], is a source of ROS, because lower levels of PO_2 induce mitochondrial ROS overproduction [3].

Thalassemic patients show chromosomal aberrations and micronuclei [8,9], which are cytogenetic biomarkers of cancer [10,11]. Iron overload is clearly associated with an increased cancer risk [7,12]. Hepatocellular carcinoma, common in hereditary hemochromatosis, is also frequently seen in thalassemia [13].

Cytokinesis-block micronucleus (CBMN) and comet assay are the main tests to assess genotoxicity in the peripheral blood lymphocytes as a biological dosimetry of DNA damage.

Micronuclei (MNi) originate from acentric chromatid/chromosome fragments or from loss of whole chromosomes during telophase. MNi frequency, other than as a genotoxicity evaluation, can be considered an early biomarker of cancer risk [11]. CBMN also detects nucleoplasmic bridges (NPBs), derived from dicentric chromosomes or telomere end fusions, and nuclear buds (NBUDs). The latter represent the amplified DNA, DNA repair complexes, and/or possibly excess chromosomes from aneuploid cells [14]. By the nuclear division index (NDI) and proportion of necrotic and apoptotic cells, CBMN assesses mitogen response and evaluates the cytotoxicity [15].

The comet assay (single-cell gel electrophoresis) is a simple, rapid, reliable, and very sensitive method to measure genotoxicity. The high sensitivity of the comet assay alkaline version is mainly attributed to the capability to recognize the temporary presence of alkali-labile abasic sites, produced by DNA glycosylase during the repair mechanism that restores the double strand integrity [16].

The aim of this study is to assess cellular oxidative damage and the related genotoxicity, analyzing lymphocytic DNA damage by CBMN and comet assay in thalassemic patients. Oxidative damage was measured by an array of parameters such as lipid peroxidation in erythrocyte lysates, whereas intracellular ROS, 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxo-dG), and mitochondrial transmembrane potential were evaluated in lymphocytes. We have used $\Delta\psi_m$ collapse as a valid biomarker of hypoxia-driven ROS increase in the respiratory chain.

Material and methods

Selection and characteristics of patients

Thalassemic patients were recruited from the Pediatrics Department of the Messina University Hospital and the Thalassemia Department of Riuniti Hospital in Reggio Calabria, Italy. The ethics committees of both hospitals approved the study.

All patients signed informed consent to study participation and completed a questionnaire, which included information about age, occupation, and lifestyle factors. Past and current drug treatment and clinical condition were collected.

One hundred thirteen thalassemic patients, age 33.8 ± 9.7 years (means $\pm$ SD), were enrolled in January–December 2009. Among these, 92 were regularly transfused and undergoing iron chelation therapy. Twenty-seven were treated with deferasirox, 25 with desferrioxamine, 16 with deferiprone, and 24 with combined chelation treatment (deferiprone–desferrioxamine).

The remaining 21 thalassemia intermedia patients had in the past been only occasionally transfused and treated with iron chelators. Ten healthy subjects, matched for age and gender with the examined patients, were enrolled as controls. Several parameters investigated in the study are part of routine examination and only 4 ml of blood from each patient was used to assess genotoxicity and cellular oxidative damage.

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Lymphocyte culture

Heparinized blood was collected by venipuncture before blood transfusion and was immediately used for lymphocyte separations in Lymphoprep separation medium (Axis-Shield, Oslo, Norway). The buffy layer was removed, washed with PBS, and collected by centrifugation. Lymphocytes were suspended in 1 ml PBS, of which 30 μl was used for comet assay, 0.5 ml for CBMN assay, and the remaining volume for the cytofluorimetric analysis. All analyses were performed in duplicate.

Assessment of lymphocytic oxidative damage

ROS, 8-oxo-dG, and $\Delta\psi_m$ were assayed by FACS analysis using a 488-nm argon laser beam for excitation (Dako, Denmark). The lymphocytes ($\sim 1 \times 10^4$ cells ml^{-1}) were suspended in PBS containing 10 mM D-glucose (pH 7.4) and three aliquots were prepared.

Intracellular ROS were evaluated using the membrane-permeative lipophilic DCF-DA (1 μM). The probe undergoes deacetylation by intracellular esterases and specifically releases green fluorescent signals (530 nm) when activated by ROS, allowing the detection of redox imbalance [17].

The 8-oxo-dG was detected by binding of the FITC-labeled avidin fluorochrome. The probe binds to 8-oxo-dG with a high specificity due to the structural analogies between the keto form of the oxidized base and biotin [18]. The method, previously adapted to flow cytofluorimetric analysis [19], was performed in lymphocytes permeabilized in methanol (15 min at −20 °C) and loaded with the avidin–FITC conjugate (1 h at 37 °C). The emission signals were collected in the green fluorescence channel.

$\Delta\psi_m$ was evaluated by measuring the incorporation of the fluorescent probe R123. The chemical properties of the cationic fluorochrome allow it to cross the mitochondrial membrane and be stored only in the matrix of functional mitochondria that possess a $\Delta\psi_m$, indicative of an active proton gradient maintained during oxidative phosphorylation [20]. The probe was added to cell suspensions (final 0.2 μM) and incubated (10 min at 37 °C). The emission signals were collected in the red fluorescence channel.

In FACS analyses, the weighted average of emission values for 100 cells was calculated and expressed in arbitrary fluorescence units (FU).

Assessment of lipid peroxidation in the erythrocyte lysates

Lipid hydroperoxides were detected in the erythrocyte lysates obtained from heparinized blood (1 ml). The analysis was performed by using the probe DPPP (Invitrogen, Milan, Italy), according to a previously reported method [19]. DPPP has a high