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Oxidative imbalance in low/intermediate-1-risk myelodysplastic syndrome patients: The influence of iron overload

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ABSTRACT

Objective: To assess the generation of reactive oxygen species (ROS) and the involvement of the main antioxidant pathways in low/intermediate-1-risk myelodysplastic syndromes (MDS) with iron overload (IOL).

Methods: We examined the levels of superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), antioxidants (glutathione, GSH; superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPx), mitochondrial membrane potential ($\Delta\Psi m$), and by-products of oxidative damage (8-isoprostanes and 8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxo-dG) in 42 MDS patients (28 without IOL at diagnosis, and 14 who developed IOL) and 20 healthy subjects.

Results: Patients with IOL showed higher $O_2\cdot^-$ levels (39.4 MFI) than normal controls (22.7 MFI, $p = 0.0356$) and patients at diagnosis (19.4 MFI, $p = 0.0049$). Antioxidant systems, except SOD activity, exhibited significant changes in IOL patients with respect to controls (CAT: 7.1 *vs* 2.7 nmol/ml/min, $p = 0.0023$; GPx: 50.9 *vs* 76.4 nmol/ml/min, $p = 0.0291$; GSH: 50.2 *vs* 24.1 MFI, $p = 0.0060$). Furthermore, mitochondrial dysfunction was only detected in IOL cases compared to controls ($\Delta\Psi m$: 3.6 *vs* 6.4 MFI, $p = 0.0225$). Finally, increased levels of 8-oxo-dG were detected in both groups of patients.

Conclusion: Oxidative stress is an important but non-static phenomenon in MDS disease, whose status is influenced by, among other factors, the presence of injurious iron.

1. Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal haematopoietic disorders characterized by an abnormal maturation of precursors, which results in ineffective haematopoiesis, peripheral blood cytopenias, and high rates of conversion to acute myeloid leukaemia. Anaemia is the clinical hallmark of these entities; it is present in the majority of MDS patients at diagnosis and deteriorates

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CMF, 5-chloromethylfluorescein diacetate; CMFDA, 5-chloromethylfluorescein diacetate; dG, deoxyguanosine; DHR, dihydrorhodamine 123; EB, ethidium bromide; GPx, glutathione peroxidase; GSH, glutathione; HE, hydroethidine; *HFE*, haemochromatosis gene; IOL, iron overload; IPSS, international prognostic score system; LIC, liver iron concentration; LPI, labile plasma iron; MDS, myelodysplastic syndrome; MFI, mean fluorescence intensity; MRI, magnetic resonance imaging; NTBI, non-transferrin bound iron; PRBCs, packed red blood cells; RARS, refractory anaemia with ringed sideroblasts; ROS, reactive oxygen species; SOD, superoxide dismutase; TMRM, tetramethylrhodamine methyl ester; TSI, transferrin saturation index; WHO, World Health Organization; $\Delta\Psi m$, mitochondrial membrane potential; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; $O_2\cdot^-$, superoxide anion; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical

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during the course of the disease. The need for continuous blood transfusions to improve anaemia symptoms leads to iron overload (IOL) [1,2]. In addition, ineffective erythropoiesis stimulates intestinal iron absorption due to the increased secretion of growth differentiation factor 15 and suppression of hepcidin production in the liver [3,4]. Thus, chronic transfusion therapy and ineffective erythropoiesis contributes to iron accumulation, especially in MDS patients with low/intermediate-1 risk [5,6].

Under normal conditions, iron circulates in plasma bound to transferrin, but in iron overload, the binding capacity of transferrin is exceeded, and the non-transferrin-bound plasma iron (NTBI) species appear [7]. Labile plasma iron (LPI) is the redox active NTBI fraction capable of permeating into cells, where it contributes to the generation of reactive oxygen species (ROS) and to the overwhelming of the antioxidant defence system. The oxidative injury results in the alteration of cell structures and organ dysfunction [8].

Previous studies showed an important role of oxidative stress in the pathogenesis of myelodysplasia. An increased level of DNA oxidative damage has been observed in MDS CD34 + bone marrow cells, suggesting their involvement in ineffective haematopoiesis by inducing apoptosis in bone marrow precursors [9,10]. Low-risk MDS patients also exhibit oxidized nucleotides in more differentiated CD34- bone marrow cell populations, which contributes to genomic instability and disease progression [11].

On the other hand, CD34 + progenitors could fail to survive because of inadequate stress defence [12]. However, little is known about the true involvement of antioxidant mechanisms in the aetiology of this group of diseases.

On the basis of these observations, the levels of several ROS, such as superoxide ($O2\cdot^-$) and hydrogen peroxide (H_2O_2), and antioxidant molecules, such as reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), were assessed in MDS patients and normal individuals to elucidate the dysfunction of the main antioxidant pathways and describe the biological role of ROS in MDS. Furthermore, the presence of DNA and lipid oxidative damage and mitochondrial injury was evaluated by measuring 8-oxo-dG, 8-isoprostanes and mitochondrial membrane potential (ΔΨm), respectively. We hypothesized that excess amounts of iron might have an effect on the oxidative stress status in low/intermediate-1 risk MDS patients.

2. Materials and methods

2.1. Patients

A total of 42 MDS patients with low/intermediate-1 risk according to the International Prognostic Scoring System (IPSS) criteria [13] were enrolled in this study. These patients were divided in two groups consisting of 28 subjects without IOL at diagnosis and 14 who developed IOL (serum ferritin > 1000 ng/ml and/or transferrin saturation index > 70%). As a control group, we used 20 healthy subjects consisting of ten males (50%) and ten females (50%) with a median age of 70 and 74 years, respectively; and normal values for biochemical and haematological parameters. All patients and controls provided their written informed consent, and the study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of our Institution.

2.2. Blood and urine sampling

Peripheral blood samples were collected in lithium heparin and EDTA tubes. Heparinized blood was centrifuged at 2500 rpm for 5 min, and the plasma fraction was aliquoted and stored at − 80 °C until analysis. Urine samples were collected in polyethylene tubes without additives and centrifuged at 4000 rpm for 20 min. The supernatant was aliquoted and frozen at − 80 °C until use.

2.3. DNA isolation and enzymatic digestion

Blood erythrocytes collected in EDTA tubes were lysed in a cell lysis buffer and centrifuged; the resulting white blood cells pellet was kept at − 80 °C until analysis. Genomic DNA was isolated according the method of Gupta [14] as modified by Muñiz et al. [15]. The isolated DNA was washed twice with 70% ethanol, dried, and dissolved in 200 ml of 10 mM Tris/HCl, 0.1 mM EDTA, and 100 mM NaCl, (pH 7.0) for enzymatic digestion. In brief, 5 mg DNA/ml was incubated with 100 units of DNase I in 40 ml 10 mM Tris/HCl and 10 ml 0.5 M MgCl2 (final concentration: 20 mM) at 37 °C for 1 h. The pH of the reaction mixture was then lowered by adding 15 ml 0.5 M sodium acetate (pH 5.1); 10 ml nuclease P1 (5 units) and 30 ml 10 mM ZnSO4 were added (final concentration: 1 mM) and the mixture was incubated for 1 h. After readjusting the pH with 100 ml 0.4 M Tris/ClH (pH 7.8), followed by the addition of 20 µl alkaline phosphate (3 units), the samples were incubated for 1 h. The enzymes were precipitated with acetone (5 vol.), removed by centrifugation, and the supernatant was dried by evaporation.

2.4. Measurement of ROS, GSH and ΔΨm in neutrophils

Whole blood cell suspensions were examined by flow cytometric analysis. The appropriate gate for leucocytes and neutrophils was determined by forward and side scatter and validated by anti-CD45-KO and anti-CD16 APC-750 (Beckman-Coulter, Miami, Florida) staining, respectively. Briefly, cells were incubated with antibodies for 20 min at room temperature in the dark (5 µl of each antibody/100 µl of whole blood), diluted with 2 ml of phosphate-buffered saline, and stained with different fluorochromes for the functional measurements. Hydroethidine (HE) (Sigma-Aldrich, St. Louis, MO) was used to determine superoxide anion generation [16]. H_2O_2 intracellular production was measured using the dihydrorhodamine 123 (DHR) assay as previously described [17]. HE and DHR were added at a final concentration of 1.59 µM and 0.29 µM, respectively. CellTracker Green (5-chloromethylfluorescein diacetate, CMFDA; Life Technologies, Carlsbad, CA) was used to detect intracellular glutathione levels. Cells were incubated with 0.05 µM CMFDA. ΔΨm was assessed using the cationic-lipophilic tetramethyl rhodamine methyl ester (TMRM) (Life Technologies, Carlsbad, CA), an indicator dye that accumulates inside the mitochondria in proportion to their membrane potential. TMRM was added at a final concentration of 12 µM (emission 574 nm).

Sample analysis was performed in a Gallios™ flow cytometer (Beckman-Coulter, Brea, CA) with the appropriated settings. Data analysis was carried out using Kaluza™ Software (Beckman-Coulter, Brea, CA). The results were expressed as the mean fluorescence intensity (MFI) in arbitrary units.

2.5. Assessment of antioxidant enzyme activities

SOD, CAT and GPx activities were measured in plasma samples. SOD activity was determined using the Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI), which utilizes a tetrazolium salt for the detection of superoxide radical generated by xantine oxidase and hypoxanthine. CAT activity was measured using the Catalase Assay Kit (Cayman Chemical Company, Ann Arbor, MI), based on the enzymatic conversion of methanol to formaldehyde in the presence of an optimal concentration of H_2O_2. To detect GPx activity, Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI) was used. This assay measures GPx activity indirectly by a coupled reaction with glutathione reductase.

2.6. 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) assay

The DNA hydrolysates were dissolved in HPLC grade water and filtered through a 0.2 mm syringe filter before the samples were applied