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ORIGINAL

Sensitivity to cisplatin-induced mutations and elevated chromosomal aberrations in lymphocytes from sickle cell disease patients

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Abstract Sickle cell disease (SCD) is an inherited disorder caused by a single nucleotide substitution in the β-globin gene. The clinical heterogeneity observed in SCD patients has been attributed to environmental and genetic factors. The patients are subjected to increased oxidative stress, particularly during vaso-occlusive crises and acute chest pain. Another possible cause of oxidative stress in SCD is the high concentration of iron in the patients' plasma. The increase in oxidative stress could be a relevant risk factor for mutagenesis and carcinogenesis. Studies on the frequency of basal chromosomal aberrations in cultured lymphocytes from SCD patients have not been reported so far. In order to contribute to the understanding of the role of the different biomarkers and their relationship with the extremely variable clinical manifestation of SCD, we investigated the frequency of chromosome damage in peripheral lymphocytes from sickle cells patients and healthy controls. We found an increased frequency of chromosome damage and percentage of aberrant metaphases in these patients when compared with control subjects, even at basal values ($p < 0.05$). In the cytogenetic sensitivity assay, the results showed that these patients presented a marked decrease in the mitotic index values compared with healthy controls. Cisplatin-induced chromosomal damage in lymphocytes from these patients was significantly higher than the frequency measured in healthy controls. The results obtained in the present study showed that more investigations are needed in order to elucidate the susceptibility to genomic instability of SCD patients.

Keywords Sickle cell · Chromosome aberrations · Cisplatin · Genotoxicity · Lymphocytes

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Introduction

Haemoglobinopathies are frequent hereditary diseases in the Brazilian population. The most common haemoglobinopathies are sickle cell disease (SCD) and thalassaemia. SCD results in a red blood cell disorder caused basically by a single base pair substitution in the β-gene.

The haemoglobin molecule that contains the substituted nucleotide induces alterations in the morphology of sickle red blood cells that lead to a wide variety of pathological and clinical manifestations [1, 2]. The clinical manifestations observed in SCD patients include anaemia, vaso-occlusion, infection, endothelial activation, strokes, acute chest syndrome and pulmonary hypertension [3].

SCD patients are subjected to increased oxidative stress, especially during acute chest syndrome [4, 5] and present higher rates of reactive oxygen species. Oxidative stress may have an important role in the pathogenesis of this disease. Red blood cells, platelets and polymorphonuclear lymphocytes are under chronic oxidative stress [6]. Oxidative modifications of plasma serum proteins have been found in SCD patients, suggesting that the post-translational modifications may be important biomarkers of disease and that pathogenesis of SCD may be modulated by oxidative stress [7]. The exacerbation of oxidative stress in SCD patients exposes the cells to a higher rate of oxidative damage in membranes, proteins and DNA. The identification of oxidative damage is important to diagnose and monitor SCD patients, in particular in anaemic patients treated with hydroxyurea.

Many studies have reported that exposure to genotoxic agents, such as reactive oxygen species, influences the cytogenetic endpoints of cultured lymphocytes, and, in addition, it determines the sensitivity of lymphocytes *in vitro* from subjects exposed to genotoxic agents such as X-rays, ultraviolet light, bleomycin or mitomycin C [8–10]. The cytogenetic characterisation and classification of the different types of chromosomal aberrations in human peripheral blood lymphocytes have an important role in human genetics. The frequency of chromosomal aberrations in peripheral blood lymphocytes is used to measure exposure to genotoxic agents. The identification of the main chromosomal damage and studies of the mechanisms of chromosome-related pathologies have proved the importance of this biomarker in the field of carcinogenesis and mutagenesis [11, 12].

The aims of the present study were to evaluate the basal and cisplatin-induced chromosomal aberration frequencies and mitotic index in peripheral blood lymphocytes of SCD patients and compare them with those of healthy controls. We have already published a cytogenetic study of the genotoxicity and cytotoxicity of hydroxyurea in SCD patients where we observed that among eight patients, one had a high rate of chromosomal aberrations [13]. This was very important as the presence of chromosomal damage in the lymphocytes of untreated patients may indicate chromosomal sensitivity. Those results prompted us to undertake this investigation with a greater number of SCD patients and compare them with healthy controls.

Material and methods

In this study we investigated a total of 30 subjects: 15 (4 males and 11 females; mean age 25.6 ± 4.51 years; range 19–34 years) patients with SCD from the Hemocentro Regional de Uberaba (Minas Gerais, Brazil) and 15 (4 males and 11 females; mean age 25.6 ± 5.24 years; range 18–35 years) healthy volunteers (control subjects). The Ethics Committee of the Universidade Federal do Triângulo Mineiro (Minas Gerais, Brazil) approved the protocol (No. 539) and written informed consent was obtained from all subjects. Exclusion criteria were smoking, previous exposure to radiation and taking medication or undergoing blood transfusions. None of the patients received or were previously exposed to hydroxyurea or any other potentially mutagenic agent.

The chromosomal aberration assay was carried out using conventional techniques [14]. One blood sample of approximately 5 ml was taken by venipuncture in heparinised tubes. Four lymphocyte cultures were initiated for each subject, two without and two with cisplatin [(*cis*-diamminedichloroplatinum(II); cDDP; CAS Nº. 15663-27-1)] obtained from Quiral Química do Brasil S.A. (Platinil), diluted with distilled water. Peripheral blood lymphocytes were cultivated for 72 h at 37°C in complete culture medium consisting of 78% RPMI 1640 (Gibco-Invitrogen), 20% of inactivated foetal bovine serum (Gibco-Invitrogen) and stimulated with 2% of phytohaemagglutinin (Gibco-Invitrogen). Cultures did not contain antibiotics. After 24 h of incubation, the cells were treated with cisplatin (0.5 μg/ml) to induce chromosomal aberrations. The concentration of cisplatin was established on the basis of the results of mitotic index analysis in preliminary experiments. Cultures were harvested 48 h after treatment. Colchicine (Sigma-Aldrich Co., USA) was added to a final concentration of 0.4 μg/ml of the culture medium, 1 h prior to harvesting. The cells were harvested by centrifugation, suspended in a hypotonic 0.075 M KCl solution, and fixed in methanol:acetic acid. Slides were stained with Giemsa (Sigma-Aldrich Co., USA). The end points analysed were mitotic index, total chromosome aberrations and percentage of aberrant metaphases. The mitotic index was determined by scoring the number of metaphases among 1000 cells per subject. Two hundred cells per subject were scored to analyse chromosomal aberrations and one hundred cells per subject were scored to analyse sensitivity to cisplatin-induced mutations. Only well spread metaphases analysed under a light microscope containing 46 ± 1 chromosomes were scored. Aberrations were classified according to ISCN [15].

Statistical analysis was used to determine whether lymphocytes from SCD patients and controls have different sensitivities and frequencies of chromosomal aberrations when exposed to cisplatin *in vitro*. The nor-

mality of variable distributions was evaluated by the Kolmogorov–Smirnov test. The data were analysed by the non-parametric Mann–Whitney *U*-test (SPSS Chicago, IL). *p*-values < 0.05 were regarded as significant.

Results

Table 1 shows the mitotic index, total chromosomal aberrations and percentage of aberrant metaphases. SCD patients showed a higher number of chromosomal aberrations when compared with healthy controls ($p < 0.05$). Even at basal values, SCD patients had statistically significant differences in the number of chromatid and chromosomal breaks when compared with controls, while the mitotic index values, used as a parameter to determine cytotoxicity, were very close (3.8% and 3.4% for controls and patients, respectively).

In vitro sensitivity of lymphocytes to cisplatin was evaluated in SCD patients and healthy controls. The treatment of the cultures with cisplatin resulted in the induction of different types of chromosomal aberrations in all subjects (Table 2). However, cisplatin-induced frequency of chromosomal damage in lymphocytes from SCD patients was significantly higher than the cisplatin-induced chromosomal aberrations observed in the controls ($p < 0.05$). Cisplatin treatment was associated with increases in the percentage of aberrant metaphases and all types of aberrations. Cisplatin also induced an increase in the number of cells with one or two aberrations. Chromatid and chromosomal breaks were the predominant type of aberrations observed. These data suggest possible chromosomal sensitivity in SCD patients. Table 2 shows the decrease in the mitotic index in SCD patients caused by cisplatin treatment (3.5% and 2.8% for controls and patients, respectively). This reduction was statistically significant ($p < 0.05$).

Discussion

This study indicates that the basal chromosomal aberration frequencies in peripheral blood lymphocytes from SCD patients are increased when compared with healthy controls with the same demographic characteristics. The basal frequency of chromosomal aberrations evaluated in the cultured blood lymphocytes from healthy controls was in line with the results obtained by Karahalil et al. [16] in healthy non-smokers. Treatment with 0.5 μg/ml of cisplatin was not cytotoxic to the control cultures, as expected from previous studies [17], but a significant decrease was detected in SCD patients. Cell cycle control is a fundamental process to regulate cell proliferation and mitosis. Analysis of cisplatin-induced chromosomal damage and its influence on mitotic index values showed that significant differences exist between SCD patients and controls. The induction of chromosomal aberrations by cisplatin was more evident in SCD patients and this suggests that lymphocytes from SCD patients are very sensitive to *in vitro* exposure to cisplatin.

Table 1 Mitotic index, distribution and total of chromosomal aberrations and percentage of aberrant metaphases (AM) in human lymphocytes from controls and SCD patients

Subjects	MI (%)	Chromosomal aberrations					Total ± SD	% of AM ± SD
		Ctg	Chg	Ctb	Chb	Ex		
All subjects ($n = 30$)	3.6	8	4	91	55	1	159 ± 5.38	2.36 ± 0.62
Controls ($n = 15$)	3.8	1	1	21	15	0	38 ± 6.38	1.10 ± 0.75
SCD patients ($n = 15$)	3.4	7	3	70	40	1	121 ± 8.02*	3.63 ± 3.18*

Two hundred cells were analysed per subject and 1000 cells per subject were analysed for mitotic index (MI). *Ctg* chromatid gaps, *Chg* chromosomal gaps, *Ctb* chromatid breaks, *Chb* chromosomal breaks, *Ex* exchanges figures

* Statistically different compared to controls (Mann–Whitney $p < 0.05$)

Table 2 Mitotic index, distribution and total of chromosomal aberrations and percentage of aberrant metaphases (AM) in human lymphocytes from controls and SCD patients treated with cisplatin (0.5 μg/ml)

Subjects	MI (%)	Chromosomal aberrations					Total ± SD	% of AM ± SD
		Ctg	Chg	Ctb	Chb	Ex		
All subjects ($n = 30$)	3.1	5	8	192	105	7	317 ± 5.20	9.03 ± 3.67
Controls ($n = 15$)	3.5	1	0	85	44	5	135 ± 4.05	7.73 ± 2.60
SCD patients ($n = 15$)	2.8*	4	8	107	61	2	182 ± 5.01*	10.33 ± 4.18*

One hundred cells were analysed per subject and 1000 cells per subject were analysed for mitotic index (MI). *Ctg* chromatid gaps, *Chg* chromosomal gaps, *Ctb* chromatid breaks, *Chb* chromosomal breaks, *Ex* exchanges figures

* Statistically different compared to controls (Mann–Whitney $p < 0.05$)

Similar results were found in patients with β-thalassaemic traits where sensitivity to 2-Gy irradiation leads to an increase in chromosomal damage measured by micronucleus frequencies in five out of nine patients studied [18]. Howell-Jolly bodies, also known as micronuclei, were analysed in the reticulocytes of a large number of children with SCD. Flow cytometry analysis showed that children with Sickle haemoglobin (HbSS) had elevated micronuclei frequencies as a consequence of chromosomal damage [19]. Data from β-thalassaemic and SCD patients suggest that haemoglobinopathies may be associated with chromosomal instability and sensitivity to genotoxic agents.

Cisplatin, like many antineoplastic drugs, exerts its cellular toxicity through induction of DNA damage. It is able to cause DNA damage either as a result of direct action or indirectly through the generation of reactive oxygen species [20]. Cisplatin is capable of generating reactive oxygen species, such as superoxide anions and hydroxyl radicals, and many of the toxic side effects of this drug are associated with the generation of free radicals by DNA interaction and lipid peroxidation [21]. In the present study, cisplatin increases sensitivity as measured by chromosomal aberration frequencies and mitotic index in lymphocytes from SCD patients when compared with healthy controls. We hypothesise that cisplatin-induced sensitisation may occur through reactive oxygen species. Cisplatin is a potent genotoxic agent, inducing sister-chromatid exchanges, chromosomal aberrations and micronucleus formation in cultured human lymphocytes [17, 22].

Previously published studies indicate that SCD patients have increased reactive oxygen species in their blood [23, 24]. Hebbel et al. [4] observed that SCD patients had increased oxidative stress, as sickle cells were able to generate greater amounts of superoxides, peroxides and hydroxyl radicals when compared with normal erythrocytes. Oxidative stress in SCD patients may result from the high concentration of iron that would lead to free radical generation through the Fenton reaction. Iron-mediated Fenton reaction facilitates hydroxyl radical generation in erythrocyte membranes [25]. Erythrocytes from SCD patients showed lower glutathione levels than normal erythrocytes and were susceptible to damage. Antioxidants, such as *N*-acetylcysteine, and vitamins C and E, reduced oxidative stress [6]. The accumulation of oxidative damage could explain the increased basal frequencies of chromosomal aberrations found in SCD patients when compared with controls. In this study, individual variation in the number of abnormal metaphases in untreated lymphocytes of SCD patients range from 0 to 24, while in the lymphocytes of healthy controls it varies from 0 to 5. Increased individual sensitivity in SCD patients can also be due to different DNA repair capacity. According to Wu et al. [9], mutagen sensitivity, determined by a chromosomal aberrations test in human lymphocytes exposed *in vitro* to known genotoxic agents, could be used as an indirect evaluation of DNA repair efficiency. The induction of chromosomal aberrations is one of the main pathways in the carcinogenesis process.

In addition to individual susceptibility, polymorphisms in genes responsible for maintaining genomic integrity are potential modifiers of disease risk. Genetic polymorphism might be involved in interindividual variations of DNA repair, associated with increased rates of DNA damage in human cells exposed to various genotoxic agents [26]. Information that chromosomal aberrations could be influenced by genetic polymorphism of DNA repair genes is emerging. The *XRCC1* gene (X-ray repair cross-complementing gene 1) is a key factor in the base excision repair and is required for an efficient repair of DNA single strand-breaks. *XRCC1* may also be involved in the repair of DNA damage induced by the antitumour agent cisplatin [27, 28].

Epidemiological studies have shown that the associations between polymorphisms in DNA repair genes result in reduced repair efficiency and increased risk of cancer. The irradiation-specific repair rates in human lymphocytes were significantly affected by the *XRCC1* polymorphism in exon 10, being lower in heterozygous 399Gln/Arg and variant 399Arg genotypes as compared with the wild-type 399Gln homozygotes [29]. In our previous study, the distribution of polymorphisms of the DNA repair gene *XRCC1* was determined in SCD patients and healthy controls. The results, in spite of the small number of patients available, indicated that the polymorphisms of the variant alleles *XRCC1* 399Gln and 194Trp were not statistically different between the subjects studied [30].

Further studies on polymorphisms of DNA repair genes in SCD patients will be able to elucidate the role of such biomarkers and their relationship with the increased chromosomal aberrations and sensitivity in SCD patients. The results obtained in the present study showed that more research is needed in order to elucidate the susceptibility to genomic instability of SCD patients. In conclusion, this investigation revealed that lymphocytes of SCD patients presented higher frequencies of basal chromosomal aberrations and were sensitive to *in vitro* cisplatin treatment when compared with healthy controls.

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Conflict of interest The authors declare that they have no conflict of interest related to the publication of this manuscript.

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