Protein Damage and Antioxidant Status Alterations Caused by Oxidative Injury in Chronic Myeloid Leukemia

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Objective: To evaluate the oxidative stress and antioxidant defense in patients with chronic myeloid leukemia.

Background: Chronic myeloid leukemia is a myeloproliferative disorder associated with a characteristic chromosomal translocation called the Philadelphia chromosome. Reactive oxygen species and other free radicals mediate phenotypic and genotypic changes leading from mutation to neoplasia in all cancers, including chronic myeloid leukemia. We evaluated patients with chronic myeloid leukemia by observing their oxidative status and antioxidant defense.

Methods: Using serum from 40 clinically diagnosed cases of chronic myeloid leukemia as well as 40 healthy controls, we measured the concentration of thiobarbituric acid, levels of protein carbonylation, total antioxidant status, catalase, superoxide dismutase, glutathione peroxidase, vitamins A and E, and the trace elements zinc, magnesium, and selenium.

Results: We found significantly increased levels of serum malonyldialdehyde and protein carbonyl in patients with chronic myeloid leukemia in comparison to healthy individuals, and significantly decreased levels of the antioxidants and micronutrients thiobarbituric acid, catalase, superoxide dismutase, glutathione peroxidase, vitamins A and E, zinc, magnesium, and selenium. These data suggest cellular damage occurring at the level of lipids and proteins.

Conclusion: These findings indicate a link between low levels of antioxidants and cellular damage in patients with chronic myeloid leukemia, supporting the idea that oxidative stress may play a role in the pathogenesis of chronic myeloid leukemia.

INTRODUCTION

Chronic myeloid leukemia (CML) was the first human malignancy shown to be consistently associated with a chromosomal abnormality, the Philadelphia chromosome (Nowell and Hungerford, 1960). At the gene level, the Philadelphia chromosome is the result of breaks on chromosomes 9 and 22 with a reciprocal translocation of the distal genetic material (Rowley, 1973). This translocation transposes the *c-abl* proto-oncogene from its normal location on chromosome 9 to a new position on chromosome 22, in proximity to the breakpoint cluster region *(bcr).* This forms the new hybrid BCR-ABL oncogene, an abnormal 8.5-kb RNA that encodes for a 210-kDa fusion protein, which, presumably through its increased tyrosine kinase activity, changes normal hematopoietic cells into CML cells (Nowell and Hungerford, 1960; Rowley, 1973; Shtivelman et al., 1985; Kurzrock et al., 2003).

Oxidative stress is a biochemical condition that occurs when intracellular antioxidants are unable to neutralize pro-oxidants such as reactive oxidant species (ROS). These ROS damage membranes, DNA, lipids, proteins, and carbohydrates, eventually causing cell injury and death. They also accelerate the development of age-related chronic diseases such as cancer and cardiovascular disease (Halliwell and Gutteridge, 1999; Galli et al., 2005; Frei, 1994). ROS contribute to several cellular functions, including the regulation of signal transduction, gene expression, and proliferation (Alvarez-Gonzalez, 1999). The biological effects of ROS vary widely in different cells, and include modulation of signaling pathways by directly altering the activity of protein kinases and protein phosphatases.

The objective of our study was to measure the intensity of oxidative damage at the cellular and molecular levels through measurements of lipid peroxidation and protein carbonyls. The study also aimed to investigate the oxidative profile in CML patients through the verification of main enzymatic antioxidant defenses (catalase [CAT] and superoxide dismutase [SOD]), nonenzymatic antioxidants (vitamins A and E), and essential micronutrients (selenium, magnesium, and zinc) in the blood of CML patients. Our study demonstrated that BCR/ABL translocation in CML may be associated with increased intracellular levels of ROS.

METHODS

Study Cohort

Our study subjects consisted of 40 clinically diagnosed CML patients who reported for treatment at the Banaras Hindu University Hospital Department of Medicine in Varanasi. Our controls consisted of 40 age- and sex-matched healthy volunteers of a socioeconomic status similar to that of the patients. None of the control subjects had any acute or chronic diseases such as diabetes, parasitosis, or immune dysfunction. All of the control

subjects had normal blood cell counts, including leukocytes, and were not undergoing any pharmacological therapy. Venous blood was collected from the patients and healthy volunteers in sterile tubes and the serum was separated for various biochemical and hematological investigations.

Presence of the characteristic Philadelphia chromosome was established by standard methods at the Chromosomal & Molecular Genetic Diagnostic Unit, Department of Zoology, Faculty of Science, Banaras Hindu University, Varanasi, India.

Written informed consent for the study was obtained from each patient and healthy subject with the approval of the ethical committee of the Institute of Medical Sciences, Banaras Hindu University.

Reagents
All chemicals and reagents used in the study were of analytical grade from Sigma-Aldrich Co. (USA).

Evaluation of Oxidative Stress, Protein Damage, and Antioxidant Status
Oxidative status of the serum of patients and controls was assessed by measuring serum lipid peroxidation levels by the thiobarbituric acid (TBARS) method (Buegeand Aust, 1978). Protein damage was assessed by measuring serum levels of protein carbonyls by a spectrophotometric detection of the reaction of 2,4-dinitrophenyl hydrazine (DNPH) with protein carbonyl to form protein hydrazine (Levine et al., 1990). Total protein content was measured using a colorimetric kit based on the biuret method. Total antioxidant status in serum was determined by a spectrophotometric method using the Randox assay kit (Miller et al., 1993).

Enzymatic Antioxidants Assay
SOD activity was measured based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol (Marklund and Marklund, 1974). Glutathione (GSH) determination was performed using Ellman's reagent (Beutler et al., 1963). CAT activity was assayed by the decomposition of hydrogen peroxide (Aebi, 1983).

Assay of Nonenzymatic Antioxidants
Vitamin A and E levels were measured by spectrophotometric standard methods (Bessey et al., 1946; Paterson and Wiggins, 1954; Quaife and Dju, 1949). Selenium levels were measured by the spectrophotometric method using azo dye (Khanna et al., 2010). Magnesium levels were measured using a colorimetric kit based on the calamagite method (Gindler and Heth, 1971). Zinc levels were also measured using a colorimetric kit (Abe et al., 1989; Makino, 1999).

Statistical Analysis
Statistical analysis was performed by SPSS 16.0. All data were expressed as the mean ± standard deviation. Statistical significance was evaluated by independent t-test. All statistical analyses were two-tailed and a value of $p < 0.05$ was considered statistically significant.

RESULTS

The characteristic features of the patients and controls are given in Table 1. The mean age of the CML patients was 36.7 ± 11.9 years. The mean leukocyte count was 80,410 per mm^3. The mean spleen size measured by ultrasonography of patients was 21.1 ± 3.5 cm, and was found to be significantly larger than controls. The Philadelphia chromosome was present in all 40 patients with CML, which confirms the BCR/ABL translocation.

Lipid peroxidation was evaluated by measuring the levels of malondialdehyde (MDA) in terms of TBARS, and protein damage was assessed by measuring protein carbonyls (PC), which are products of irreversible nonenzymatic protein oxidation. The mean level of MDA was significantly higher in CML cases as compared to control cases. The elevated levels of MDA in patients are a marker of increased lipid peroxidation or oxidative stress. The levels of protein carbonyl were also elevated significantly in comparison to the normal controls, as shown in Table 2. On the other hand, the levels of serum total antioxidant status (TAS) in CML cases were lower than in the

TABLE 1. CHARACTERISTICS OF CML PATIENTS AND CONTROL CASES

Characteristics	Control	Cases
Male	23	23
Female	17	17
Mean Age (year)	37.3 ± 11.6	36.7 ± 11.9
Mean Duration of Symptoms (months)	N/A	3.4 ± 1.9
Mean Spleen Size (cm)	10 ± 0.5	21.1 ± 3.5
Mean Hemoglobin (gm/dl)	12.5 ± 1.2	11.14 ± 1.68
Mean Leukocyte Count (per mm^3)	7,600	80,410
Mean Creatinine (mg/dl)	0.90 ± 0.19	0.85 ± 0.12
Ph Chromosomes(+)	N/A	40 (100%)

TABLE 2. LEVELS OF OXIDATIVE STRESS AND PROTEIN CARBONYLS IN CML PATIENTS AND CONTROL CASES

Parameters	Control (n=40)	Cases (n=40)	p-value*
Malonaldehyde (mmol/l)	0.02 ± 0.18	3.91 ± 0.203	0.002
Protein Carbonyl (nmol/mg of protein)	1.32 ± 0.76	1.79 ± 0.13	0.003

Values are given as mean ± SEM. *Statistical analysis was done by independent Student's t-test. All statistical analyses were two-tailed and a value of $p < 0.05$ was considered statistically significant.

TABLE 3. ENZYMATIC ANTIOXIDANT, NONENZYMATIC ANTIOXIDANT, AND TRACE-ELEMENT ANTIOXIDANT LEVELS IN CML PATIENTS AND CONTROL CASES

Parameters	Control (n=40)	Cases (n=40)	p-value*
TAS (mmol/l)	1.81 ± 0.06	1.49 ± 0.09	0.007
Enzymatic Antioxidants			
Catalase (units/ml)	33.32 ± 1.49	27.43 ± 1.43	0.006
SOD (units/ml)	149.79 ± 6.26	87.62 ± 6.59	0.0001
GPx (units/dl of hemolysate)	51.25 ± 2.96	38.80 ± 3.66	0.01
Nonenzymatic Antioxidants			
Vitamin A (µg/dl)	45.84 ±1.63	38.24 ± 2.52	0.01
Vitamin E (mg/l)	13.09 ± 0.51	10.96 ± 0.71	0.01
Trace Elements			
Zinc (µg/dl)	87.69 ± 3.67	75.96 ± 2.50	0.01
Magnesium (mg/l)	31.05 ± 0.97	27.44 ± 0.072	0.004
Selenium (µg/l)	38.90 ± 4.67	25.52 ± 0.795	0.006

Values are given as mean ± SEM. *Statistical analysis was done by independent Student *t*-test.
All statistical analyses were two-tailed and a value of p <0.05 was considered statistically significant.

control group. The enzymatic antioxidants SOD, reduced GPx, and CAT likewise were found to be lower in patients relative to controls, as shown in Table 3.

The levels of vitamins A and E were significantly lower in CML cases compared to controls, although values for both the CML cases and the controls were within normal reference intervals. Serum levels of the important trace elements selenium, magnesium, and zinc were significantly lower in CML patients as compared to controls.

These observations suggest that oxidative stress can release catalytic trace elements from tissues. This may initiate a vicious cycle of free-radical generation, causing imbalance in enzymatic and nonenzymatic antioxidant defense mechanisms.

DISCUSSION

Free radicals are implicated in the pathogenesis of tissue injury in many human diseases. The disturbance of the pro-oxidant/antioxidant balance, resulting from increased free-radical production, antioxidant enzyme inactivation, and excessive antioxidant consumption, is the causative factor in oxidative damage. These free radicals set up a chain reaction that changes cell permeability, denatures proteins, alters enzyme activity, decreases neurotransmitter transmission, causes breakage of DNA, and degrades structural proteins. Thus, free radicals are directly responsible for causing genetic mutations and carcinogenesis. The body has multiple defense mechanisms against free radical–mediated tissue damage. Among them, antioxidants are the primary lines of defense. Under normal circumstances, there is a critical balance between pro-oxidizing and antioxidizing forces. Antioxidants interact with free radicals in several ways, controlling damaging chain reactions. Examples of antioxidants include vitamins A and E, SOD, CAT, GPx, bilirubin, and uric acid (Shtivelman et al., 1985).

Cancer cells under sustained ROS stress tend to heavily utilize adaptation mechanisms and may exhaust cellular ROS-buffering capacity. A relationship between leukemia and oxidative stress has been observed. Leukemic cells produce higher amounts of ROS than nonleukemic cells because the former are experiencing sustained oxidative blockade (Al-Gayyar et al., 2007).

It has been suggested that ROS exert their cytotoxic effect by carbonylating proteins, leading to a loss of protein function. Protein dysfunction is considered to be a widespread marker of severe oxidative stress, damage, and disease. ROS also cause peroxidation of membrane phospholipids, changing the permeability of the cellular membrane, increasing membrane fluidity and rigidity, and in some cases increasing the risk of membrane rupture. Our findings are in broad agreement with an earlier study carried out on CML patients that suggests that accumulation of ROS may result in significantly increased lipid peroxidation and protein damage at the cellular and molecular levels (Singh et al., 2009; Tandon et al.et al., 2002).

Our study also found that CML patients were lacking in key antioxidant enzymes and essential micronutrients, which act as the first line of defense against free radicals and ROS. SOD efficiently removes the superoxide radical by converting it to H_2O_2, which in turn is metabolized by GPx and CAT to molecular oxygen and water. With reduced antioxidant enzyme activities, the body is unable to dispose of the superoxide radical and H_2O_2, which can attack biological molecules, leading to extensive tissue damage. Divalent ions of transition metals can

promote lipid peroxidation in vitro, and much attention is currently focused on lipid peroxidation in the pathogenesis of metal toxicity. The mechanism of pathogenesis could be mediated by direct effects of trace elements such as zinc, magnesium, and selenium on the formation of hydroxyl free radicals from hydrogen peroxide and superoxide via the Fenton and Haber-Weiss reactions (Zuo et al., 2006; Dalle-Donne et al., 2006; Prasad, 1991).

Many antioxidants are micronutrients or depend on micronutrients for their activity. These include vitamins A and E as well as trace elements such as zinc, magnesium, and selenium, which act as cofactors for antioxidant enzymes. In our study, we observed that the activity of SOD, CAT, GPx, trace elements, and vitamins was significantly decreased in CML patients as compared to controls. These findings suggest that alterations in antioxidant defenses, which normally protect biological tissues from ROS, may be related to leukemia progression. Alternatively, it is possible that the cancer process itself causes the observed dysfunction of the antioxidant system.

In conclusion, although reactive species are well recognized for playing a dual role—deleterious and beneficial—excessive accumulation of ROS contributes to antioxidant depletion and dysfunction. Genomic instability is manifested by the accumulation of chromosomal aberrations and mutations leading to the malignant progression of disease. Research evidence suggests that abnormalities in protein translation, modification (mainly phosphorylation), and degradation of DNA play critical roles in the initiation, development, and induction of the BCR/ABL transformation. Although these modifications can be removed efficiently by DNA repair mechanisms, a persistent increase in ROS could lead to an accumulation of mutations (Spencer et al., 1995). Increased levels of oxidative damage and decreased levels of the antioxidant system in the CML patients in the present study suggest that BCR/ABL transformed leukemia cells display higher levels of oxidative stress. In addition, it is possible that the BCR/ABL transformation affects the protein levels or enzymatic activity of one or more enzymes that regulate ROS.

Thus, there may be a link between decreased antioxidant levels and increased cellular alterations due to oxidative damage, supporting the possibility of the persistence of oxidative stress in CML. More studies are necessary to confirm whether these alterations are the cause or the consequence of carcinogenesis.

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