SUPEROXIDE DISMUTASE, CATALASE AND GLUTATHIONE PEROXIDASE ACTIVITIES IN LYMPHOCITES AND IN SERUM OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

Ivanka Zelen1, Predrag Djurdjevic2, Suzana Popovic3, Szezana Radivojic4, Marina Stojanovic1, Dejan Baskic2 and Nebojsa Arsenijevic3

1Department of Biochemistry, School of Medicine, University of Kragujevac, 2Department of Pathophysiology, School of Medicine, University of Kragujevac, 3Department of Microbiology and Immunology, School of Medicine, University of Kragujevac, 4Clinical Center Kragujevac

ABSTRACT
Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by clonal expansion of relatively mature B-lymphocytes with high percentage of cells being arrested in the nonproliferative G0/G1 cell cycle phase. The main feature of the disease is an accumulation of malignant B-cells with low proliferation activity that escape the fate of programmed cell death by a variety of mechanisms. There are many evidences for oxidative stress involvement in both initiation and promotion of multistage carcinogenesis. Overproduction of reactive oxygen intermediates above functional capability of cellular antioxidants may result in the instability of important macromolecules, and it represents the molecular basis of many diseases including inflammation processes, cardiovascular alterations and cancer. The measurement of anti-oxidents is one way of analyzing the involvement of oxidative stress in the course of CLL and other diseases.

In the present study we investigated the activities of antioxidant enzymes in both lymphocytes and plasma of CLL patients of early and advanced stage of disease and compared them to those of healthy subjects of similar age. The aim was to determine their predictive, i.e. prognostic role for disease outcome. Such studies may provide clues on the mechanism through which oxidative stress may influence tumor growth and its clinical progression.

Our result show that SOD activity in lymphocytes was significantly reduced in both CLL-groups: the decrease is about 40% in early stage and more than 60% in advanced stage. Catalase activity was decreased more than 35% in early stage and more than 65% in advanced stage, while Gpx activity was decreased about 50% in early stage and about 80% in advanced stage. Slightly increased plasma SOD and Gpx activities were not significantly different from the controls, while plasma CAT activity was increased more than 8% compared with normal, healthy subject. Therefore, the analysis of antioxidant enzymes activities of CLL patients, may be applied as a good predictive factor for the disease outcome.

Key words: chronic lymphocytic leukemia, oxidative stress, antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, lymphocytes, plasma

INTRODUCTION
Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by clonal expansion of relatively mature B-lymphocytes with high percentage of cells being arrested in the nonproliferative G0/G1 cell cycle phase. The main feature of the disease is an accumulation of malignant B-cells with low proliferation activity that escape the fate of programmed cell death (PCD or apoptosis) by a variety of mechanisms (1). The major causes of disease are not exactly known nor there is a detailed understanding about how the elusive origin may relate to clinical expression, basic biological mechanisms or pathogenesis (2). A variety of chromosomal aberrations have been described as possible causes of the CLL. However, it is unknown which, if any, of these chromosomal alterations is the initial transforming event in CLL. It has been proposed that molecular defects such as oxidative stress, although by yet unknown mechanism, may induce the constitutive activation of several transmembrane signaling pathways that regulate the differentiation, cell cycle progression and apoptosis of B-cells.

There are many evidences for oxidative stress involvement in both initiation and promotion of multistage carcinogenesis (3). Overproduction of reactive oxygen...
intermediates above functional capability of cellular antioxidants may result in the instability of cellular macromolecules, and it represents the molecular basis of many diseases including inflammation processes, cardiovascular alterations and cancer (4). Reactive oxygen species (ROS) are chemically active molecules generated endogenously during various cellular metabolic activities. In mammalian cells, mitochondria are the major intracellular source of ROS generation (4). The overproduction of ROS can result in detrimental cellular damage including lipid peroxidation, DNA adduct formation, protein oxidation and enzyme inactivation that ultimately lead to cell death. Diverse cellular ROS, such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH · ), have long been held as the harmful by-products of life of an aerobic environment. ROS are highly reactive and potentially toxic compounds capable of modifying and damaging several types of cellular macromolecules including lipids, proteins and DNA, ultimately leading to cytotoxicity and mutagenesis (4). Therefore, cells have evolved elaborate defense systems to counteract the toxic effect of ROS. These include both nonenzymatic (glutathione, pyridine nucleotides, ascorbate, retinoic acid, thioredoxin and tocopherol) and enzymatic (such as superoxide dismutase, catalase, glutathione peroxidase and peroxiredoxin) pathways that limit the rate of oxidation and thereby protect cells from oxidative stress (5, 6). Notwithstanding, evidence is emerging that ROS also act as signals or mediators in many cellular processes, such as cell proliferation, differentiation, apoptosis and senescence (7). The redox environment of a cell may alter the balance between apoptosis and mitosis by affecting gene expression and enzyme activity (8). Consequently, cellular redox state is increasingly accepted key mediator of multiple metabolic, signaling and transcriptional pathways essential for normal cellular function, cell survival and apoptosis (9).

The measurement of anti-oxidants is one way of analyzing the involvement of oxidative stress in the course of CLL and other diseases. Anti-oxidant defenses comprise enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). Superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalyzes the dismutation of the O$_2^-$ by successive oxidation and reduction of the transition metal ion at the active site in a ping-pong type mechanism with remarkably high reaction rates (10).

In humans, there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (11). EC-SOD is a secretory, tetrameric, Cu and Zn containing glycoprotein found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph and sinovial fluid (12). CAT is a tetrameric haemin-enzyme that catalyzes the reaction with H$_2$O$_2$ to form 2H$_2$O and O$_2$. It has been suggested that the action of CAT is confined in peroxisomes, and that the enzyme does not contribute to the regulation of cellular redox potential (13). However, the enzyme may also be released into the extracellular environment where it has the potential to function as a potent antioxidant and thereby regulate cell survival (14). Glutathione peroxidase (GPx) is a selenoprotein, which reduces lipid or nonlipid hydroperoxides as well as H$_2$O$_2$ while oxidizing glutathione, thereby protecting mammalian cells against oxidative damage. There are at least five GPx isoenzymes found in mammals (15). Although GPx shares H$_2$O$_2$ as a substrate with CAT, it alone can react effectively with lipid and other organic hydroperoxides. The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas CAT becomes more significant in protecting against severe oxidant stress (16).

There are some evidences to suggest that the antioxidant systems allow cells to undergo normal differentiation (17). Alterations in the enzymatic system, in particular, are believed to play a central role in this process. Considering all previous findings, in the present study we investigated the activities of antioxidant enzymes in lymphocytes and in plasma of both CLL patients and healthy subjects in order to gain a comprehensive view about the status of their antioxidant defenses machinery.

**MATERIAL AND METHODS**

**Patients and controls**

Forty-six B-CLL patients were included in the study (22 males, 14 females, range 51–82 years: mean ± SD, 66.5 ± 6.9 years) and 32 healthy subjects were used as a control group (17 males, 15 females, range 49–80 years: mean ± SD, 65.3 ± 7.6 years). B-CLL was diagnosed according to standard clinical and laboratory criteria. CLL patients were staged according to the Rai’s classification (18), 10 were in stage 0, 15 in stage I, 13 in stage II and 8 in stage III. CLL patients were divided into two groups, early (0 + I; 25 patients, range 54 - 82 years: mean ± SD, 68.7 ± 9.2 years) and advanced stages (II + III; 21 patients, range 51 - 78 years: mean ± SD, 64.6 ± 3.5 years). Most of the patients were newly diagnosed cases. Early diagnosed patients did not receive any antileukemic therapy at least six months prior to the investigation. In order to prevent any possible alteration and influence on the outcome of the study, patients having either infectious disease or other conditions were excluded. All subjects (both patients and controls) were non-smokers who did not receive any systemic or topical treatment/drug prior to initiation of investigation that could have affected defense mechanism of antioxidant. None of the patients and controls had alcohol abuse problems nor performed regular exercise other than daily activities. The local Ethics Committee approved the study and all subjects gave the written informed consent prior to the study, according to the Declaration of Helsinki.

**Blood sampling**

All blood samples were obtained in the morning at the same hour, following 12 hours of fasting, and collected in polystyrene tubes. Heparinized venous blood (10 ml)
was collected in vacutainers and centrifuged at 3000xg for 10 minutes to separate plasma and cells. The plasma aliquots were stored at -80°C until analysis. Hemolyzed samples were excluded. Mononuclear cells/lymphocytes/ were isolated by lymphoprep centrifugation as described earlier (Lymphoprep, Nicomed Pharma AS, Oslo, Norway) (19). Shortly, lymphocytes suspension with known cells number was prepared in deionized water following three washes in RPMI – 1640 medium. Samples were stored at -80°C until their analysis. Just before analysis, lymphocytes suspension was lysed three times by freezing – thawing cycles.

**Methods**

**Determination of antioxidant enzymes.** Cu-Zn SOD and GPx activities were estimated using Ransod and Ransel kits, supplied by Randox Laboratories, Ardmore, Northern Ireland, UK. The samples were processed differently for the two enzymes according to the instructions of the manufacturer. Method of Goth (20) was used to spectrophotometrically estimate catalase (CAT) activity in the samples. **Determination of Cu-ZnSOD.** This assay employs xanthine and xanthine oxidase to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to form red formazan dye. Superoxide dismutase (SOD) activity is then measured by the degree of inhibition of this reaction. One unit of SOD activity is defined as the amount of enzyme that causes a 50% inhibition of the rate of reduction of INT observed in the blank. Reagent blank was assayed with 0.01M phosphate buffer pH 7.0. The standard curve was prepared using serial dilutions of 4.0 U/mL SOD. SOD activity was then measured at 37°C at 505 nm (Olympus AU 600) and expressed as specific enzyme activity (U/g) for serum samples and as enzyme activity per number of cells (U/20 x 106 Ly) for lymphocytes lysates.

**Determination of GPx.** The assay was performed according to the method of Paglia and Valentine (21). GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and xantine oxidase to generate superoxide anion - which reacts with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to form red formazan dye. Superoxide dismutase (SOD) activity is then measured by the degree of inhibition of this reaction. One unit of SOD activity is defined as the amount of enzyme that causes a 50% inhibition of the rate of reduction of INT observed in the blank. Reagent blank was assayed with 0.01M phosphate buffer pH 7.0. The standard curve was prepared using serial dilutions of 4.0 U/mL SOD. SOD activity was then measured at 37°C at 505 nm (Olympus AU 600) and expressed as specific enzyme activity (U/g) for serum samples and as enzyme activity per number of cells (U/20 x 106 Ly) for lymphocytes lysates.

**Determination of CAT activity.** Method of Goth (20) was used to spectrophotometrically estimate catalase (CAT) activity in the samples. Determination of CAT activity is based on the ability of CAT to break two molecules of hydrogen peroxide into two molecules of water and oxygen:

\[ \text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} \text{O}_2 + 2\text{H}_2\text{O} \]

**RESULTS**

**Table 1.** Antioxidant enzyme activities in lymphocytes of CLL patients and control subjects.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>controls</th>
<th>early stage</th>
<th>advanced stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/20x106 Ly)</td>
<td>1.76 ± 0.78</td>
<td>1.22 ± 0.51a</td>
<td>0.69 ± 0.41c</td>
</tr>
<tr>
<td>CAT (U/1x106 Ly)</td>
<td>0.66 ± 0.09</td>
<td>0.43 ± 0.13a</td>
<td>0.23 ± 0.15b</td>
</tr>
<tr>
<td>GPx (U/1x106 Ly)</td>
<td>5.46 ± 1.22</td>
<td>2.78 ± 2.16b</td>
<td>1.98 ± 1.01c</td>
</tr>
</tbody>
</table>

All values are represented by mean ± SD comparing CLL with control values

<table>
<thead>
<tr>
<th>enzyme</th>
<th>controls</th>
<th>early stage</th>
<th>advanced stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/g)</td>
<td>93.83 ± 11.88</td>
<td>100.29 ± 14.36</td>
<td>98.83 ± 11.32</td>
</tr>
<tr>
<td>CAT (kU/g)</td>
<td>0.295 ± 0.18</td>
<td>0.33 ± 0.22</td>
<td>0.554 ± 0.32b</td>
</tr>
<tr>
<td>GPx (kU/g)</td>
<td>9.68 ± 2.37</td>
<td>9.77 ± 2.64</td>
<td>9.94 ± 3.09</td>
</tr>
</tbody>
</table>

All values are represented by mean ± SD comparing CLL with control values

**Table 2.** Antioxidant enzyme activities in plasma of CLL patients and control subjects.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>controls</th>
<th>early stage</th>
<th>advanced stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/g)</td>
<td>93.83 ± 11.88</td>
<td>100.29 ± 14.36</td>
<td>98.83 ± 11.32</td>
</tr>
<tr>
<td>CAT (kU/g)</td>
<td>0.295 ± 0.18</td>
<td>0.33 ± 0.22</td>
<td>0.554 ± 0.32b</td>
</tr>
<tr>
<td>GPx (kU/g)</td>
<td>9.68 ± 2.37</td>
<td>9.77 ± 2.64</td>
<td>9.94 ± 3.09</td>
</tr>
</tbody>
</table>
To understand the overall status of antioxidant enzyme machinery in both CLL patients and control subjects, we studied the activities of antioxidant enzymes in both lymphocytes and plasma from both groups. The results obtained in patients and control group were summarized in Table 1 and Table 2, respectively. As seen from Table 1, there were important changes in the activities of antioxidant enzyme in lymphocytes comparing CLL patients with controls. SOD activity was significantly reduced in both CLL-groups: the decrease is about 40% in early stage and more than 60% in advanced stage. Catalase activity was decreased more than 35% in early stage and more than 65% in advanced stage, while Gpx activity was decreased about 50% in early stage and about 80% in advanced stage. The results for plasma measurement of antioxidant enzyme activities were shown in Table 2. Slightly increased plasma SOD and Gpx activities in both stages were not significantly different from the controls, while plasma CAT activity was increased in advanced CLL stage more than 85% compared to normal, healthy subject.

DISCUSSION

A disturbance of oxidative metabolism is a common feature of transformed tumor cells (23). Both the alterations of antioxidant enzymes and increases in the production of oxygen reactive species have been described to contribute to tumorigenesis (24). As a result, higher rates of lipid peroxidation (25) and different forms of DNA base lesions (26) have been found in the majority of neoplastic tissues. Thus, the higher susceptibility of tumor tissues to oxidative stress as compared to the normal cells is supported by the increase of lipid peroxidation, DNA damage and by the decrease of antioxidant enzyme activities (27). Although some possible mechanisms through which oxidative stress exerts a regulatory role in tumor growth and progression, including genomic instability, oncogene activation (28) and angiogenesis (29) are known, several important questions remain unanswered. It is not clearly known whether both oxidative stress and tumor result from increased oxidative production or from failure of antioxidant systems (23, 30, 31). Although important changes in cellular redox homeostasis have been documented during tumor growth in experimental models, such variations have not yet been demonstrated in humans. Most of the difficulties encountered in these studies are related to the complexity of the biochemical pathways that regulate the cellular redox balance (32). A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter the redox balance in the cell. The data reported in the literature on antioxidant molecule and enzymes in different human cancer types are controversial.

The present study investigated the SOD, CAT and Gpx activities in both lymphocytes and plasma of CLL patients in order to obtain a comprehensive view of the cancer patient antioxidant enzyme machinery. The aim was to determine their predictive, i.e. prognostic role for disease outcome. Such studies may provide clues on the mechanism through which oxidative stress may influence tumor growth and its clinical progression. The result of the present study showed that activities of antioxidant enzymes of interest were significantly lower in lymphocytes of CLL patients while there was an important increase in plasma CAT activity. Also, there is an increasing trend of activities of SOD and of Gpx in plasma, but of no statistical importance.

The levels of all antioxidant enzymes investigated were lower in lymphocytes in both CLL groups compared to control subjects. This result is in accordance with the observed decrease of two enzymes, SOD and CAT, as demonstrated earlier in CLL (27), in children with acute lymphocytic leukemia (26), in human colorectal carcinoma and other tumors (33). However, the decreased activity of Gpx in lymphocytes demonstrated in our results is in contrast to the previous research (27, 34). The observed decrease in enzyme activities in lymphocytes was found in both early and advanced stages of disease. Therefore, the decrease in activities is progressively enhanced by the disease stage. Thus, the lower enzyme activity is directly proportional to the increased stage of the disease. Decreased levels of SOD, CAT and Gpx may cause the accumulation of superoxide anions and $\text{H}_2\text{O}_2$ in tumor cells (33, 35). Moreover, lipid peroxidation (LP), as one of the main effect of oxidative stress in cells, induces the increase of MDA concentrations. In addition, LP can be generated either due to an excessive action of pro-oxidants or due to the reduced functional activity of antioxidant defense machinery (24). Due to the decreased activity of antioxidant enzymes in lymphocytes, specifically Gpx, there is an increase in LP in malignant cells leading to the rise of the MDA concentrations in plasma, as already documented in the our previous work (35). It has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent due to its high cytotoxicity and inhibitory action on protective enzymes (36, 37). Therefore, the possible explanation for the decrease of the activities of SOD, CAT and Gpx in lymphocytes of CLL patients could be due to the inhibitory effect of MDA on protective enzymes. On the other hand, in the case of severe oxidative stress there is an intensive production of ROS that overcome antioxidant capacity of the cells. This phenomena consequently leads to the breakdown of the all antioxidative defense machineries that is manifested as a decrease of the activities of protective enzymes, including SOD, CAT, Gpx. Alternatively, it is possible that the antioxidant system is impaired as a consequence of an abnormality in the antioxidative metabolism due to the cancer process (32).

To date, no previous studies have ever demonstrated the activities of the antioxidant enzymes both in lymphocytes and plasma of CLL patients. Thus, in order to obtain a comprehensive view of the antioxidant enzyme machinery in CLL patients, besides the usual measurement of antioxidant enzyme activities in lymphocytes, we have also examined their activities (SOD, CAT and Gpx) in plasma of CLL patients of both early and advanced stage and compared them to healthy subjects of similar age.
The results from our study demonstrated the increase in the activities of antioxidant enzymes in plasma, mainly activity of CAT which was significantly elevated. In addition, there was an increasing trend of activities of SOD and of GPx in plasma, but of no statistical importance. Our findings, however, are different from some previous reports. Results from Bakan (38) show a decrease in GSH concentration, GPx and SOD activities in serum of CLL patients. One of the possible explanations for the elevated activities of the antioxidant enzymes in plasma may lay in the fact that a few particular enzymes could normally be present in plasma, such as CAT, even though it is primarily intracellular enzymes. (39). Moreover, it is very well known about the extracellular isoform of SOD (15). On the other hand, it could also be possible that antioxidant enzymes in plasma, such as SOD and CAT, may originate from different type of cells than lymphocytes, including endothelial cells and other blood cells (40–43).

Our understanding of endogenous mechanisms of carcinogenesis by oxidative processes had advanced greatly in the last decade, yet the description of the molecular action of carcinogenesis must be further elucidated to prevent and treat neoplastic disease properly (44). Therefore, the analysis of antioxidant enzymes activities in lymphocytes of CLL patients, including SOD, CAT, and GPx, may be applied as a good predictive factor for the disease outcome. However, determination of protective enzymes activities in plasma of CLL patients is a poor prognostic factor, since the presence of these enzymes in plasma may originate from other cell types than lymphocytes. Furthermore, the identification of an adequate oxidative markers for tumor cell metabolism may be useful for early diagnosis as well as for assessment of tumor progression (45).

ACKNOWLEDGMENTS

The study was fully supported by School of Medicine, University of Kragujevac.

REFERENCES


