OXIDATIVE STRESS MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Predrag Djurdjević1, Ivanka Zeleni2, Petar Ristic3, Ivan Jovanović1, Vladimir Jakovljević1, Dejan Baskić1, Suzana Popović4 and Nebojša Arsenijević2
1Department of Pathophysiology, Faculty of Medicine, University of Kragujevac, 2Department of Biochemistry, Faculty of Medicine, University of Kragujevac, 3Department of Microbiology and Immunology, Faculty of Medicine, University of Kragujevac, 4Department of Physiology, Faculty of Medicine, University of Kragujevac, 5Public Health Institute, Kragujevac

MARKERI OKSIDATIVNOG STRESA U HRONIČNOJ LIMFOCITNOJ LEUKEMIJI

Predrag Djurdjević1, Ivanka Zeleni2, Petar Ristic3, Ivan Jovanović1, Vladimir Jakovljević1, Dejan Baskić1, Suzana Popović4 and Nebojša Arsenijević2
1Katedra za patofiziologiju, Medicinski fakultet, Univerzitet u Kragujevcu, 2Katedra za biohemiju, Medicinski fakultet, Univerzitet u Kragujevcu, 3Katedra za microbiologiju i imunologiju, Medicinski fakultet, Univerzitet u Kragujevcu, 4Katedra za fiziologiju, Medicinski fakultet, Univerzitet u Kragujevcu, 5Institut za zaštitu zdravlja, Kragujevac

Received/Primljen: 03.02.2006. Accepted/Prhvaćen: 09.06.2006.

ABSTRACT

Chronic lymphocytic leukemia is characterized by the progressive accumulation of small immature lymphocytes which do not proliferate and that remain predominantly (more than 95%) in the G0 phase of the cell cycle. Expansion of malignant cell clone appears to be due to an underlying defect in its ability to undergo apoptosis. One of the potential mechanisms of defective apoptosis could be irregular oxidative stress. The goal of our investigation was to determine the plasma level of nitric oxide, superoxide anion and malondialdehyde in patients with chronic lymphocytic leukemia as markers of oxidative stress. Thirty patients with untreated chronic lymphocytic leukemia in the A stage of the disease classified by Binet and thirty healthy volunteers were examined. Nitric oxide (its stable metabolites, nitrite (NO2−) and nitrate (NO3−), superoxide anion and malondialdehyde were measured by spectrophotometry in plasma of both investigated groups which were obtained from heparinized whole blood after centrifugation.

Our results showed that the plasma levels of nitrate/nitrite (32.21 ± 7.20 vs 28.42 ± 7.27 nmol/ml, p < 0.01), superoxide anion (10.34 ± 9.40 vs 8.52 ± 6.29 nmol/ml, p < 0.01) and malondialdehyde (2.76 ± 1.61 vs 1.37 ± 0.90 nmol/ml, p < 0.01) were increased in patients with chronic lymphocytic leukemia than in the group of healthy volunteers.

These data suggest that there is more intensive oxidative stress in patients with chronic lymphocytic leukemia. It could be one of the potential mechanisms in the pathogenesis of irregular apoptosis of malignant lymphocytes and it could take part in the genesis of chronic lymphocytic leukemia.

Key words: chronic lymphocytic leukemia, nitric oxide, superoxide anion, malondialdehyde.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is predominantly clonal B-cell neoplasm of small, resting, long-lived B-cells. Despite recent advances in understanding of genetics (1), biology (2), clinical behavior (3) and treatment (4), CLL has not been cured yet and its progression and outcome are highly unpredictable. CLL cells co-express low levels of surface membrane molecules CD5, CD23 and weak CD22 and surface membrane immunoglobulin (5). Expansion of these malignant cells leads to the accumulation in the peripheral blood, bone marrow and many tissues. These cells are functionally defective and immunologically distinct from normal B cells (6). The clinical course of B-CLL is highly heterogeneous, ranging from less than two years in symptomatic patients with the advanced disease to more than twenty years for patients with an early stage and non-progressive disease (7). Although the pathogenesis of B-CLL has not been fully elucidated, the progressive increase of lymphocyte count coupled with the very low proportion of proliferating cells has led to the notion that B-CLL may be determined by defective apoptosis (8). Precise mechanisms underlying apoptosis have still remained unknown. Dysregulation of the p-53, c-myc and bcl-2 oncogenes can be the cause of defective apoptosis in B-CLL and even though the B-CLL cell molecular alterations involving different oncogenes and tumor suppressor genes have been established, the role of oxidative stress in the pathogenesis of this disease is poorly understood and it is a matter of interest (9).

Oxidative stress is a well-known phenomenon in the body which plays an important role in the pathogenesis of various diseases and syndromes (10). Reactive oxygen species (ROS) consist of superoxide anion (O2−),
hydrogen peroxide (H₂O₂) and hydroxyl radical (OH⁻) which are generated by cells in some physiological and pathological conditions. Any rearrangement between pro-oxidants and antioxidants in which pro-oxidants prevail, is known as oxidative stress. ROS can react with all macromolecules, particularly with polyunsaturated fatty acid on the cell membrane and this process is named lipid peroxidation. The secondary product of this process is malondialdehyde which is a useful indicator for this reaction (11). After the beginning of an initial reaction with ROS a continuing chain reaction starts followed by cell injury (12). One of the most abundant free radicals in the body is nitric oxide (NO) and its excess production can cause inhibition of mitochondrial respiratory enzymes (13). All these radicals can interact with the nucleic acid, proteins and lipids causing cellular dysfunction and even death. Interaction with the nucleic acids can lead to genetic disturbances which may cause irregular B lymphocyte apoptosis and that can be a critical event in the pathogenesis of B-CLL.

According to these facts, the aim of our investigation was to determine the plasma level of nitric oxide species (nitrates and nitrites), superoxide anion, hydrogen peroxide and malondialdehyde in CLL patients as parameters of oxidative stress.

MATERIAL AND METHODS

Patients and controls

Thirty B-CLL patients (13 females and 17 males, median age 67.8 years, range 54-82 years) and thirty healthy control subjects (15 females and 15 males, median age 63.8 years, range 49-77 years) were included in the study. B-CLL was diagnosed according to standard clinical and laboratory criteria. Only patients with the stage A, according to Binet’s clinical classification of the disease were studied. None of the patients had been treated with any kind of cytoreductive therapy at least 6 months at the time samples were obtained. Patients with infectious diseases or other conditions which could alter results and consequently influence the outcomes of our study were excluded.

The controls were healthy volunteers without known acute and chronic diseases. Exclusion criteria were positive parameters of the systemic inflammation (high erythrocyte sedimentation rate, high serum fibrinogen level, high serum C-reactive protein level) due to other etiologies or positive anamnestic data for other illnesses (e.g. autoimmune diseases, acute and chronic infections, systemic and local inflammations etc) that might have influenced the investigated parameters.

All subjects (patients and controls) were non-smokers, none of whom received any systemic and topical treatment such as corticosteroids, cyclosporine A and similar drugs which might affect oxidative stress parameters within six months prior to initiation of the investigation. None of the patients and controls had alcohol abuse problems and none of them performed regular exercise other than daily activities. The local Ethics Committee approved the study and prior to initiation, the written informed consent was obtained from all subjects according to the Declaration of Helsinki.

Methods

All blood samples were obtained after 12 hours of fasting, in the morning, at the same time and were collected in polystyrene tubes. Nitric oxide, superoxide anion and malondialdehyde were investigated in the plasma samples. The plasma samples of both groups were stored at -20°C until assayed.

NO determination

Before testing, the plasma samples were deproteinized by using acid solution. In 1500μL tubes, 100 μL of 3M perchloric acid, 400 μL of 20mM EDTA and 200 μL of serum were added. Extracts were incubated in ice for 20 minutes with occasional mixing and then centrifugated at 15000rpm for 5 minutes. The supernatants were removed into other tubes and 120μL 2M potassium-carbonate was added to neutralize the extracts. The neutralized extracts were stored at -20°C until testing. Just before the use, extracts were defrosted and centrifuged in order to reduce presence of potassium-perchlorate particles.

NO values in plasma were measured in the form of nitrates (NO₃⁻) and nitrites (NO₂⁻) as final stable products of NO metabolism. The method for detection the plasma nitrate and nitrite levels was based on the Griess reaction modified by Baskic et al (14). Griss reagent was prepared ex tempore just before the experiment by mixing equal amounts of stocks: 2% (w/v) sulfanilamide dissolved in 5% HCl and 0.1% (w/v) aqueous solution of N-1-naphthyl-ethylene-diamine-dihydrochloride (N-NEDA). Nitrate and nitrite solutions in H₂O (10mM) were prepared fresh, daily. Nitrate standard solution was serially diluted (final concentration after adding other reagents 0.5-67 μM) in a 96-well, flat-bottom polystyrene microtiter plate in final volume of 100μL. The destilated aqua (diluting medium) was used as the standard blank. After loading the plate with samples (100μl), addition of VC₁₃ (100μl) to each well was rapidly followed by addition of the Griess reagents (100μl). Blank consisted of the diluting medium and Griess reagent. Nitrite was measured in a similar manner except samples and nitrite standards that were exposed only to the Griess reagent. In either case the absorbance was measured at 540nm (Multiplate Reader 230S, Organon) following 30 minutes of incubation. Concentration of NOx (NO₂⁻ + NO₃⁻) was determined by using Xia software for data analysing based on the standard curve which was obtained by linear regression of absorbance values for each standard reduced for blank values. Results were expressed as nanomoles per milliliter (nmol/mL).

Superoxide anion determination

Superoxide anion values in plasma were measured by using the method of Auclair et Voisin (15). Actually, determination was performed by spectrophotometric method.
based on the reaction of superoxide anion with Nitro Blue Tetrazolium (NBT) which formed nitrophormazan blue. As a standard solution, we used adequate amount of Krebbs-Hensenleite solution. Absorbance was registered at 550nm with appliance of spectrophotometer Multiplate Reader 230S, Organon. Concentration of superoxide anion was calculated by using mathematical formula which was noted in the method. Results were expressed as nanomoles per milliliter (nmol/mL).

**MDA determination**

Lipid peroxidation product malonildialdehyde (MDA) concentration in plasma was determined by thiobarbituric acid assay according to the protocol of Ohkawa (16). At high temperature and low pH value, malondialdehyde reacts with 2-thiobarbituric acid via nucleophytic addition. The product of this reaction is a colored substance and concentration of MDA correlates with the color intensity of this mixture. Extracts of lipid peroxides were obtained from plasma by using 0.5 vol 28% TCA (threchlor acetate). Interval of extraction was 30 minutes in ice container with sporadic stirring. Prior to performing the tests, TCA treated extracts were stored at the temperature -20°C. Defrosted extracts were centrifuged at 15000 rpm (Eppendorf 5415 D) for 4 minutes. TBA was added to translucent supernatants thus creating mixture with volumetric ratio 4:1 (extract:TBA). For this purpose we applied 0.1% TBA dissolved in 0.05 M NaOH. These mixtures were then incubated for 15 minutes at 95°C. Color intensity was determined by using spectrophotometric method (LKB Biochrom Ultrospec 4050). Absorbance was measured at 532 nm. Molar absorbance coefficient for MDA:TBA complex is $1.56 \times 10^5 \text{mol} \text{L}^{-1} \text{cm}^{-1}$. For apparatus calibration, the blank sample was used. It consisted of all required reagents except the plasma sample which was replaced by distilled water. Concentration of thiobarbituric acid reactans (TRARs) was calculated according to formula:

$$C_{MDA} = \left[ \frac{\Delta A \times TV \times D \times V_{\text{plasm}}}{E \times D \times V_{\text{blank}}} \right] \times R \times F$$

\(\Delta A\) – absorbance of serum sample excluding absorbance of blank sample
\(E\) – molar absorbance coefficient for MDA:TBA complex
\(1.56 \times 10^5 \text{ mol} \text{ L}^{-1} \text{ cm}^{-1}\)
\(D\) – optical path length
\(TV\) – amount of incubated mixture
\(V_{\text{plasm}}\) – amount of plasma sample
\(R\) – dispersion of plasma sample
\(F\) – multiplying factor ($x10^6$) for conversion from mol/L to nmol/mL.

**Statistical analysis**

All values are expressed as mean ± standard deviation (SD). Commercial SPSS (Statistical Package for the Social Sciences) version 11.0. for Windows was used for the statistical analysis. Statistical evaluation was performed by Student’s T test for paired observations, one-factorial and two-factorial analysis of variance. The differences were considered to be significant when p value was less than 0.05 and highly significant when p value was less than 0.01.

**RESULTS**

We found that NO plasma values were higher in CLL patients than in the control group. Average NO value in patients with B-CLL was $32.21 \pm 7.26 \text{ nmol/ml}$ and in the control subjects, it was $28.42 \pm 7.27 \text{ nmol/ml}$ (p<0.01) (Figure 1).

**Figure 1.** NO plasma level in B-CLL patients and in the control group

Superoxide anion plasma level in B-CLL patients was higher than in the control subjects (Figure 2). Difference in superoxide anion concentration between the tested groups was highly statistically significant ($10.34 \pm 9.40$ vs $8.52 \pm 6.29 \text{ nmol/ml}$; p<0.01).

**Figure 2.** Superoxide anion plasma level in B-CLL patients and in the control group.

We found similar results in concentration of MDA (figure 3). MDA plasma level in B-CLL patients was higher than in the control group and the observed difference was highly statistically significant ($2.76 \pm 1.61$ vs $1.37 \pm 0.90 \text{ nmol/ml}$, p<0.001).

**Figure 3.** MDA concentration in B-CLL patients and in healthy volunteers.
DISCUSSION

For organisms living in an aerobic environment, exposure to ROS is continuous and unavoidable. ROS are potentially toxic products of cellular metabolism that are generated during the production of adenosine triphosphate by aerobic metabolism in the mitochondria where electrons escaping from the respiratory complexes I and III can react with molecular oxygen to produce oxygen radicals (17). According to their reactive chemical properties, ROS may cause various types of tissue injury. Overproduction of ROS above capability of naturally produced antioxidants may represent a molecular basis for initiation and promotion of multistage carcinogenesis. Highly reactive ROS may interact with DNA inducing a multitude of oxidative modifications and they are implicated in the mutagenesis by protooncogene activation and tumor suppressor gene inhibition (18).

Our results indicate that oxidative stress and lipid peroxidation are accelerated in patients with B-CLL. We demonstrated that, although the plasma levels were heterogeneous, there was a significant increase in the plasma NO value in B-CLL patients. In the study of Bakan et al (19) similar results were demonstrated as well as there was no significant difference in NO level on the basic disease stages. NO exerts different effects on cell death depending on conditions (20). Effects on apoptosis depend on NO concentration, flux and cell type (21). In some situation NO activates the transduction pathways which leads to apoptosis whereas in other cases NO protects cells against spontaneous or induced apoptosis (20, 21). Many mechanisms for inhibition of apoptosis have not been known yet. NO inactivates caspases (such as caspase-3) through oxidation and nitrosylation of the active cystein (20), stimulates cGMP-dependent protein kinase, modulates the member bcl-2/bax family oncogenes, induces synthesis of heat shock protein 70 and takes part in the ceramide pathways (21). Otherwise NO is synthesized from L-arginine by three isofoms of NO synthase (NOS), two of which (endothelial and neurological NOS) are constitutively expressed and are regulated by calcium/calmodulin and phosphorylation while the third (inducible NOS) synthase is induced during different pathological events and it produces higher level of NO (22). Inducible isoform of NO is spontaneously expressed in B-CLL cells (23, 24) which is the reason for increased generation of NO by B-CLL. This spontaneous expression of iNOS is caused by cytokines IL-4 and interferon-gamma which prevent spontaneous in vitro apoptosis of B-CLL cells (25). Flavones and polyphenols can down-regulate iNOS and consequently decrease the endogenous NO production which induces apoptosis of B-CLL cells in vitro (26). Inhibition of the iNOS pathway leads to the increased apoptosis of tumor cells in vitro indicating that endogenous production of NO increases malignant cell resistance to the normal apoptotic process. All factors which induce high expression of iNOS in vivo in the B-CLL cells have not been identified yet. Engagement of CD23 stimulates iNOS activation in malignant cells suggesting that in vivo interaction of CD23 with one of its ligands may contribute to iNOS induction. Interaction of CD40-CD40 ligand may also be involved in the inhibition of caspase activities (24).

Mitochondrial respiration is the major biochemical pathway for production of superoxide anion in cells. We showed that the plasma level of \( \text{O}_2^- \) was significantly higher in B-CLL patients than in the control group. Recent studies have demonstrated similar results. The accumulation of \( \text{O}_2^- \) leads to the free-radical-mediated damage of mitochondrial membranes and activation of apoptotic cascade (27). CLL cells generate increased levels of \( \text{O}_2^- \) which is associated with mitochondrial DNA mutations (28). Accumulation of this radical in cells was associated with morphological and biochemical changes typical of apoptosis (29). Leukemia cells from different B-CLL patients have different rates of \( \text{O}_2^- \) generation (29). Inhibition of superoxide dismutase (SOD) led to the accumulation of \( \text{O}_2^- \) (27, 29). Cell contents of \( \text{O}_2^- \) depend on previously used therapeutic agents. Actually, the \( \text{O}_2^- \) level was significantly higher in CLL cells isolated from previously treated patients with various treatment than the CLL cells from previously untreated patients (29). CLL cells at different stages of the disease may have different metabolic activities and thus produce various levels of \( \text{O}_2^- \) depending on the energy requirement by leukemia cells (29). Using agents for ROS production and SOD inhibition in CLL cells may be a new strategy to enhance therapeutic activity (30).

The process of lipid peroxidation is one of oxidative conversions of polyunsaturated fatty acids which are important for normal function of most mammalian cells. MDA is one of end-products of lipid peroxidation induced by ROS and well-correlated with the degree of lipid peroxidation (31). Lipid peroxidation of the cellular structure may play an important role in the pathogenesis of many pathological processes such as carcinogenesis. In our study, significantly higher MDA plasma level in B-CLL patients than in the control group indicates the intensive lipid peroxidation process which can be the pathogenetic basis of the disease. There are different results of serum MDA level in B-CLL patients in literature. Devi et al (32) showed that plasma lipid peroxidation products in untreated leukemia patients (such as B-CLL) were in normal range. Our results are in accordance with some other studies (19, 33). There is also extensive lipid peroxidation in malignant B-cells as indicated by an increased MDA value in these cells which is in accordance with different forms of DNA bases lesions (33). MDA level and degree of DNA bases damages in transformed lymphocytes are positively correlated with the duration of disease (33).

Our findings suggest that extensive oxidative stress caused by ROS may be related to the pathogenesis of CLL. The identification of adequate oxidative markers for tumor cell metabolism may be useful for early diagnosis and assessment of tumor progression. Understanding of endogenous mechanisms of carcinogenesis by serious
oxidative stress and molecular action of carcinogens must be further elucidated.

REFERENCES


5. Mainou-Fowler T, Miller s, Proctor SJ, Dickinson AM. The levels of TNFα, IL-4 and IL10 production by T-cells in B-cell chronic lymphocytic leukemia (B-CLL). Leukemia Res 2001; 25: 157-63.
27. Sankarapandi S, Zweier JL. Evidence against the generation of free hydroxyl radicals from the interaction of copper, zinc su-