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ORIGINAL ARTICLE

Oxidative stress accelerates spontaneous apoptosis of B-chronic lymphocytic leukemia lymphocytes

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Summary

Purpose: *B-chronic lymphocytic leukemia (B-CLL) is characterized by the progressive accumulation of small immature B lymphocytes which do not undergo apoptosis due to an underlying defect. One potential mechanism of defective apoptosis could be irregular oxidative stress. The goal of our investigation was to determine in vitro production of oxidative stress markers by lymphocytes of B-CLL patients.*

Patients and methods: *30 untreated stage A B-CLL patients, as well as 20 stage B and C patients and 30 healthy volunteers as a control group were examined. Nitric oxide (NO), superoxide anion, hydrogen peroxide and malondialdehyde (MDA) were measured by spectrophotometry in supernatants of lymphocytes cultures of all 3 investigational groups. The method applied for detecting apoptosis was fluorescence microscopic analysis using acridine orange/ethidium bromide (AO/EB) double staining.*

Results: *In vitro lymphocyte production of superoxide anion, hydrogen peroxide and MDA was increased in B-CLL patients, while there were no statistical significantly differences of NO production among the tested groups. Compared with the spontaneous apoptosis observed in control subjects lymphocytes, B-CLL lymphocytes showed increased percentages of apoptotic cells after incubation for 24 h. Disease progression was not followed with significant differences in spontaneous apoptosis of B-CLL lymphocytes.*

Conclusion: *This intensive oxidative stress markers production in cultures of B-CLL lymphocytes could be one of the potential mechanisms in the pathogenesis of abnormal apoptosis.*

Key words: apoptosis, chronic lymphocytic leukemia, hydrogen peroxide, malondialdehyde, nitric oxide, superoxide anion

Introduction

B-CLL is characterized by the accumulation of monoclonal, functionally immature $CD5^+$ B lymphocytes [1]. 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 originated by defective apoptosis [2].

Apoptosis is the physiological process accompanied with many morphological and biochemical changes whereby most cells, including B lymphocytes, are eliminated; this process leads to homeostasis. It is well known that the majority of B-CLL cells have a long lifespan *in vivo,* but such cells rapidly undergo spontaneous apoptosis *in vitro* [3,4]. *In vitro* poor survival may be a result of lack of accessible growth factors because the addition of several cytokines, including interleukin-4, interleukin-6, interleukin-8 and interferons α and γ, has been shown to promote CLL cells survival *in vitro* [5]. The role of oxidative stress in the pathogenesis of this disease is poorly understood and a matter of interest.

Oxidative stress, a well known phenomenon characterized with overproduction of chemically active free

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radicals (FR) plays an important role in the pathogenesis of various diseases and syndromes and is thought to be involved in both initiation and promotion of carcinogenesis [6,7]. FR act as signals or mediators in many cellular processes, such as cell proliferation, differentiation, apoptosis and senescence [8]. On the other hand, cells have evolved defense systems consisted of nonenzymatic and enzymatic pathways to counteract with FR, thereby protecting cells from oxidative stress [9,10]. The cell redox environment may alter genes' expression and enzyme activity and induce the constitutive activation of several transmembrane signaling pathways which regulate the differentiation, cell cycle progression and apoptosis of lymphocytes [11,12].

The aim of our investigation was to determine the *in vitro* production by B-CLL lymphocytes of NO, superoxide anion, hydrogen peroxide and MDA as oxidative stress markers and correlate them with spontaneous apoptosis of B-CLL lymphocytes.

Patients and methods

Patients and controls

The local Ethics Committee approved the study and prior to initiation written informed consent was obtained from all subjects according to the Declaration of Helsinki. B-CLL was diagnosed by established clinical criteria and confirmed by immunophenotypic analysis for the expression of CD5, CD19 and monoclonal immunoglobulin in accordance with NCI Working Group Guidelines [13]. The clinical staging was based on the Binet system [1]. Thirty stage A B-CLL patients (13 females and 17 males, median age 67.8 years, range 54-82), 20 B and C stage B-CLL patients (9 females and 11 males, median age 63.6 years, range 58-78) and 30 healthy control subjects (15 females and 15 males, median age 64.8 years, range 49-77) were included into the study. Most of the patients were newly diagnosed cases. Previously diagnosed patients had not received antileukemic treatment at least 6 months prior to the investigation. The controls were healthy volunteers without known acute and chronic diseases. Exclusion criteria for all subjects were positive parameters of systemic inflammation (erythrocyte sedimentation rate, serum fibrinogen and C-reactive protein levels) due to other etiologies or positive anamnestic data for other illnesses that might have influenced to investigated parameters (e.g. autoimmune diseases, acute and chronic infections, systemic and local inflammations, etc). All subjects (patients and controls) were nonsmokers, without alcohol abuse problems, and none of them performed regular exercise other than daily activities. Also, nobody was receiving any systemic and topical treatment (corticosteroids, cyclosporine A, etc) within 6 months prior to the initiation of the investigation.

Isolation of peripheral blood lymphocytes

All blood samples were obtained in the morning and collected in polystyrene tubes. Heparinized peripheral blood (10 ml) was centrifuged at 400 g for 10 min to separate plasma and cells. Peripheral blood mononuclear and polymorphonuclear cells were separated by single-step continuous density-gradient centrifugation with Lymphoprep (Lymphoprep 1.077, Nicomed Pharma AS, Oslo, Norway). The separated mononuclear cells were washed 3 times with culture medium RPMI 1640 without phenol red (Sigma, Cat. No. R4130, Germany). The monocytes were removed by adhesion on plastic Petri dishes [14]. Cell number and viability were determined using trypan blue and acridine orange/ethidium bromide staining (all from Sigma, Germany).

Incubation

Purified patient and control subject lymphocytes were cultured in RPMI 1640 medium without phenol red (Sigma, Cat. No. R4130, Germany) supplemented with 10% fetal bovine serum (FBS; Seromed, Berlin, Germany), 2 mM L-glutamine (Merck, Germany), 100 IU/ml penicillin G (ICN Galenika, Zemun, Serbia) and 100 mg/ml streptomycin (ICN Galenika, Zemun, Serbia) at 37° C and 5% CO_2 in fully humidified atmosphere for 24 h at a concentration 2×10^6/ml. The investigated FR levels were determined in the supernatants of lymphocytes cultures. After 24 h of incubation the supernatants were removed using centrifugation at 3000 g for 10 min and stored at –80° C until assayed. Cell pellets were used for apoptosis determination.

Detection of apoptosis

After 24 h incubation, the lymphocytes were harvested by centrifugation, suspended in PBS (pH 7.2) and aliquoted for stain procedure. Fluorescent assay with AO/EB double staining was used [15]. One microlitre of dye mixture (100 mg/ml AO and 100 mg/ml EB in distilled water) was mixed with 9 microlitres of cell suspension on a clean microscope slide and examined immediately (fast uptake) by fluorescence microscopy (Polywar, Reinchard Jung, Vienna, Austria) at 400×magnification. A minimum of 500 cells was counted in every sample. The percentage of apoptotic

cells was defined as the average number of apoptotic cells per 100 counted cells.

NO determination

NO values in supernatants of lymphocytes' cultures were measured in the form of nitrites (NO_2^-) and nitrates (NO_3^-) as final stable products of NO metabolism. The method for detection supernatants nitrate and nitrite levels was based on the Griess reaction modified method of Miranda et al. [16]. The concentration of NO was determined using Xia software for data analyzing based on standard curvature which was got by linear regression of absorbance values for each standard reduced for blank values. Results were expressed as nanomoles per millilitre (nmol/ml).

Superoxide anion and hydrogen peroxide determination

The lymphocytes' culture supernatants values of superoxide anion and hydrogen peroxide were measured using sprectrophotometric methods [17,18]. Absorbances were registered using spectrophotometer (LKB Biochrom Ultrospec 4050, Cambridge, UK). Concentrations of both FR were calculated using mathematic formulas [17,18] and results were expressed as nanomoles per millilitre (nmol/mL).

MDA determination

The lipid peroxidation product MDA concentration in culture supernatants was determined by thiobarbituric acid assay according to the protocol of Ohkawa et al. [19] using spectrophotometer (LKB Biochrom Ultrospec 4050, Cambridge, UK). Concentration of thiobarbituric acid reactants (TBARs) was calculated according to relevant mathematic formulas [19].

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Commercial SPSS version 11.0. for Windows (SPSS version 11.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The distributions of data were evaluated for normality using the Kolmogorov-Smirnov test and then retested using chi-square test. Statistical evaluation was performed by Student's t-test for paired observations, one-factorial and two factorial analysis of variance. For nonparametric variables, differences between two independent groups were determined by the Mann-Whitney U-test. Comparisons between three groups of nonparametric variables were performed by the Kruskall-Wallis test. P values less than 0.05 were considered significant and less than 0.01 were considered highly significant.

Results

The results of lymphocytes' spontaneous apoptosis are presented in all Figures. Compared with control subjects the lymphocytes' spontaneous apoptosis of stage A, B and C showed significantly increased percentages of apoptotic cells. Progression of disease was not followed with significant differences in spontaneous apoptosis of lymphocytes after incubation for 24 h (8.27±1.53% vs. 9.31±1.74%; $p > 0.05$).

No significant differences were observed in NO supernatants values between patients and healthy controls ($p > 0.05$; Figure 1). The average NO value in stage A B-CLL patients was less (15.54±4.41 nmol/ml) than in the control subjects (18.47±5.45 nmol/ml), while the group with B and C stages patients had the highest NO value (19.89 ±5.61 nmol/ml; Figure 1).

On the contrary, superoxide anion level in supernatants in the group of B and C stages patients was significantly increased compared with the control group (9.86±1.29 vs. 4.12±1.61 nmol/ml; $p < 0.05$), whereas there were no differences between A stage B-CLL patients and healthy volunteers (5.56±2.13 vs. 4.29±1.61 nmol/ml; $p > 0.05$) (Figure 2). Interestingly enough, the progression of disease was followed with

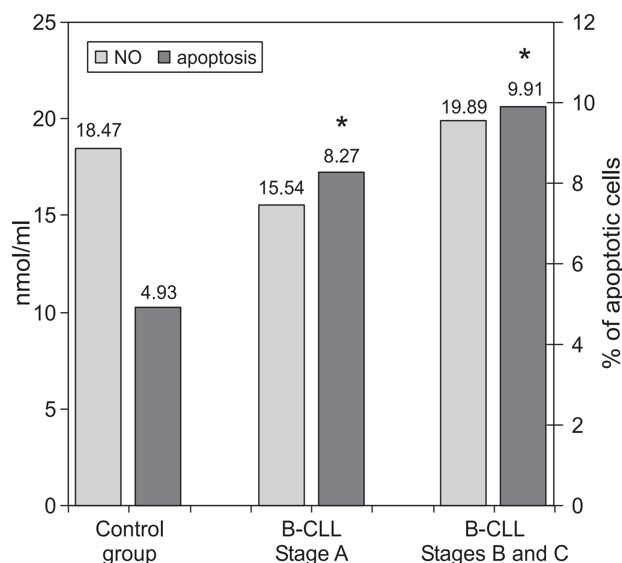

Figure 1. NO levels in lymphocyte culture supernatants are represented by mean of B-CLL patients vs. control ($p > 0.05$). The percentages of apoptotic lymphocytes of both investigated groups of B-CLL patients vs. control after incubation for 24 h (*$p < 0.05$ significant when compared to control group).

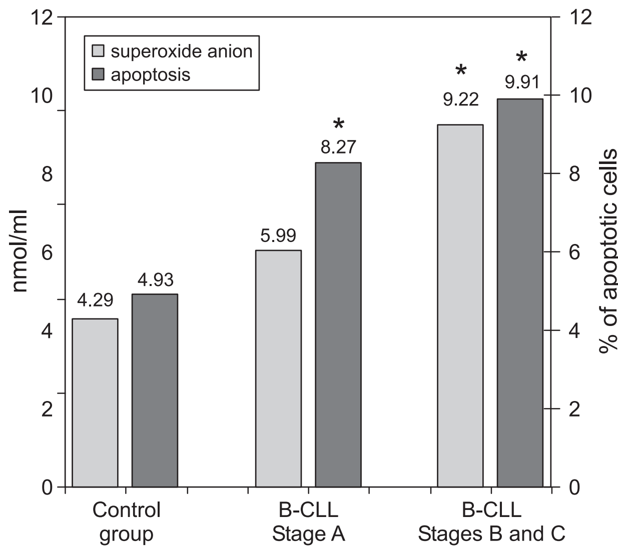

Figure 2. Superoxide anion levels in B-CLL patient lymphocyte culture supernatants are increased compared with the control group. The percentages of apoptotic lymphocytes of both investigated groups of B-CLL patients vs. control after incubation for 24h (*p <0.05 significant when compared to control group).

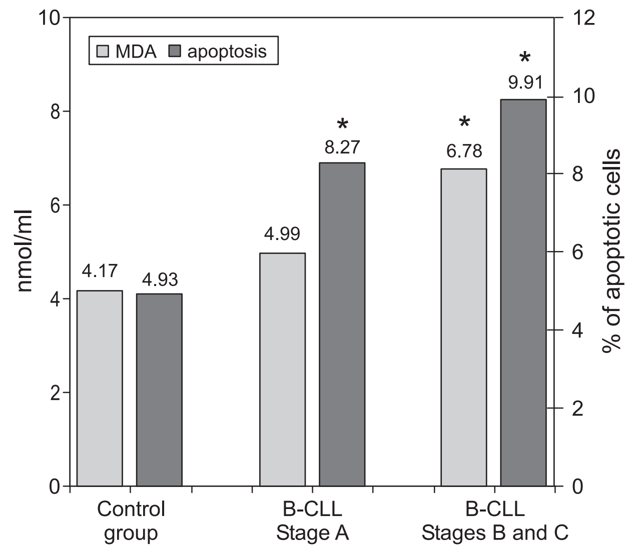

Figure 4. MDA values in supernatants of cultured lymphocytes are represented by mean of both groups of B-CLL patients compared with control. The percentages of apoptotic lymphocytes of both investigated groups of B-CLL patients vs. control after incubation for 24h (*p <0.05 significant when compared to control group).

significant increase of superoxide anion production *in vitro* (9.86±1.29 vs. 5.56±2.13 nmol/ml; $p<0.05$).

As seen in Figure 3, we obtained similar results comparing hydrogen peroxide level in lymphocytes' culture supernatants. Namely, hydrogen peroxide values in supernatants of B and C stages group of patients were significantly increased compared with the control group (0.70±0.08 vs. 0.50±0.09 nmol/ml; $p<0.05$), without differences between A stage B-CLL patients and controls (0.63±0.19 vs. 0.50±0.09 nmol/ml; $p>0.05$). There were no significant differences of hydrogen peroxide values in supernatants of both investigated groups of B-CLL patients.

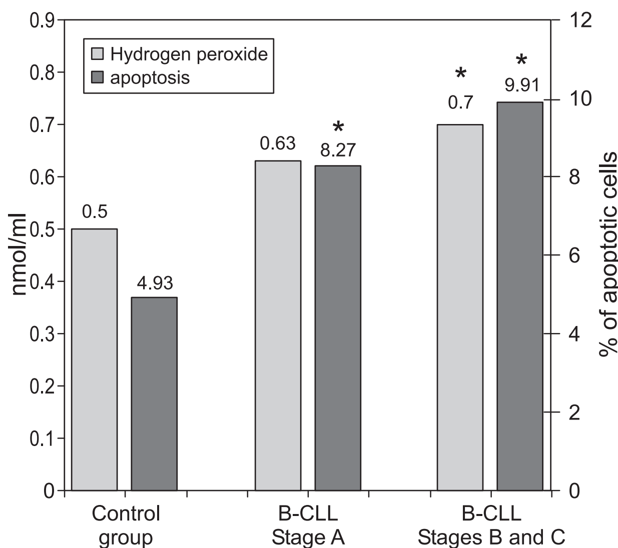

Figure 3. Hydrogen peroxide values in supernatants of cultured lymphocytes are represented by mean of both groups of B-CLL patients compared with control. The percentages of apoptotic lymphocytes of both investigated groups of B-CLL patients vs. control after incubation for 24h (*p <0.05 significant when compared to control group).

The intensity of lipid peroxidation, as a marker of peroxidation status, was measured in supernatants of cultured leukemic and normal lymphocytes. The obtained results showed significant differences between the supernatants level of MDA in stage B and C patients compared with the controls (Figure 4). However, no differences were found in the level of MDA of stage A patients compared with controls or between both groups of B-CLL patients.

Discussion

For organisms living in an aerobic environment exposure to FR is continuous and unavoidable. Most FR are potentially toxic products of cellular metabolism and are generally produced intracellularly during the production of adenosine triphosphate in the mitochondria [8]. FR transient fluctuations serve important regulatory functions but FR overproduction above the capability of naturally produced antioxidant defense system may represent a molecular basis for oxidative stress. At the cellular level oxidant injury elicits a wide spectrum of responses ranging from proliferation to senescence, to apoptosis or ne-

crosis [8]. Although some possible mechanisms through which oxidative stress exerts a regulatory role in tumor growth and progression, including genomic instability, oncogene activation and angiogenesis, several other important questions remain unanswered [8]. It is not clearly known whether both oxidative stress and tumor result from increased oxidant production or from failure of the antioxidant systems. Although important changes in cellular redox homeostasis have been documented during tumor growth in experimental models, such variations have not yet been well 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.

The present study investigated the *in vitro* production of oxidative stress markers by cultured lymphocytes of B-CLL patients in order to obtain a comprehensive view of the FR machinery. We also wanted to demonstrate whether oxidative stress markers might influence the spontaneous apoptosis of lymphocytes *in vitro*. The results of the present study showed that, besides the heterogeneity of supernatant levels, there were increased FR values in lymphocytes' cultures of B-CLL patients compared with the control subjects.

Our findings indicate that the increased percentage of apoptotic B-CLL patients' lymphocytes in cultures after incubation is not associated with significantly changed NO production *in vitro.*

NO is an important pleiotropic molecule involved in neurotransmission, regulation of vascular homeostasis, mitochondrial respiration, signal transduction and proliferation. NO is synthesized from L-arginine by 3 isoforms of NO synthase (NOS). Endothelial and neuronal NOS are constitutively expressed while the inducible NOS is generally expressed during different pathological events. So inducible NOS (iNOS) is spontaneously expressed in B-CLL lymphocytes followed by increased generation of NO [20]. This spontaneous expression of iNOS might be caused by the cytokines IL-4 and interferon-gamma *in vivo,* which could be the reason for elevated NO plasma level demonstrated in other studies [21]. Moreover, it is well known that supplementation of these cytokines prevents spontaneous apoptosis of B-CLL lymphocytes *in vitro* [22]. This could explain why no significant differences were seen in NO supernatant level *in vitro* between the tested groups in our study. On the contrary, inhibition of the iNOS pathway leads to increased apoptosis of tumor cells *in vitro,* indicating that endogenous production of NO contributes to malignant cell resistance to the normal apoptotic process [23]. Unfortunately, all factors which induce *in vivo* high expression of iNOS in the B-CLL lymphocytes are not yet identified.

NO exerts different effects on cell death depending on conditions. Effects on apoptosis depend of NO concentration, flux and cell type. In some situations NO activates the transduction pathways which lead to apoptosis, whereas in other cases NO protects cells against spontaneous or induced apoptosis. The anti-apoptotic action of NO seems mostly due to its capacity to inactivate caspases (such as caspase-3) through oxidation and nitrosylation of the cysteine present in the active sites of these enzymes. Also NO stimulates cGMP-dependent protein kinase, modulates the members bcl-2/bax family oncogenes, induces synthesis of heat shock protein 70 and takes part in ceramide apoptotic pathways.

Mitochondrial respiration is the major biochemical pathway for producing superoxide anion in the cells. Since B-CLL lymphocytes contain significantly more mitochondria than normal B lymphocytes [24] we believe that B-CLL lymphocytes would have to have intensive superoxide anion (O_2^-) production *in vitro*. The results of our study showed that the supernatant levels of O_2^- in lymphocytes' cultures were significantly higher in group B and C stages patients than in healthy subjects and group A stage B-CLL patients. So increase of superoxide anion values in the supernatants of lymphocytes' cultures correlates with disease progression. Recent studies have demonstrated similar results. The accumulation of O_2^- leads to the free radical-mediated damage of mitochondrial membranes, mitochondrial DNA mutations and activation of the apoptotic cascade with morphological and biochemical changes typical for apoptosis [24]. Interestingly leukemic cells from different patients have different rates of O_2^- generation which could be explained by supposing that B-CLL lymphocytes at different disease stages may have different metabolic activities and thus produce various level of O_2^- depending on the energy requirement by the cells [24]. Also it is well known that superoxide dismutase (SOD), the first line of defense against oxygen-derived FR, 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 [24]. The inhibition of SOD described in recent studies leads to accumulation of O_2^- [24]. Nevertheless, lymphocytes' contents of O_2^- depend on previously used therapeutic agents. Actually, the O_2^- levels were significantly higher in B-CLL lymphocytes isolated from previously treated patients with various treatments than in the leukemic cells from previously untreated patients [24]. Therefore, using agents for FR production and SOD inhibition in B-CLL lymphocytes may be a new strategy to attain improved therapeutic results.

Among FR, hydrogen peroxide is the longest-lived and freely membrane permeable which rapidly

established a transmembrane gradient with intracellular concentration below extracellular. Some previous reports illustrated cellular responses after external application of hydrogen peroxide [25]. In our study we investigated the *in vitro* hydrogen peroxide production by cultured lymphocytes and the results demonstrated that its level was significantly higher in the group B and C stages patients compared with the control group. Progression of disease is accompanied with increase of hydrogen peroxide level, but without statistical significance. It is unclear whether overproduction of hydrogen peroxide or failure of antioxidant defense system in cultured lymphocytes resulted in higher supernatants values. Catalase is a tetrameric haemin enzyme that catalyzes the reaction with H_2O_2 to form $2H_2O$ and O_2. The excess amount of hydrogen peroxide could be caused by decreased catalase activity in B-CLL cells with progressive enhancement with disease progression [26]. A possible explanation for the decrease of SOD and catalase activities in B-CLL lymphocytes could be attributed to the inhibitory effect of MDA on protective enzymes [26]. On the contrary, in conditions followed with an intensive FR production the antioxidant capacity of the cells would be overcome, which, in turn, leads to decreased activities of the protective enzymes such as SOD and catalase. Alternatively, it is possible that the antioxidant system is impaired as a consequence of an abnormality in the antioxidative metabolism due to the malignant process [26].

Lipid peroxidation, as one of the main effects of the oxidative stress in cells, can be generated either by an excessive action of pro-oxidants or by reduced functional activity of the antioxidant defense machinery. MDA is one of the end-products of lipid peroxidation induced by FR and is also well correlated with the degree of lipid peroxidation. It has been claimed that MDA acts as a tumor promoter and co-carcinogenic factor due to its high cytotoxicity and inhibitory action on protective enzymes [26]. In our study significantly higher MDA supernatant level in the group of patients with B and C stages compared with the control group indicates intensive lipid peroxidation process. Although MDA supernatant level was slightly increased with disease progression, the difference was not significant. Our results are in concordance with some other studies. The increased MDA value in malignant lymphocytes is in accordance with different forms of DNA base lesions which are positively correlated with the duration of disease [26]. The decreased activity of antioxidant enzymes in lymphocytes, specifically glutathione peroxidase, could be correlated with the rise of the MDA levels in plasma and supernatants of lymphocytes cultures [21].

Apoptosis, as an essential process for the normal functioning and homeostasis, can be induced by a range of intrinsic and extrinsic stimuli as the cellular decision to live or die is made by the coordinated action and balancing of many different pro- and antiapoptotic factors. Defects in the control of this coordination and balance may contribute to a variety of human malignancies including B-CLL. All of the mechanisms controlling apoptosis remain obscure, but there is growing consensus that oxidative stress and redox state of the cell may play a pivotal role in regulating apoptosis [27].

Our results indicated that production of most FR is accelerated in cultured B-CLL patients' lymphocytes and might indirectly suggest that the high levels of FR in supernatants of lymphocyte cultures are associated with high percentages of spontaneous apoptosis of B-CLL lymphocytes.

Recent studies showed that FR had been proposed as a common mediator of apoptosis. Evidence for such a role includes the findings that FR can be detected in many forms of apoptosis, antioxidants prevent most forms of apoptosis and mitochondria, as a major source of FR, play a critical role for the programmed cell death [27]. Also exogenously applied FR induce apoptosis, especially hydrogen peroxide at low concentration, while higher hydrogen peroxide concentration induced by chemotherapeutic drugs can cause necrosis or inhibit apoptosis [28]. It is possible that there are only a few pathways involving FR in controlling apoptosis. One pathway involves the direct induction of molecular damage by FR which is followed with activation of p53/p21 and generation of signals that trigger apoptosis [27]. Also modification of intracellular thiol pools may disturb signal transduction pathways which can be perceived by the cell as abnormal and may trigger apoptosis [27]. Recent studies have shown that tumor necrosis factor-alpha (TNF-α), constitutively produced by B-CLL lymphocytes, induces apoptosis via overproduction and accumulation of FR [29]. TNF-α related FR cytotoxicity is mediated through activation of the c-Jun-N-terminal kinase (JNK) cascade [30]. Accordingly, antioxidant agents effectively block sustained activation of JNK pathway and consecutively inhibit programmed cell death [30].

Although there are numerous evidences of the critical role of FR in apoptosis, data also exist indicating that FR is not crucial. FR cannot be detected after all apoptotic inducers and antioxidants can induce apoptosis in some systems [27]. So FR can trigger apoptosis but are not a final common mediator of this form of cell death. Our findings suggest that extensive oxidative stress caused by FR might be related to the pathogenesis and progression of B-CLL. Understand-

ing the endogenous mechanisms of carcinogenesis by serious oxidative stress and the molecular action of carcinogens must be further elucidated.

Acknowledgements

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