Reactive oxygen and nitrogen species in the course of B-CLL

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Abstract

Purpose: The study objective was to investigate the production of NO, cGMP and superoxide anion radical by neutrophils, and to examine alterations in serum or plasma total nitric oxide, MDA and cGMP levels in B-CLL patients.

Material and methods: PMNs were isolated from 20 patients with B-CLL. Total nitrite was measured in cell supernatants and serum by Griess method. The generation of superoxide anion radical by cells was estimated using cytochrom-c reduction test. The cGMP level in cell supernatants and plasma was assessed by ELISA kit whereas serum MDA level using a spectrophotometric assay by Guege and Aus.

Results: The PMNs in B-CLL patients were characterised by impaired NO generation and enhanced cGMP production. Contrary to the control group, no significant effect was found of rhIL-15 and rhIL-18 on the release of these mediators by PMNs. Superoxide anion radical release by PMNs was decrease. Serum MDA and plasma cGMP levels were elevated in B-CLL patients as compared to the controls.

Conclusions: The reduced production of nitric oxide and superoxide anion radicals by PMNs in B-CLL may impair the cytotoxic effect of neutrophils on leukaemic B cells. Low secretion of nitric oxide by PMNs and high levels of cGMP in PMN supernatants suggest that activation of guanyl cyclase (sGC) in these patients may occur in the presence of agents other than nitric oxide. Moreover, the findings indicate the enhancement of lipid peroxidation with the progression of the neoplastic process in B-CLL patients.

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Introduction

Chronic B cell lymphocytic leukaemia (B-CLL) is a neoplastic disease characterised by proliferation and accumulation of B cells arrested in the early phase of cell division [1,2].

Disturbances can be observed in cellular and humoral defence mechanisms. These changes may also affect non-specific response cells – neutrophils. Recent reports seem to indicate a significant role of these cells, particularly in the early phase of the antitumour response [3].

Activated neutrophils have the potential to destroy cancer cells through a direct cytotoxic action. In the vicinity of cancer cells, a rapid rise is observed in oxygen metabolism of neutrophils, resulting in the formation of numerous reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical, hydrogen peroxide and nitric oxide (NO). Reactive oxygen and nitrogen species exert an effect on cell membrane lipids and organelles, by changing their fluidity, signal transmission, cellular transport and accelerating cell apoptosis [3-5].

Nitric oxide is produced during the conversion of L-arginine to L-citrulline, the reaction being catalysed by NO synthase (NOS). Induced NOS (iNOS), controlled by a series of cytokines, is found in neutrophils (PMNs) [5,6]. Previously, we observed enhanced expression of iNOS in PMNs from healthy persons under the influence of rhIL-15 and rhIL-18 [7].

Cyclic guanosine monophosphate (cGMP) is an indicator of the amount of generated NO and has been known as a "secondary transmitter" of information in the cell, activated by nitric oxide [8-10]. Lipid oxidation by nitric oxide and by proteinated form of superoxide anion radical, i.e. hydrogen peroxide (hydroperoxy radical), results in the production of toxic malondialdehyde (MDA), another "secondary transmitter" with a significant role in neoplastic promotion [11,12].

The study objective was to investigate the production of NO, cGMP and superoxide anion radical by neutrophils, and to examine alterations in serum or plasma total nitric oxide, MDA and cGMP levels in patients with chronic B cell lymphocytic leukaemia.

Material

Twenty patients with B-CLL hospitalized in the Department of Haematology, Medical University of Białystok (13 men and 7 women), mean age 58, were recruited for the study. Ten patients had stage I B-CLL and the remaining ten were in stage III in the Rai classification.

Stage I of inactive disease was referred to as the initial phase due to higher detectability than in stage 0. In Rai stage III, the disease is advanced, but patients are in better general condition than in stage IV.

The diagnosis was based on clinical observation, peripheral blood morphology tests, bone marrow punctate, trepanobiopsy, lymph node biopsy and cytochemical examinations. A flow cytometer EPIX XL (Coulter, USA) was used to identify immunophenotypes of leukaemia cells. The monoclonal antibody panels of CD19 and CD20 for B cells and CD3, CD7 and CD8 for T cells were applied to differentiate between these cells.

All the study patients underwent the investigations prior to treatment with cytostatics and corticosteroids. The patients who had accompanying acute inflammatory bacterial, viral, mycotic or allergic conditions were excluded from the study.

The control group consisted of 15 healthy age-matched subjects (6 women and 9 men).

Methods

Cells were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient 1.115 g/ml (Polfa) by Zemman et al. [13]. Plasma was also obtained from heparinized (10 U/ml) whole blood. Serum was received from whole blood without additives.

Polymorphonuclear cells (PMNs) were suspended in the culture medium (HBSS) to provide $5x10^6$ cells/ml and the cells were incubated in flat-bottomed 96-well plates (Microtest III-Falcon) for 4 h at 37ºC in a humidified incubator with 5% CO_2 (NUAIRETM). rhIL-15 (50 ng/ml; R&D) and/or rhIL-18 (50 ng/ml; R&D Systems) were tested to stimulate secretion by PMN.

Determination of total nitric oxide (NO_3^-/NO_2^-) concentration in cell cultures and serum

Nitric oxide produced in cells in the presence of superoxide anion radical is rapidly converted to nitrate and nitrite (NO_3^-, NO_2^-). Nitrate and nitrite are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrate and nitrite concentrations. NO production by PMN was determined using an indirect method based on measurement of NO_2^- ion concentration in culture supernatants and serum according to Griess's reaction. In the samples analyzed, nitrates were reduced to nitrites in the presence of cadmium, and then converted to nitric acid that gave a colour reaction with Griess's reagent [14]. NO_2^- ion concentrations were determined by spectrophotometric analysis at λ=540 nm with reference to a standard curve.

Analysis of generation of superoxide anion radical by PMN using cytochrom-c reduction test

"Oxygen burst" in neutrophils was explored by detecting the production of O_2^-* by these cells according to Mc Cord's method, in Bhuyan's modification [15], which utilizes differences in light absorbance between solutions containing non-reduced and reduced cytochrome-c. Cytochrome-c does not permeate through the plasmic membrane and thus its reduction by superoxide anion radical in PMN supernatants indicates O_2^- * release outside of the cell.

Cytochrome-c solution in phosphate buffer (KH2PO4/K2HPO4), pH=7.8, containing 0.1 mM EDTA, was added to two parallel samples with isolated neutrophils. Cytochrome-c concentration was 15 mg/ml. Superoxide dismutase (SOD), 5000 U/ml activity, was added to the reference sample, while buffer to the study sample. Next, after addition of LPS (10 μg/ml) to both test-tubes, the samples were incubated at 37ºC, and then absorbance was read at λ=550 nm in the presence of deionized water. The result was presented as nontitrated F index expressed by the absorbance ratio of reference sample to reduced sample.

$$F = \frac{\text{Abs Cyt-c +SOD}}{\text{Abs Cyt-c}}$$

Determination of cGMP concentration in the PMN supernatants and plasma

The cGMP level in the cell supernatants and plasma was assessed using ELISA kit (R&D Systems).

Determination of MDA concentration in serum

MDA level in serum was assessed using a spectrophotometric assay by Guege and Aus [16].

Statistical evaluation

The results obtained were analyzed statistically using Microsoft Excel spreadsheet and Statistica 5.1 suite. Data are expressed as mean ±standard deviation (SD). Normal distribution of data was assessed by the Kolmogorov-Smirnov test. Since the data were not normally distributed, U-Mann-Whitney nonparametric tests for unrelated results were used to compare differences between the groups. A p value of <0.05 was accepted as statistically significant.

Results

1. Concentration of total nitric oxide (NO_3^-/NO_2^-) in PMN supernatants of B-CLL patients

In stage I and III B-CLL patients, both unstimulated and stimulated PMNs were found to release smaller amounts of NO as compared to the control group (*Tab. 1*).

Total NO levels in unstimulated PMN supernatants were significantly higher in stage III patients, as compared to stage

***Table 1*. Concentrations of total nitric oxide (NO_3^-/NO_2^-) in PMN supernatants of B-CLL patients**

PMN	Concentrations of total NO (NO_3^-/NO_2^-) (μM/5x10^6 cells/ml)		
	Control subjects n=15	Patients in stage I n=10	Patients in stage III n=10
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
Unstimulated	20.87±3.05	14.15*±2.86	16.8^{b}*±3.1
LPS stimulated	30.01^{a}±5.25	14.69*±2.72	19.33^{ab} *±3.25
rhIL-15 stimulated	24.19^{a}±3.16	15.75*±3.7	17.66*±4.01
rhIL-18 stimulated	26.45^{a}±3.44	15.4*±2.17	17.76*±4.32

* – statistical differences as compared to control subjects ($p<0.05$); ^{a} – statistical differences between unstimulated and stimulated cells ($p<0.05$); ^{b} – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

***Table 2*. Generation of superoxide anion radical by neutrophils in B-CLL patients**

PMN	Control subjects n=15	Patients in stage I n=10	Patients in stage III n=10
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
Unstimulated	1.27±0.23	0.67*±0.08	0.78*^{b}±0.05
LPS stimulated	2.91^{a}±0.97	0.91*±0.39	0.82*±0.35
rhIL-15 stimulated	2.69^{a}±1.21	0.88*±0.29	0.95*±0.48
rhIL-18 stimulated	2.78^{a}±0.97	0.77*±0.24	0.97*±0.36

* – statistical differences as compared to control subjects ($p<0.05$); ^{b} – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

I patients. PMN stimulation with LPS in stage III led to higher production of NO as compared to unstimulated cells in stage I patients (*Tab. 1*).

No differences were observed in total NO levels between unstimulated and rhIL-15 and rhIL-18 stimulated PMNs both in stage I and III B-CLL.

2. Concentration of total nitric oxide (NO_3^-/NO_2^-) in the serum of B-CLL patients

The analysis of total serum NO levels in B-CLL patients (14.29 μM±1.4) showed no changes as compared to the control group (14.26 μM±1.56; $p>0.05$).

Total serum NO concentrations in stage III patients was higher in comparison to stage I patients (15.7 μM±1.8; 12.88 μM ±1.4; $p<0.05$).

No correlation was observed between total NO levels in unstimulated PMN supernatants and serum concentration in B-CLL patients.

3. Generation of superoxide anion radical by neutrophils in B-CLL patients

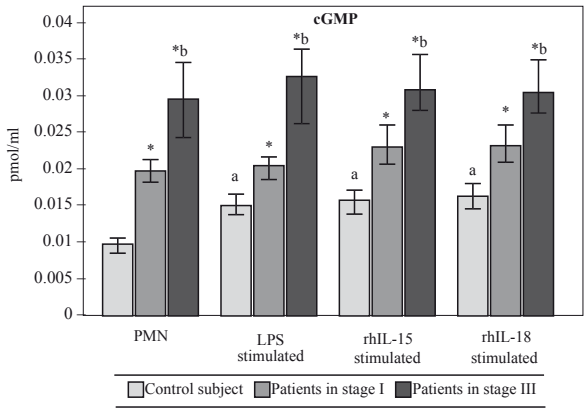
The ability of neutrophils to generate superoxide anion radical was found to be much lower in both B-CLL groups as compared to the control group (*Tab. 2*).

In stage III patients, PMNs had greater potential to produce superoxide anion radical in comparison with stage I patients (*Tab. 2*).

4. Concentrations of cGMP in PMN supernatants and in the plasma of B-CLL patients

In all patients, cGMP levels were higher than in control subjects. Moreover, in stage III, leukocytes released more cGMP than in stage I (*Fig. 1*).

***Figure 1*. Concentrations of cGMP in PMN supernatants of B-CLL patients**

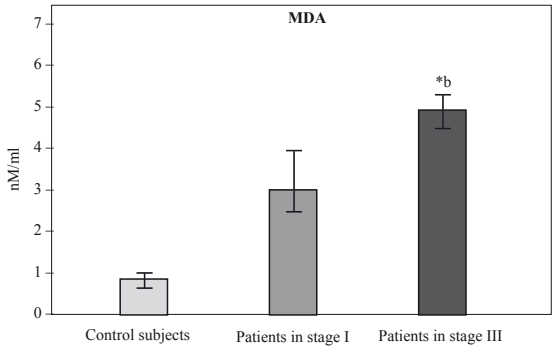


* – statistical differences as compared to control subjects ($p<0.05$); a – statistical differences between unstimulated and stimulated cells ($p<0.05$); b – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

However, unlike in control subjects, cGMP levels in LPS, rhIL-15 or rhIL-18 stimulated leukocyte supernatants in stage I and III patients were not significantly higher as compared to unstimulated cells (*Fig. 1*).

Higher plasma cGMP levels were found in stage I and III patients (0.46 pmol/ml±0.14 and 0.46 pmol/ml±0.17 respectively) in comparison with the control values (0.27 pmol/ml±0.09).

Figure 2. **Concentrations of MDA in the serum of B-CLL patients**



* – statistical differences as compared to control subjects ($p<0.05$); b – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

5. Malondialdehyde level (MDA) in the serum of B-CLL patients

Serum MDA levels in stage I and III patients were significantly higher than in the control group (*Fig. 2*). Moreover, serum MDA concentration was found to increase with the disease advancement. It was significantly higher in stage III than in stage I patients.

Discussion

In the current study, we have demonstrated that the production of nitric oxide and superoxide anion radical by peripheral blood neutrophils is impaired in B-CLL patients. This may have a significant implication for these patients. The reduced production of nitric oxide and superoxide anion radical by PMNs of B-CLL patients indicates a reduced cytotoxic effect of neutrophils on cancer cells. There is evidence that low NO concentrations may activate matrix metalloproteinases (MMPs), the enzymes which function as remodelling factors of the extracellular matrix components, enhancing cancer cell growth, migration and invasion [17]. Given the above, the impaired NO production by neutrophils may play an important role in tumour progression in B-CLL patients. Additionally, low levels of NO have been reported to exert angiogenic effects in various tumour models [18]. One the other hand, there are data suggesting that inhibition of the iNOS pathway leads to increased apoptosis of tumour cells *in vitro* [4].

Low concentrations of nitric oxide, undetectable by Griess method applied in the current study, may be due to a rapid reaction with superoxide anion radical resulting in the formation of toxic peroxynitrite. Peroxynitrite exhibits a cytotoxic effect associated with superoxide dismutase nitrolysis, which may eventually lead to disturbances in the reductive state of the cell and promote neoplastic progression [19].

Peroxynitrite may also contribute to the activation of cGMP synthesis in cells, which has been confirmed by our own findings indicating the enhanced synthesis of cyclic guanosine monophosphate by PMNs in B-CLL patients [20].

The process of cGMP synthesis is controlled by various extra- and intracellular mediators, including cytokines [8]. Shindo et al. *in vitro* showed an increase in cGMP production in fibroblasts and myocardial cells under the influence of IL-1β and LPS, but not when exposed to TNF-α, IL-2, IL-6, IL-8 and TGF-β [21]. We observed no significant effect of rhIL-15 and rhIL-18 on cGMP release by PMNs in B-CLL patients. Moreover, high cGMP concentrations in PMN supernatants coexisting with low nitric oxide production by PMNs suggest that guanyl cyclase activation (sGC) in patients with chronic B-CLL is likely to occur in the presence of biologically active agents other than nitric oxide.

Changes in NO released by PMNs can affect its blood serum level. However, total serum NO levels in patients with B-CLL did not differ from the control, but there was difference in NO levels between patients in stage III and in stage I.

Different results have been reported by Bakan et al. who found no significant differences in NO concentrations between stage I and stage III patients [22].

The increased serum NO level may directly affect cell structural components, which is reflected in, e.g., MDA production [22]. We found higher serum MDA levels in B-CLL patients than in control subjects. Elevated serum MDA concentrations observed in stage III patients as compared to stage I indicate enhancement of lipid peroxidation with the progression of the neoplastic process.

High MDA levels despite low serum total nitric oxide concentrations can be explained by the fact that other compounds such as sulfur dioxide, hydroxyl radical, radical cation and hydroperoxyl radical exhibit lipid peroxidation potential. Besides, the method used to estimate MDA concentration, based on reactions with thiobarbituric acid and employed in our study, is nonspecific. Apart from MDA, it also detects such compounds as bilirubin, sialic acid, products of degradation of saccharide and other aldehydes, whose concentration can also change in the course of the neoplastic processs [16].

When elevated, MDA may react with thiol groups and amino acid proteins, lipids, amino saccharides and nitric bases constituting nucleic acids. Modification of physical properties of the cellular membranes by MDA via increased permeability for H+ and other polar substances may lead to changes in the electric potential difference on both sides of the membranes and eventually to the loss of integrity and inhibition of the activity of protein-transporting enzymes [15,16,23].

Similarly, Ghalaut et al. and Oltra et al. observed high serum MDA levels accompanied by reduced activity of antioxidant enzymes, such as superoxide dismutase and catalase [24,25]. However, Devi et al. found no changes in serum MDA in leukaemic patients despite increased production of superoxide anion radical by neutrophils and elevated levels of antioxidant enzymes [26].

The study has revealed that changes in NO in PMN supernatants and in serum, in cGMP in PMN supernatants and in serum MDA are more characteristic of stage III than stage I patients with B-CLL. The above observations seem to suggest that the research on reactive oxygen and nitrogen species and their indirect markers may have significant diagnostic and prognostic implications for the assessment of oxidative processes in patients with B cell lymphocytic leukaemia.

References

1. Adams DJ, Levesque MC, Weinberg JB, Smith KL, Flowers JL, Moore J, Colvin OM, Silber R. Nitric oxide enhancement of fludarabine cytotoxicity for B-CLL lymphocytes. Leukemia, 2001; 15: 1852-9.

2. Madej JA. Etiologia i patogeneza nowotworów. α-medica press 1999. Wydanie II.

3. Di Carlo E, Forni G, Lollini PL, Colombo MP, Modesti A, Musiani P. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood, 2001; 97: 339-45.

4. Kolb JP, Roman V, Mentz F, Zhao H, Rouillard D, Dugas N, Dugas B, Sigaux F. Contribution of nitric oxide to the apoptotic process in human B cell chronic lymphocytic leukaemia. Leuk Lymphoma, 2001; 40: 243-57.

5. Kroncke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol Chem Hoppe Seyler, 1995; 376: 327-43.

6. de Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM, Billiar TR, Geller DA. Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. PNAS, 1996; 93: 1054-59.

7. Jabłońska E, Kiersnowska-Rogowska B, Pużewska W, Rogowski F, Parfieńczyk A. Tlenek azotu w nadsączach hodowli leukocytów i surowicy krwi u pacjentów z przewlekłą białaczką limfatyczną B-komórkową (pbl-B). Przegl Lek, 2006; 12.

8. Koesling D, Bohme E, Schultz G. Guanylyl cyclases, a growing family of signal-transducing enzymes. FASEB J, 1991; 5: 2785-91.

9. Stroop SD, Beavo JA. Structure and function studies of the cGMP-stimulated phosphodiesterase. J Biol Chem, 1991; 266: 23802-9.

10. Vigne P, Feolde E, Ladoux A, Duval D, Frelin C. Contributions of NO synthase and heme oxygenase to cGMP formation by cytokine and hemin treated brain capillary endothelial cells. Biochem Biophys Res Commun, 1995; 214: 1-5.

11. Alagol H, Erdem E, Sancak B, Turkmen G, Camlibel M, Bugdayci G. Nitric oxide biosynthesis and malondialdehyde levels in advanced breast cancer. Aust N Z J Surg, 1999; 69: 647-50.

12. Bakan E, Taysi S, Polat MF, Dalga S, Umudum Z, Bakan N, Gumus M. Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. Jpn J Clin Oncol, 2002; 32: 162-6.

13. Zeman K, Tchórzewski H, Majewska E. Prosta i szybka metoda jednoczesnej izolacji limfocytów i granulocytów krwi obwodowej. Immunol Pl, 1988; 13: 217-25.

14. Schulz K, Kerber S, Kelm M. Reevaluation of the Griess method for determining NO/NO_2^- in aqueous and protein-containing samples. Nitric Oxide, 1999; 3: 225-34.

15. Bhuyan DK, Bhuyan KC. Assessment of oxidative stress to eye in animal model for cataract. Methods Enzymol, 1994; 233: 630-9.

16. Bartosz G. Druga twarz tlenu. Wydawnictwo Naukowe PWN, 2003.

17. Śliwowska I, Kopczyński Z. Metaloproteinazy macierzy zewnątrzkomórkowej – charakterystyka biochemiczna i kliniczna wartość oznaczania u chorych na raka piersi. Współ Onkol, 2005; 9: 327-35.

18. Lirk P, Hoffmann G, Riedel J. Inducible nitric oxide synthase-time for reappraisal. Drug Targets Inflamm Allergy, 2002; 1: 89-108.

19. Svingen BA, Buege JA, O'Neal FO, Aust SD. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. J Biol Chem, 1979; 254: 5892-9.

20. Tarpey MM, Beckman JS, Ischiropoulos H, Gore JZ, Brock TA. Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis. FEBS Lett, 1995; 364: 314-8.

21. Shindo T, Ikeda U, Ohkawa F, Kawahara Y, Yokoyama M, Shimada K. Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. Cardiovasc Res, 1995; 29: 813-9.

22. Bakan N, Taysi S, Yilmaz O, Bakan E, Kuskay S, Uzun N, Gundogdu M. Glutathione peroxidase, glutathione reductase, Cu-Zn superoxide dismutase activities, glutathione, nitric oxide, and malondialdehyde concentrations in serum of patients with chronic lymphocytic leukemia. Clin Chim Acta, 2003; 338: 143-9.

23. Gerber M, Astre C, Segala C, Saintot M, Scali J, Simony-Lafontaine J, Grenier J, Pujo H. Oxidant-antioxidant status alterations in cancer patients: relationship to tumor progression. J Nutr, 1996; 126: 1201S-7S.

24. Ghalaut VS, Ghalaut PS, Singh S. Lipid peroxidation in leukaemia. J Assoc Physicians India, 1999; 47: 403-5.

25. Oltra AM, Carbonell F, Tormos C, Iradi A, Saez GT. Antioxidant enzyme activities and the production of MDA and 8-oxo-dG in chronic lymphocytic leukemia. Free Radic Biol Med, 2001; 30: 1286-92.

26. Devi GS, Prasad MH, Saraswathi I, Raghu D, Rao DN, Reddy PP. Free radicals antioxidant enzymes and lipid peroxidation in different types of leukemias. Clin Chim Acta, 2000; 293: 53-62.