Cytokine and adhesion molecule concentrations in blood of patients with B-cell chronic lymphocytic leukaemia with regard to disease progression

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Abstract

Purpose: **To examine concentrations of IL-8, E-selectin and VCAM-1 in blood plasma, culture supernatant and isolated broken lympocytes in patients with chronic lymphocytic leukaemia (CLL), at I and III stage of the disease according to Rai's classification and in healthy individuals constituting controls. Two types of lymphocyte cultures were carried out – nonstimulated and stimulated with mitogen.**

Material and methods: **A concentration assay of substances mentioned above was made using ready immunoenzymatic sets of the ELISA type.**

Results: **In all cases, plasma concentrations of IL-8, E-selectin and VCAM-1 were the highest in III stage of CLL and moderately increased in I stage comparing to control. The concentrations of IL-8 in culture supernatants and in broken lymphocytes and concentration of VCAM-1 in lymphocytes were increased in both stages of CLL. In contrast concentrations of E-selectin were decreased in lymphocytes in both types of culture. Significant decrease of E-selectin and VCAM-1 concentrations were observed in supernatants of nonstimmulated cultures.**

Conclusion: **Significant increase of E-selectin, IL-8 and VCAM-1 concentration in blood plasma may support higher activity and suggest the higher ability of leukaemic lymphocytes to migration. Increased IL-8 concentration in isolated, broken, stimulated lymphocytes may be a characteristic feature of the biochemical processes of leukaemic lymphocytes. The lowest values of E-selectin and VCAM-1 concentrations in culture supernatants and broken lymphocytes may suggest their degradation during in culture.**

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Introduction

B-cell chronic lymphocytic leukaemia (CLL) belongs to the malignant lymphomas and originates from the immunological system. Its etiopathogenesis has not been fully explained yet. At present the scientists incline to the genetic theory. This states the point at which mutation is generated idiopathically or due to the activity of external agents, such as toxic chemical substances, organic substances, certain medications, and physical agents. The aberrations of chromosomes responsible for primary blastic lymphocyte transformation ultimately induce B-cell chronic lymphocytic leukaemia (B-CLL). These aberrations refer most frequently to a long arm of chromosome 13 (13 q). They can also be produced by the surplus of long arm of the chromosome 14 (14q+). Neoplastic lymphocytes are blocked at the stage of G_0 division cycle, which protects them against maturation and death through apoptosis [1-3].

Interleukin-8 (IL-8) belongs to the chemokines, so-called substances having the characteristics of both cytokines and chemotactic agents. IL-8 is characterized by high biological activity and has IL-8R specific receptors belonging to a superfamily combining with G protein that binds the guanylic nucleotide. This plays a vital role in neoplastic invasiveness and metastasis [4-6]. E-selectin (endothelial leukocyte adhesion molecule 1; ELAM-1) takes part mainly in the first stage of migration i.e. leukocyte rolling. It is of interest that IL-8 is absent from nonstimulated endothelial cells, and the induction of its expression requires a new protein [7-9].

Vascular cell adhesion molecule 1 (VCAM-1) takes part in leukocyte adhesion to endothelial capillaries during inflammatory processes. An increase in its expression has been observed after IL-1β, IL-4 and IL-14 stimulation. Very-late-activated integrin 4 (VLAI-1) is a ligand for VCAM-1 [10,11].

IL-8 and VCAM-1 actively participate in lymphocyte migration, directly or indirectly, by means of appropriate

Table 1. Comparison of mean concentrations of IL-8 in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of IL-8 (pg/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
	X±SD p	Supernatant (N0) X±SD p	Broken lymphocytes (L0) X±SD p	Supernatant (NS) X±SD p	Broken lymphocytes (LS) X±SD p
Control group N=9 (C)	7.888±1.682	7.889±1.64	250.18±12.53	7.889±3.45	262.66±20.3
Patients in I stage of CLL n=9 (I)	12.76±1.684 I : C (p<0.02)	12.35±1.7 I : C (p<0.02)	512.86±95.54 I : C (p<0.02)	16.684±2.43 I : C (p<0.02)	558.74±92.7 I : C (p<0.02)
Patients in III stage of CLL n=9 (III)	15.851±1,691 III : C (p<0.02)	14.72±2.54 III : C (p<0.02)	873.82±253 III : C (p<0.0001)	17.03±2 III : C (p<0.02)	1318.7±231.7 III : C (p<0.0002)

ligands. Therefore the aim of this study was the determination of concentrations of IL-8 and VCAM-1 in the plasma, cell culture supernatants and broken lymphocytes.

Material and methods

The study material included: plasma, culture supernatant and isolated and broken lymphocytes obtained from patients with B-CLL cell (9 individuals in stage I and 9 in stage III according to Rai's classification) [12] and from 9 healthy volunteers of the same age (workers and students of the Medical University of Białystok).

The diagnosis of leukaemia was established on the basis of clinical observation, morphological composition of peripheral blood, marrow puncture and cytochemical tests, such as the reaction of alpha-naphtyl and black Sudan-B to peroxidases (POX), PAS and esterases. Leukaemic cell immunophenotyping was performed by means of a coulter flow cytometer EPICS XL using a group of antibodies to discriminate between B and T lymphocytes; CD19 and CD20 for B lymphocytes, and CD3, CD7 and CD8 for T lymphocytes, respectively.

In patients with CLL, examinations were carried out before treatment with cytostatics, corticosteroids, blood transfusions or possibly RTG therapy, 3-4 weeks after conventional treatment in patients at stage III of the disease. Patients with acute inflammation conditions caused by both bacterial and viral infections and allergies, were excluded from the studies.

The assay of E-selectin, IL-8 and VCAM-1 concentrations was performed on blood plasma, culture supernatant and broken lymphocytes with immunoenzymatic ELISA kits from the R&D Europe Systems (UK) and DPC Biermann GmbH (Germany). The assay procedure was not radically different from the instructions enclosed by the manufacturer. Lymphocytes were isolated using G Gradisol according to the method described by Zeman et al. [13]. Two types of cultures were always grown from the blood collected from each patient. These were nonstimulated (0) and Neupogen – stimulated in pairs. Roche Neupogen (granulocyte colony stimulating factor) in doses of 1 μl/4 ml was used to activate lymphocytes and to provoke them to blastic transformation. In each case the assay was performed in: a) the blood plasma, b) the culture supernatant without mitogens-(N0), c) a culture supernatant with Neupogen-(NS), d) broken mitogen-non-stimulated lymphocytes-(L0) and e) broken Neupogen stimulated – (LS) lymphocytes.

In order to eliminate erytrocyte impurities, hemolytic shock was induced using buffered, hypotonic liquid PBS without Ca^{++} and Mg^{++} ions. Intravital staining with trypan blue resulted in 92-98% viable cells for examination.

An isolated leukocyte culture was performed for 4 hours on the Parker medium according to a Kuhn and Gallin's method [14]. The isolated lymphocyte count with regard to the donor was between $4x10^6$ and $6x10^6$ of cells/1 ml of culture.

After incubation, the cells were washed and centrifuged, then broken by means of a Vibra Cell ultrasound homogenizer (Sonics Material, USA) at a power of 50 W with a frequency above 80 Mhz for a 20 seconds.

All data are displayed as mean ±SD. Comparisons between groups were performed using Student's t-test. A value of $p<0.05$ was deemed significant.

Results

The determination of IL-8 concentrations in the plasma of B-CLL patients showed their significant increase at stages I and III of the disease. The highest increase was in plasma at stage III of B-CLL ($p<0.02$) (*Tab. 1*).

In the culture supernatant, a significant increase was observed in IL-8 concentrations at the stages I and III of B-CLL patients in non-stimulated groups: (N0 I) ($p<0.02$), (N0 III) ($p<0.02$) and in stimulated (NS I) ($p<0.02$), (NS III) ($p<0.02$) in comparison to control groups. IL-8 concentrations

Table 2. Comparison of mean concentrations of E-selectin in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of E-selectin (ng/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
	X±SD p	Supernatant (N0) X±SD p	Broken lymphocytes (L0) X±SD p	Supernatant (NS) X±SD p	Broken lymphocytes (LS) X±SD p
Control group N=9 (C)	3.7548±0.9277	4.1702±0.308	0.832±0.136	0.498±0.123	0.409±0.094
Patients in I stage of CLL n=9 (I)	3.7284±0.6662 I : C N.S.	0.1564±0.015 I : C (p<0.002)	0.624±0.099 I : C N.S.	0.44±0.12 I : C N.S.	0.084±0.021 I : C (p<0.02)
Patients in III stage of CLL n=9 (III)	7.08±1.22 III : C (p<0.02)	0.3976±0.159 III : C (p<0.002)	0.63±0.099 III : C N.S.	0.44±0.12 III : C N.S.	0.084±0.021 III : C (p<0.02)

Table 3. Comparison of mean concentrations of VCAM-1 in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of VCAM-1 (ng/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
	X±SD p	Supernatant (N0) X±SD p	Broken lymphocytes (L0) X±SD p	Supernatant (NS) X±SD p	Broken lymphocytes (LS) X±SD p
Control group N=9 (C)	13.712±3.04	1.022±0.019	0.4718±0.051	0.1698±0.0315	0.359±0.067
Patients in I stage of CLL n=9 (I)	26.752±2.515 I : C (p<0.02)	0.247±0.033 I : C (p<0.002)	0.4478±0.029 I : C N.S.	0.2952±0.133 I : C N.S.	4.1±0.39 I : C (p<0.002)
Patients in III stage of CLL n=9 (III)	29.74±2.941 III : C (p<0.02)	0.247±0.103 III : C (p<0.002)	0.483±0.115 III : C N.S.	3.005±0.711 III : C (p<0.002)	4.24±0.13 III : C (p<0.002)

were found to be about twofold higher in stage III before stimulation and I and stage III of B-CLL after stimulation. The moderate increase was in B-CLL-patients at stage I in comparison to the control group.

IL-8 values in isolated, broken lymphocytes were generally higher in comparison to the IL-8 concentrations in the control groups; the highest level – sixfold higher – occuring at stage III of B-CLL after stimulation (LS III) ($p<0.0002$). A moderate increase – four-fold – was observed in lymphocytes at stage III non-stimulated ($p<0.0001$). Concentrations of IL-8 were about two-fold higher at stage I of B-CLL patients before ($p<0.02$), and after stimulation ($p<0.02$).

Concentrations of E-selectin were slightly higher in the plasma of B-CLL patients at stage I and approximately two-fold higher in stage III of B-CLL ($p<0.002$) in comparison to the control group (*Tab. 2*).

A significant decrease was observed in E-selectin concentrations in non-stimulated lymphocyte culture supernatant: (N0 I), (N0 III) ($p<0.002$) and similar in stimulated (NS I), (NS III) in comparison to the levels from the control groups. The lowest decrease in this selectin's concentrations was observed in the supernatant from non-stimulated culture of leukaemia cells.

E-selectin values in non-stimulated, isolated, broken lymphocytes of stage I and III B-CLL patients were lower in comparison to the control group. E-selectin concentrations were significantly lower in the stimulated group than in non-stimulated groups ($p<0.02$).

Concentrations of VCAM-1 were higher in the plasma of B-CLL patients at stages I and III of the disease ($p<0.02$) in comparison to concentrations in control group (*Tab. 3*).

A significant decrease was observed in VCAM-1 concentrations in (N0 I), (N0 III) ($p<0.02$) non-stimulated lymphocyte

culture supernatant in comparison to control groups. In contrast, a significant increase was noted at stage III of B-CLL after stimulation (NS III) ($p<0.002$).

VCAM-1 concentrations were seven-fold higher in isolated, broken lymphocytes after stimulation: (LS I), (LS III) ($p<0.002$) and only slightly higher in B-CLL at stage III before stimulation in comparison with the levels of stimulated lymphocytes in the controls.

Discussion

Chemokines and adhesion molecules actively participate in the lymphocyte migration from the blood to the surrounding tissue. They play especially important role in all stages of leukocyte migration, directly or indirectly by means of appropriate ligands. This process is observed wherever there are capillaries. However lymphocyte permeation takes place in the specialized capillary venules with high endothelium existing in lymphatic organs. This migration is possible due to chemokines, adhesion molecules and their receptors [1,6,15].

Chemotaxis and the second stage of migration depends on the lymphocytes being activated by chemokines. These are small proteins about 8-10 kDa in weight. Protein G is the basic link for this stage of migration [4]. IL-8 belongs to the most potent chemotactic factors of the immune system, having great importance in neoplastic invasions and formation of metastases. Protein G catalyzes changes of GTP to GDP (joined with protein G subunit α) after ligand conjunction with a specific receptor. Cytoplasmatic proteins after activation are transported through the cell to the nucleus. They play an important role in the activation of gene transcription [4,5,16]. The stimulus causes the activation of the genes; after that, it causes stimulation of the biosynthesis of biologically active proteins. This allowes leukocyte migration to the region of the homeostatic disturbance almost instantanously.

Therefore, we can see some tendency of the paradoxical activation of the leukocytes to synthesis and excretion substances like IL-8 in the course of CLL. Permanent activation of the lymphocytes by these interleukins creates a situation where leukocytes respond even to the smallest stimuli [17].

The results of our examinations show a significant increase of IL-8 concentrations in the blood plasma, culture supernatants and in isolated, broken lymphocytes. They may indicate intensification of IL-8 synthesis and excretion processes by the leukaemic lymphocytes. The conditions of the culture allow for the separation of lymphocytes from other types of cells. So most probably, the significant increase of IL-8 concentrations observed in our study corresponds to the excretory function of lymphocytes. It is interesting that processes of synthesis and excretion subside after a mitogene reaction. This may suggest the particular participation of external factors in the onset and progression of leukaemic processes.

On the surface of the endothelium vessel are adressins, which are ligands to lay receptors found on the surface of the lymphocyte. Coupling these ligands initiates different processes leading to migration through the vessel wall. Migration begins at the moment the cell approaches the vessel wall. Selectins L, E and P take part in stage I of adhesion – leukocyte rolling – along the internal wall of blood vessels and stage IV – diapedesis [2,6,17].

E-selectin manifests itself only on the surface of endothelium after stimulation, for example by IL-1, bacterial endotoxin (LPS), or tumor necrosis factor-α (TNF-α). In the advanced stages of CLL, its overproduction is most likely to occur. The soluble isoforms of this selectin can be expressed in the blood plasma at all times. The expression of sE-selectin is recognized as a final indicator of endothelium activation.

The increased E-selectin concentration found in the blood plasma is supported by some authors who discuss the activation of endothelium in the course of CLL [1,4,6,8]. A significant decrease in E-selectin concentration in I and III stages of CLL in culture supernatants and in broken lymphocytes has been found which may indicate degradation of E-selectin's soluble forms in the case of the culture. In the group after stimulation, a significantly decreased E-selectin concentration in comparison with nonstimulated groups seems to be of interest. The study of this dependence will allow us to find some way to understand the specific humoral activity of the endothelial cells.

Stage III – strong adhesion – takes part among other molecules from the immunoglobulin superfamily, which are co--receptors for integrin β_2 and are participants in the intercellular adhesion [18].

Soluble VCAM-1 is liberated from previously activated endothelial cells. It is known from the literature that concentrations of sVCAM-1 are higher in patients with inflammation, arteriosclerosis, coronary artery disease and leukaemia [19].

Our studies show a significant increase in sVCAM-1 concentrations in the blood plasma of CLL-patients which appears to confirm this assumption. We also observed a significant decrease of VCAM-1 concentrations in the culture supernatant (N0) in CLL-patients. It is proposed that this molecule was subjected to some biodegradation in the course of the culture. Also it may give evidence of the less than physiological resistance of this substance to exogenous agents. For example: temperature, partial pressure of CO_2 or a lack in the culture medium are factors which consolidate the structures of VCAM-1 to protect against degradation.

A significant increase of VCAM-1 concentrations in isolated, broken lymphocytes after Neupogen stimulation may indicate the tearing off and inactivation of the molecules bound with the membrane receptors of lymphocytes during the course of mitogenic action.

Changes in substances related to the cytokine network are characterized by proliferative disorders. Enhanced values of interleukins and adhesion molecules observed in our examination may give evidence of their increase during excretion. There are no dysfunctional regulation systems of lymphocyte function during early stage of CLL. Permanently ongoing processes act in order to prevent and control the growth of cells and their excessive migration to the surrounding tissues. In vivo cytokines may undergo decomposition, inactivation or destruction by substances located in the circulatory system [20-24]. Our studies appear to confirm this assumption. In blood plasma we observed the highest concentrations of cytokines in stage III in comparison with stage I of CLL. The repair processes take place only in the patient, where many cell lines react simultaneously. In isolated cultures, with specific conditions, we can

study the excretion function of only one type of cell. The significant increase of the investigated interleukin's concentrations in nonstimulated supernatant cultures in CLL may suggest the greatest ability of leukaemic lymphocytes to excrete them. The addition of Neupogens to the cultures, imitates the effect of mitogenes in a CLL-patient's body. A significant increase of IL-8 and VCAM-1 concentrations may support the essential role of mitogens in cell multiplication processes. The significant decrease of concentrations of E-selectin in nonstimulated groups is interesting. It is probably connected with the activation of degradation processes.

In conclusion, significant increase of E-selectin, IL-8 and VCAM-1 concentration in blood plasma may support higher activity and suggest the higher ability of leukaemic lymphocytes to migration. Increased IL-8 concentration in isolated, broken, stimulated lymphocytes may be a characteristic feature of the biochemical processes of leukaemic lymphocytes. The lowest values of E-selectin and VCAM-1 concentrations in culture supernatants and broken lymphocytes may suggest their degradation during of the culture.

These investigations may appear useful in the future as an additional diagnostic index of lymphocyte migration.

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