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Evaluation of TNF superfamily molecules release by neutrophils and B leukemic cells of patients with chronic B – cell lymphocytic leukemia

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It was demonstrated that TNF superfamily proteins may affect significantly the time of leukemic cells' survival in the course of B-cell chronic lymphocytic leukemia (B-CLL).

The aim of our study was to evaluate the expression and release of BAFF (B-cell activating factor), APRIL (a proliferation-inducing ligand) and TRAIL (TNF-related apoptosis inducing ligand) molecules belonging to the cytokines of the superfamily of the tumor necrosis factor (TNF) by neutrophils (PMNs) and, for comparison, B cells isolated from the blood of patients with B-CLL vs. their concentration in the blood serum. 40 patients suffering from B-CLL and a control group of 15 healthy subjects were included in the study. Cytoplasmic fractions of PMNs and B cells were analyzed with the use of western blotting for the presence of TRAIL, BAFF and APRIL. Soluble TRAIL, BAFF and APRIL in the culture supernatants and the serum were assessed using ELISA kits.

PMNs and B cells of patients with B-CLL before treatment demonstrated the statistically significantly higher expression of APRIL and BAFF proteins when compared with the control group of healthy subjects. In contrast, the expression of TRAIL protein in both types of cells of patients was statistically significantly lower than its expression in the control cells.

In the supernatants of PMN and B lymphocytes of patients the decreased concentrations of sBAFF, unchanged of APRIL and increased of sTRAIL molecules were demonstrated.

The results of studies carried out in patients with B-CLL before treatment indicate that the relations demonstrated between APRIL, BAFF and TRAIL molecules, released by neutrophils and B cells and relations between their concentrations in the serum can significantly influence the development of B-CLL.

Key words: B-cell chronic lymphocytic leukemia (B-CLL), tumor necrosis factor (TNF), B-cell activating factor (BAFF), a proliferation-inducing ligand (APRIL), TNF-related apoptosis-inducing ligand (TRAIL)

BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) molecules belong to the cytokines of the superfamily of the tumor necrosis factor (TNF) [1-3]. According to various authors' studies, BAFF and APRIL molecules control homeostasis of B lymphocytes, their differentiation, maturation and survival in the physiological and pathological states, including neoplasms. It was proved that the increased expression of these molecules induced the development of B cells in chronic B-cell lymphocytic leukemia (B-CLL) [4, 5]. TRAIL molecule was also found to play a role in the survival of B cells in B-CLL [6].

BAFF (BLyS) has been detected both as a type-II membrane-bound form (mBAFF, 32kDa) and a soluble form (sBAFF, 17kDa). BAFF molecule has been proved to have its biological effects via binding specific receptors, such as TACI, BCMA and BAFF-R [5, 7]. APRIL protein, known as TNFSF13, also binds to TACI and BCMA, but not to BAFF-R. A specific receptor for APRIL has not been identified so far. APRIL molecule is present only in a soluble form [7, 8].

TRAIL (TNF-related apoptosis inducing ligand) molecule, also belonging to TNF family proteins, shows an opposite effect to BAFF and APRIL and plays a role in the elimination of neoplastic cells, including leukemic cells, in 60% of cases [2, 3]. TRAIL, also known as Apo-2L, can be biologically effective as an integral membrane protein (mTRAIL, 32kDa), as well as a soluble cytokine (sTRAIL, 24kDa) [3]. TRAIL exerts its activity by interacting with a system of two death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) and three decoy receptors, which are devoid of functional death do-

mains (TRAIL-R3/DcR1 and TRAIL-R4/DcR2) or produced as a secreted protein (osteoprotegerin) [2, 3, 9].

Our earlier studies carried out in patients with B-CLL showed a decreased level of TRAIL molecule in the serum of patients with B-CLL before treatment, which may lead to impaired apoptosis of leukemic B cells [10]. The evaluation of other molecules of this superfamily having properties promoting the survival of B cells may contribute to the better knowledge of B-CLL pathomechanism.

BAFF, APRIL and TRAIL molecules were detected in monocytes /macrophages, dendrite cells and activated T lymphocytes [1, 2, 7]. Neutrophils, taking part in an early stage of an anti-cancer response, are also a significant source of these molecules [11-13].

The changes in BAFF, APRIL and TRAIL levels secreted by these cells may affect significantly the time of leukemic cells' survival in the course of B-CLL [6, 14, 15, 16].

The aim of our study was to evaluate the expression and release of BAFF, APRIL and TRAIL proteins by neutrophils and, for comparison, B lymphocytes isolated from the blood of patients with B-CLL vs. their concentration in the blood serum.

The examinations can define the role of both cell groups in the secretion of proteins of TNF superfamily in patients with B-CLL, which may markedly widen the knowledge about the pathomechanism of this disease and form the basis for development of potential methods of immunotherapy. Simultaneous assessment of sBAFF, APRIL and sTRAIL concentrations in the blood serum can indicate a significant source of these proteins in the circulation of the study patients.

Materials and methods

Patients. Serum samples were collected from 40 patients with B-CLL in stage I, II, III and IV according to the Rai classification, hospitalized in the Department of Hematology, Medical University of Bialystok (aged 40-75 years, mean 62.1). The samples were investigated before and after treatment. The diagnosis of leukemia was based on clinical observation, morphological composition of the peripheral blood, bone marrow puncture, trepanobiopsy, lymph node biopsy and cytochemical examinations. A flow cytometer EPIX XL (Coulter, USA) was used to identify immunophenotypes of leukemic cells. The monoclonal antibody panels of CD5, CD19, CD23 and CD20 were applied to differentiate B cells. In all analysed samples B lymphocytes presented more than 55% of positive $CD19^+$/ $CD/5^+$. Patients were graded according to the Rai staging system as follows: low-risk disease (stage 0), intermediate-risk (stage I and II) and high-risk (stage III and IV) [17, 18]. Patients with accompanying acute inflammatory bacterial, viral, mycotic or allergic states were excluded from the study.

Control group. The control group consisted of 15 healthy subjects (10 women and 5 men) of the same age (employers of Medical University of Bialystok).

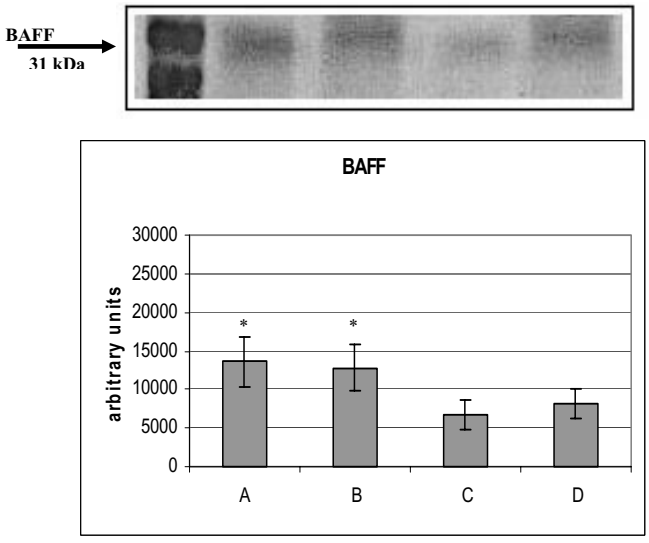
Preparation of neutrophils (PMNs) and B cells. PMNs and B cells were isolated from the whole blood treated with EDTA by density centrifugation, using Polymorphoprep (Axis-Shield, Oslo, Norway) (density – 1.113g/ml). This method enables simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMCs), involving B lymphocytes and polymorphonuclear cells, involving neutrophils. Neutrophils and B cells were separated by positive selection using the MidiMACS magnetic separation system (Miltenyi Biotec, Germany). MicroBeads conjugated to monoclonal anti-human CD16 antibodies were used to separate neutrophils in the magnetic field of a MACS Separator. MicroBeads conjugated to monoclonal anti-human CD19 antibodies were used to separate B cells in the magnetic field of a MACS Separator. The purity of isolated PMNs and B cells was determined by May-Grunewald-Giemsa-staining.

Western blot analysis Cytoplasmic protein fractions of PMNs and B lymphocytes B were analyzed with the use of western blotting for the presence of TRAIL, BAFF and APRIL. Cells were lysed directly in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich, CHEMIE GmbH P.O. Steinheim, Germany) by sonication using Vibra-Cell Ultrasonic Processor (Sonics&Materials, Inc., USA). Protein fractions were suspended in Laemmli buffer (Bio-Rad Laboratories, Herkules CA, USA) and next electrophoresed on SDS-PAGE. The resolved protein was transferred onto 0.2 μm pore-sized nitrocellulose (Bio-Rad Laboratories, Hercules CA, USA). The nitrocellulose was incubated at +4 °C for 20h with the primary polyclonal antibody anti-TRAIL, anti-BAFF, and anti-APRIL (R&D Systems). After washing in 0.1% TBS-T, the membrane was incubated at room temperature for 1h with alkaline phosphatase anti-mouse IgG Abs (Vector Laboratories, Burlingame, CA, USA). Immunoreactive protein bands were visualized following the addition of AP-Conjugate Substrate Kit (Bio-Rad Laboratories, Herkules CA, USA). Bands intensity was quantified using LabImage 1 D gel software.

Cytokine measurement by ELISA assays. Soluble TRAIL, BAFF and APRIL in the culture supernatants of PMNs and lymphocytes B were confronted with their serum levels. Soluble TRAIL and APRIL concentrations were assessed using ELISA kits R&D Systems (Minneapolis, USA), soluble BAFF was assessed using ELISA kit Bender MedSystems (GmbH, Austria).

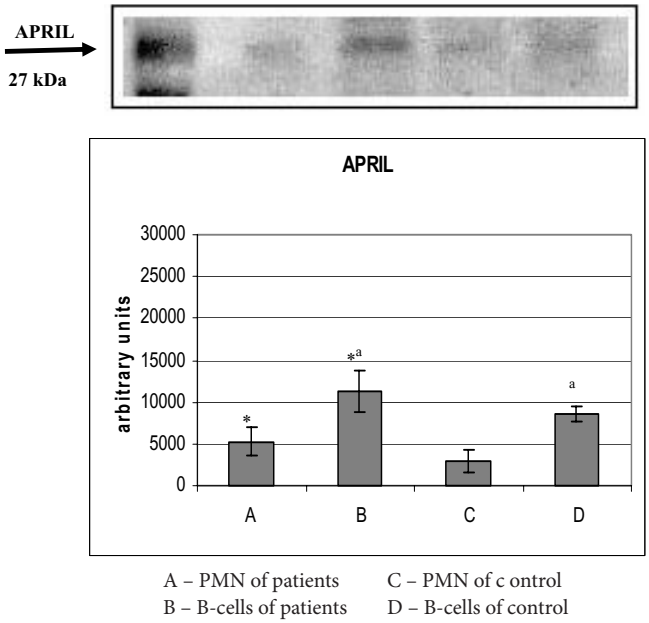
Results

Expression of BAFF, APRIL and TRAIL in PMNs and B cells of patients with B-CLL. Western blot analysis showed that the lysates of all examined groups of cells contained a 31 kDa protein that was stained by anti-BAFF polyclonal antibody (Fig. 1). PMNs of patients with B-CLL before treatment demonstrated the statistically significantly higher expression of BAFF protein in comparison to the control group of healthy subjects. Results obtained revealed also a statistically significantly higher expres-



* – significant difference with control (p<0.05)

Figure 1. Western blot analysis of cellular BAFF in PMNs and B cells from control and patients with B-CLL.



* – significant difference with control (p<0.05)
[a] – significant difference between B-cells and PMN (p<0.05)

Figure 2. Western blot analysis of cellular APRIL in PMNs and B cells from control and patients with B-CLL.

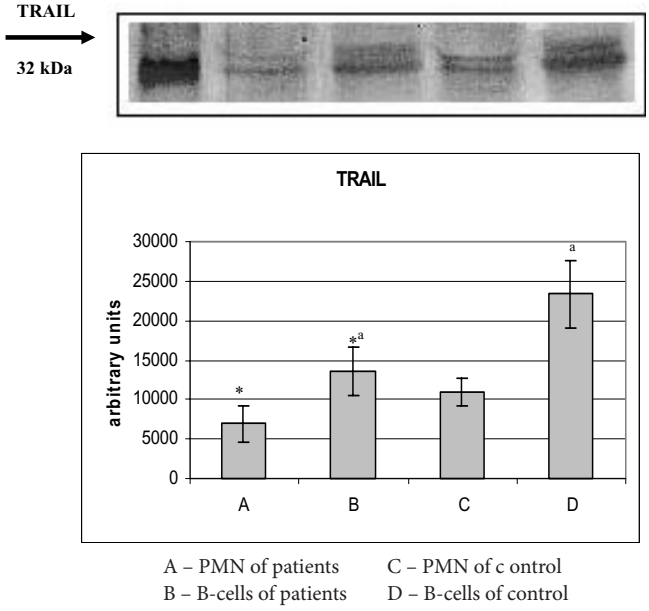
sion of BAFF protein in $CD19^+$ – sorted B cells of patients as compared to the expression in the control group.

The presence of cellular APRIL protein, a 27 kDa, in lysates of all examined cells was confirmed with the use of specific anti-APRIL polyclonal antibody. PMNs and B cells of patients showed the statistically significantly higher expression of APRIL in comparison to the control group (Fig. 2). Similarly to control, PMNs of patients revealed the lower expression of this protein than in B cells.

The presence of cellular TRAIL molecule, a 32 kDa, in lysates of all examined cells, was confirmed also by using anti-TRAIL polyclonal antibody (Fig. 3). The expression of TRAIL protein in both types of patients' cells was statistically significantly lower than its expression in the control cells. Similarly to the control, PMNs of patients showed lower expression of TRAIL than in B cells.

The concentration of BAFF, APRIL and TRAIL in supernatants of PMNs and B cells and the serum by ELISA. In the supernatants of PMNs isolated from the blood of patients with B-CLL in all stages, lower concentrations of sBAFF molecule and unchanged concentrations of APRIL were determined compared to the values obtained in controls (Table 1). There were no differences in their concentrations between patients in different stages. The concentrations of sTRAIL in the supernatants of PMNs of patients in all stages were statistically significantly higher than in the control.

In the supernatants of autologous $CD19^+$B cells of patients, sBAFF concentrations were significantly lower than those in controls and unchanged concentrations of APRIL molecules



* – significant difference with control (p<0.05)
[a] – significant difference between B-cells and PMN (p<0.05)

Figure 3. Western blot analysis of cellular TRAIL in PMNs and B cells from control and patients with B-CLL.

(Table 2). In contrast, sTRAIL concentrations in the supernatants of these cells were significantly higher in all patients compared to controls.

Table 1. Concentrations of APRIL, sBAFF and sTRAIL in the supernatants of PMNs of patients with B-CLL.

		PMNs		
		APRIL(ng/ml) Median±Min./Max.	**sBAFF**(ng/ml) Median±Min./Max.	**sTRAIL**(ng/ml) Median±Min./Max.
Control		**2.71±1.46/5.31**	**0.12±0.085/0.18**	**0.17±0.12/0.28**
Patients	**Stage 0**	**2.21±1.89/2.44**	**0.074*±0.05/0.21**	**0.34*±0.25/0.50**
	Stage I/II	**2.91±1.22/5.94**	**0.061*±0.05/0.13**	**0.35*±0.11/0.46**
	Stage III/IV	**2.45±1.89/2.91**	**0.071*±0.02/0.15**	**0.48*±0.12/0.49**

*- significant difference with control ($p<0.05$)

Table 2. Concentrations of APRIL, sBAFF and sTRAIL in the supernatants of B cells of patients with B-CLL.

		B cells		
		APRIL(ng/ml) Median±Min./Max.	**sBAFF**(ng/ml) Median±Min./Max.	**sTRAIL**(ng/ml) Median±Min./Max.
Control		**2.67±1.63/5.15**	**0.13±0.08/0.21**	**0.13±0.06/0.29**
Patients	**Stage 0**	**3.61±1.30/5.64**	**0.064*±0.04/0.17**	**0.46*±0.18/0.63**
	Stage I/II	**3.58±1.11/6.61**	**0.052*±0.03/0.05**	**0.41*±0.01/0.48**
	Stage III/IV	**3.89±1.59/3.72**	**0.044*±0.01/0.34**	**0.39*±0.12/0.69**

*- significant difference with control ($p<0.05$)

Table 3. Concentrations of APRIL, sBAFF and sTRAIL in the serum of patients with B-CLL.

		Serum		
		APRIL(ng/ml) Median±Min./Max.	**sBAFF**(ng/ml) Median±Min./Max.	**sTRAIL**(ng/ml) Median±Min./Max.
Control		**2.17±1.04/6.51**	**0.98±0.39/1.68**	**0.30±0.13/0.49**
Patients	**Stage 0**	**5.06*±1.39/8.79**	**0.42*±0.21/0.72**	**0.61*±0.50/0.75**
	Stage I/II	**6.81*±0.16/9.56**	**0.32*±0.19/0.43**	**0.50*±0.02/0.81**
	Stage III/IV	**7.91*±2.13/9.72**	**0.57*±0.16/0.98**	**0.63*±0.03/0.71**

*- significant difference with control ($p<0.05$)

No significant differences in the study of the molecules secretion were found between PMN and B cells, both in controls and patients.

The evaluation of the examined proteins concentrations in the blood serum of patients with B-CLL proved a significant increase in the concentrations of APRIL and sTRAIL molecules in comparison with controls, whereas the concentrations of sBAFF molecule were significantly lower when compared to controls (Table 3).

The analysis of relations between concentrations of the molecules study in cell supernatants and their values in the blood serum proved a correlation between concentrations of BAFF molecule in PMN supernatants and the blood serum ($r=0.648$, $p<0.05$). Similarly, such a correlation was observed between BAFF concentrations in B cells supernatants and in the blood serum of patients ($r=0.849$, $p<0.001$).

Discussion

There are numerous proofs that changes in the production and release of cytokins by the immune system cells may influence the development of the neoplastic process [4, 13, 16].

Our studies carried out in patients with B-CLL proved the altered ability of their neutrophils and leukemic B cells to secrete APRIL, sBAFF and sTRAIL molecules. No changes in the secretion of APRIL and, unexpectedly, an increase in the release of sTRAIL molecules accompanied the lower secretion of sBAFF by these cells. However, the results obtained by the Western blot method indicated different expression of designated proteins in comparison with their secretion by both cell groups. The high expression of cellular APRIL and BAFF in PMNs and leukemic B cells at their unchanged or decreased secretion may be due to the deficiency of factors influencing

their release. The studies of other researchers proved that in case of neutrophils, APRIL and BAFF forms were transformed intracellularly into biologically active soluble forms by means of furin convertase associated with the Golgi apparatus and were released outside the cell in the presence of some cytokines [7, 8, 12].

IFN-γ is a known factor influencing the expression and release of APRIL and BAFF, whose secretion by lymphocytes in the course of B-CLL is decreased [19]. A low level of IFN-γ may affect also the impaired intracellular accumulation of TRAIL in PMNs, which is connected with its increased secretion in the soluble form (sTRAIL) [20].

Taking into consideration biologic properties of the molecules, the changes observed may lead to significant clinical implications in patients. The decreased secretion of sBAFF and the unchanged secretion of APRIL by neutrophils, molecules influencing the growth and proliferation of neoplastic B cells, indicate a favorable role of these cells in all study patients. This was confirmed by the observation regarding the simultaneous increase in the release of the molecule sTRAIL by neutrophils, initiating apopotosis in neoplastic cells [3]**.** Since neutrophils are the cells of an early phase of the immune response, the changes mentioned above may play a special role in the early stages of cancer development [13, 20, 21]. On the other hand, the quantitative, ten-fold-higher predominance of APRIL molecule over sTRAIL molecule may balance a favorable activity of TRAIL and favor the development of B-CLL, especially, because leukemic B cells are characterized by the resistance to TRAIL activity [5].

Similar changes, with regard to APRIL, sBAFF and sTRAIL molecules, were observed in case of authologic leukemic B cells. Although unchanged APRIL secretion accompanied the increased sTRAIL release, the quantitative predominance of APRIL over sTRAIL may prolong the survival time of leukemic cells in the autocrine and /or paracrine way [1, 16, 22]. There are available data indicating the expression of receptors for APRIL on B cells, which if bind to an appropriate ligand, contribute to their survival, which was observed *in vitro* conditions [16].

Though a specific receptor for APRIL has not been identified so far, the examinations proved that apart from the capability of binding to the specific receptors for BAFF – TACI and BCMA, APRIL showed the ability to bind to heparin proteoglycan (HSPG) present in neoplastic cells [8]. It was established that APRIL-HSPG interaction play a key role in the activity promoting tumor growth.

The more detailed studies proved that the extended time of B cells survival by APRIL was associated with the inhibition of these cells apoptosis in the way dependent on the activation of the classical way of NF-κB. Endo's et. al. studies confirmed that in leukemic B cells, the molecule of APRIL was able to activate the classical pathway of NF-κB activation in the course of B-CLL [15].

Conversely to numerous other cytokines released by neutrophils, the molecules of TNF superfamily synthesized and released by them reached the level comparable to the level observed in mononuclear cells such as monocytes or dendritic cells [12].

The results of our study indicated that the expression and secretion of these proteins in neutrophils and in normal and leukemic B cells were at the same level. The findings mentioned above confirmed a considerable role of neutrophils as a source of these molecules, both in the physiological state and in the course of B-CLL.

The changes in the secretion of the study molecules of the TNF superfamily by neutrophils and leukemic B cells may influence their level in the circulation. Our study proved high concentrations of APRIL molecule and low concentrations of sBAFF molecule in the serum of patients with B-CLL compared to healthy controls. Similar results were obtained by Bojarska's et al. and Planelles et al., who observed an increase in the concentrations of circulating APRIL molecule and the decreased concentrations of sBAFF molecule in the same group of patients [14, 16]. Patients with high concentrations of APRIL had significantly worse prognosis compared to patients with lower concentrations of this molecule [16].

Apart from the changes in APRIL and sBAFF concentrations, a high concentration of sTRAIL was determined in the serum of the patients with B-CLL, which was in accordance with the results of our previous studies [10].

The relations between these molecules in the serum of patients seem to reflect the relations between them observed in neutrophils and leukemic B cells. The correlations proved between BAFF secretion by both groups of cells and its concentration in blood serum obviously indicated that they were a significant source of BAFF molecule in the patients' circulation.

Summing up the results of studies carried out in patients with B-CLL before treatment, the relations demonstrated between APRIL, BAFF and TRAIL molecules, in the supernatants of neutrophils and B cells and in the serum can significantly influence the development of B-CLL. Further studies including the analysis of the study of parameters after treatment are required to evaluate a potential diagnostics and prognostic significance of the findings. The results obtained may contribute to the development of new methods of supportive immunotherapy based on the modulation of expression and release of the study molecules of the superfamily of TNF not only by leukemic B cells but authologic neutrophils as well.

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