Overexpression of B cell-activating factor (BAFF) in neutrophils of oral cavity cancer patients – preliminary study

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In the present study the expression of tumor-promoting B cell-activating factor (BAFF), a member of the tumor necrosis factor superfamily (TNF) in neutrophils from oral cavity cancer patients, was examined by real-time PCR. For the purpose of comparison, the expression of BAFF protein was assessed in autologous peripheral blood mononuclear cells (PBMCs). An important question of this study has also been to explain the role of NF-κB in the induction of BAFF molecule.

The increased expression of BAFF at the mRNA and protein levels in neutrophils and mononuclear cells of patients before and after treatment were accompanied by the increased expression of phospho-IκB protein level. Demonstrated excessive expression and secretion of BAFF by examined leukocytes suggest a tumor-promoting activity of those cells in oral cavity cancer patients. The overexpression of BAFF, observed at mRNA and protein levels in PMNs and PBMCs, as well as the secretion of soluble form of sBAFF by these cells, accompany the increased concentrations of sBAFF in the serum of patients.

Observations above suggest that the modulation of BAFF molecules in examined leukocytes and the levels thereof in the serum may have future implications for immunotherapy of oral cavity cancer patients.

Key words: oral cavity cancer, BAFF, PMNs, PBMCs

There is a number of observations indicating that inflammation triggered by tumor-infiltrating host leukocytes influences the tumor development [1,2]. A number of solid tumors are infiltrated by neutrophils that secrete different molecules and cytokines, modulating the effector cells' activity within the tumor microenvironment, as well as directly stimulating or inhibiting tumor growth [3,4,5]. It is known that neutrophils are able to produce ligands of the TNF-superfamily proteins, such as TNF-α, Fas ligand (FasL), CD30 ligand (CD30L), B cell-activating factor (BAFF), TNF (Tumor Necrosis Factor)-related apoptosis-inducing ligand (TRAIL) and a proliferation-inducing ligand (APRIL). Alterations in the production of those TNF-superfamily proteins by neutrophils can play a role in the proliferation, growth and survival of normal and transformed cells [5,6,7].

The previous study on patients with oral cavity cancer revealed high secretion of TNF-α and its natural inhibitor, the soluble receptor sTNFRp75, as well as deficit in the secretion of soluble TRAIL by neutrophils and mononuclear cells of peripheral blood [8,9]. The restriction of TNF-α biological availability by sTNFRp55 and insufficient secretion of tumor-inhibiting sTRAIL may be the factors responsible for development of malignant process in those patients. The deficit of TRAIL appears to have particular importance because the oral squamous carcinoma cells express TRAIL receptors, making them sensitive to TRAIL-induced apoptosis [10,11].

The ultimate effect of TNF-α and TRAIL expression in patients with oral cavity cancer may result from the relation to the expression of other TNF superfamily proteins, acting as tumor-promoting factor, such as BAFF, produced in large amounts by human neutrophils [5].

BAFF (also known as BLyS, TALL-1, THANK and TNFSF-13B) has been detected both as a type-II membrane-bound form (mBAFF, 32kDa) and a soluble form (sBAFF, 17kDa) [12,13]. In neutrophils, BAFF is processed intracellulary by a Golgi-associated furine pro-protein convertase, prior to the secretion of the biologically active sBAFF [5,14].

BAFF exerts its activity by interacting with three receptors: transmembrane activator and CAML interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R) [12,13]. The expression of TACI was discovered predominantly on CD27+ memory B cells, BCMA on plasma cells, plasmablasts and tonsillar germinal center B cells and BAFF-R, on all peripheral T and B cells [13].

The physiological role of BAFF is mainly associated with B-cell maturation and survival. [12,13,15]. Study by Hase et al. on B cells showed that BAFF up-regulates Pax5 activity and its downstream target CD19, a component of the B cell co-receptor complex that plays an important role in B-cell differentiation [15]. The role of BAFF as a survival factor for B cells was confirmed by research, which indicated that BAFF induces expression of anti-apoptotic molecules in B cells [12]. Additionally, BAFF acts as a proliferation factor of activated B cell and promote Ig switching of IgD+B cells [16].

BAFF has also been shown to co-stimulate human T-cell activation and to regulate T-cell responses [12,13,17]. Sutherland et al. have demonstrated that BAFF augments Th1 responses, enhancing inflammatory response [17].

Various clinical studies have demonstrated that interaction between BAFF and its receptors is capable of regulating the survival and proliferation of different malignant cells [13]. A tumor-promoting function of BAFF has been reported particularly in various B-cell malignancies, as well as in human breast or esophageal cancers [12,18,19].

In order to better understand the mechanism responsible for the development of oral cavity cancer we took interest in the expression and secretion of BAFF molecule by leukocytes, such as neutrophils. Available data indicated that cytokine generation in human neutrophils is influenced by distinct MAPK and NF-κB pathways, but NF-κB activation appears to be a central event in the constitutive and inducible expression of various cytokines in these cells [20].

Taking the above into consideration, we examined the BAFF expression in neutrophils and, for comparison, in mononuclear cells of patients with oral cavity cancer. Furthermore, we assessed the involvement of NF-κB pathway in this molecule induction. The obtained results may be helpful for better understanding of the role of neutrophils in tumor development in confrontation with mononuclear leukocytes and indicate new perspectives for cancer immunotherapy based on the modulation of TNF superfamily proteins.

Table 1. Concentrations of sBAFF in the supernatants of PMNs, PBMCs and the serum of patients with oral cavity cancer.

	sBAFF (ng/ml)		
	Control	Patients	
		Before treatment	After treatment
PMNs	0.12 ± 0.04	0.29* ± 0.09	0.32* ± 0.11
PBMCs	0.15 ± 0.05	0.33* ± 0.13	0.41* ± 0.19
Serum	1.01 ± 0.34	2.25* ± 0.97	2.75* ± 1.1

* – statistical differences with control (p<0.05)

Materials and methods

Patients. Examined were 21 patients, aged 45 to 49, with squamous cell carcinoma of oral cavity treated in the Maxillofacial and Plastic Surgery Clinic at the Medical University of Bialystok (Table 1). Examinations were carried out on patients before the treatment and 3 weeks after the surgical removal of the tumor mass. Patients did not receive any treatment or medication before the examination. No clinical signs of infection were observed in patients. Furthermore, patients did not receive the radiotherapy or chemiotherapy during 2 months after surgery. Patients had no significantly increased leukocytosis. Control subjects (n=15) were healthy people aged from 30 to 53 years (average: 41.5 years old).

The study was approved by the Ethics Committee of the Medical University of Bialystok and all patients submitted their consent in writing.

Preparation of PMNs and PBMCs. Cells were isolated from whole blood treated with EDTA by density centrifugation, using Polymorphprep (Axis-Shield, Oslo, Norway) (density – 1.113g/ml). This method enables simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMCs) and polymorphonuclear cells, involving neutrophils (PMNs). The purity of isolated PMNs and PBMCs was determined by May-Grunewald-Giemsa-staining.

CD16 positive PMNs separation. PMNs were separated by positive selection using Midi MACS magnetic separation system (Miltenyi Biotec, Germany). For the separation, MicroBeads conjugated to monoclonal anti-human CD16 antibodies were used. The MACS Column was placed in the magnetic field of a suitable MACS Separator and rinsing of MACS Buffer. Isolated PMNs were suspended in MACS Buffer (up to 5×10^7 total cells) and incubated with CD16 MicroBeads for 30 min at 4-8°C. After washing, the cells were suspended in MACS Buffer.

RNA isolation and cDNA synthesis. For real-time PCR, total RNA was isolated from 10^7 untreated and stimulated neutrophils or peripheral blood mononuclear cells (RNeasy Mini Kit, Qiagen, Germany) was used to isolate total RNA from PMNs and PBMCs, according to the manufacturer's specification. The amount of RNA was measured by spectrophotometry (QuantGen Biopharmacia). RNA integrity was verified by 1.5% agarose gel electrophoresis identifies by ethidium bromide staining and $OD_{260/280}$ absorbance ratio >1.95. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using SuperScript TM First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's specification in the MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

Real-time PCR. The levels of transcripts were measured by real-time PCR using human genes QuantiTec Hs_TNFSF113B_2_SG Assay (Qiagen) and QuantiTec Hs_PRS18_1_SG Assay (s18) (Qiagen) as normalizer.

Real-time PCR was performed in duplicate in 20μl using the QuantiTect SYBR Green PCR Master Mix (Qiagen) following the manufacturer's instruction and carried out in the Chromo4
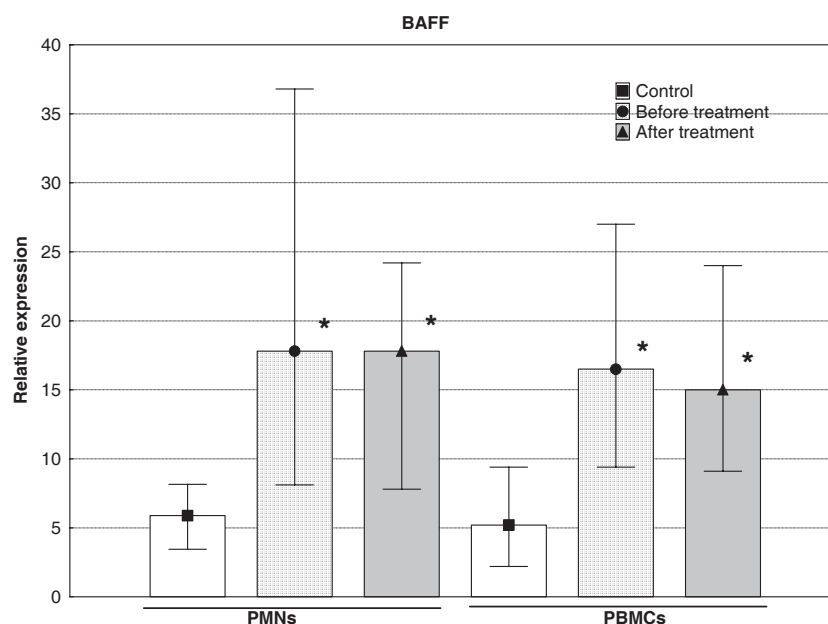
Figure 1. Real-Time PCR was performed on neutrophils and mononuclear cells from peripheral blood of patients and the control. mRNA of BAFF levels was normalized to house-keeping gene – s18. * – a statistically significant differences with control (p<0.05).

Real-time PCR Detector (BIO-RAD, USA). The thermal cycling conditions included an initial activation step at 95°C for 15 min, followed by 40 cycles of denaturation, annealing and amplification (95°C for 30, 55°C for 30 s, 72°C for 30 s). At the end of the amplification phase a melting curve analysis was carried out on the product formed. The fluorescent data collection was performed during the annealing step.

A standard curve construction was generated employing a serial of four dilutions of cDNA derived from unstimulated cells in reaction with the house-keeping gene – s18. Based on these curves, the levels of total *TNFSF13B* transcripts were calculated after normalization of *TNFSF13B* products to s18. The value of C_T was determined by the first cycle number at which florescence was greater that the set threshold value. To calculate our data, we used the comparative C_T method for relative quantification (ΔC_T method).

Western blot analysis. Cytoplasmic protein fractions of PMNs and PBMCs were analyzed with the use of Western blot for the presence of BAFF and phospho-IκB as a sign of NF-κB activation. 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 electrophorezed 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-BAFF or monoclonal antibody anti-phospho-IκB (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 BCIP/NBT Liquid Substrate System (Sigma-Aldrich, Steinheim, Germany). Band intensity was quantified using ImageJ method and estimated by arbitrary units (AU).

The antibody against beta-actin (1:1000; Santa Cruz Biotechnology), which detects the expression of beta-actin in cells lysates, was used as an internal control.

ELISA method. Soluble BAFF concentrations in the supernatants of PMNs and PBMCs were confronted with the serum levels using ELISA kit by Bender MedSystems (GmbH, Austria).

Statistical analysis. Statistical analysis was performed with a statistic package – Statistica 6.0 software. Nonparametric U Mann-Whitney test was used. Results were expressed as median, minimum, and maximum values. For analysis correlation Pearson's linear correlation was used. The p-values below 0.05 were considered statistically significant.

Results

mRNA expression of BAFF analyzed by real-time PCR. The real-time PCR analysis revealed increased mRNA levels of BAFF in neutrophils in patients before treatment, in comparison with control cells (Fig. 1). BAFF was overexpressed in 17 out of 21 patients. It is important to note that the expression of BAFF-mRNA in the cells of patients after surgical treatment remained on the same levels. High expression of BAFF was found in 15/21 examined patients after treatment.
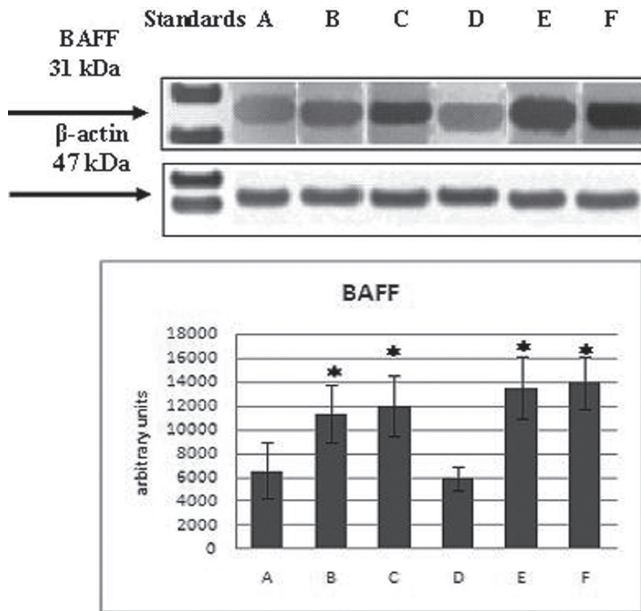
Figure 2. Western blot analysis of BAFF in PMNs and PBMCs cells from patients with oral cavity cancer before and after treatment and control subjects. Representative of five independent samples of blood are shown. A – PMNs of control, B – PMNs of patients before treatment, C – PMNs of patients after treatment, D – PBMCs of control, E – PBMCs of patients before treatment, F – PBMCs of patients after treatment. * – statistical differences with control (p<0.05).
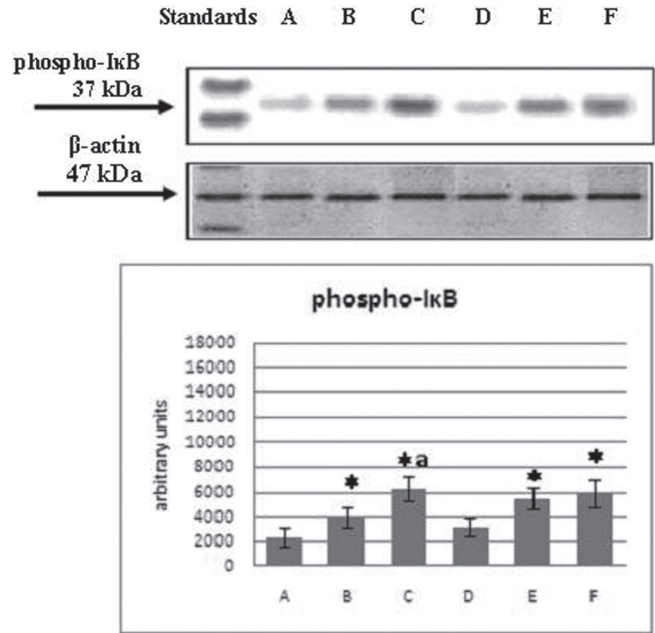
Figure 3. Western blot analysis of phospho-IκB protein expression in PMNs and PBMCs of patients before and after treatment and control subjects. Representative of five independent samples of blood are shown. A – PMNs of control, B – PMNs of patients before treatment, C – PMNs of patients after treatment, D – PBMCs of control, E – PBMCs of patients before treatment, F – PBMCs of patients after treatment. * – statistical differences with control (p<0.05), a – statistical differences between patients before and after treatment (p<0.05).

Similarly to PMNs, a significant increase in the mRNA levels of BAFF in autologous PBMCs of patients before treatment was also observed (Fig. 1). Overexpression of BAFF was found in 16 out of 21 patients. In patients after treatment those cells exerted the same high levels of BAFF-mRNA expression (17/21 patients).

There were no significant differences in the mRNA expression of BAFF between PMNs and PBMCs of patients and the control (Fig. 1).

No significant differences were found in the expression of BAFF-mRNA in PMNs and PBMCs with respect to disease advancement and location (data not presented).

BAFF and phospho-IκB expressions in PMNs and PBMCs analyzed by Western blot . Western blot analysis showed that the lysates of PMNs and PBMCs of cancer patients and the control contained 31 kDa protein, stained by anti-BAFF polyclonal antibody (Fig. 2). PMNs and PBMCs of patients before and after treatment demonstrated higher expression of BAFF protein in comparison with the cells of the control group (Fig. 2). The analysis of BAFF protein expression between PMNs and PBMCs lysates collected from patients revealed the same expression of BAFF in both types of cells (Fig. 2).

The expression of phospho-IκB protein (37 kDa), as a sign of NF-κB activation, was found in PMNs and PBMCs of cancer patients and the control (Fig. 3). Enhanced expression of

phospho-IκB was observed in the cells of patients before and after treatment, as compared to control. There were no differences in the expression of phospho-IκB between examined leukocytes of patients before and after treatment.

sBAFF concentrations in the supernatants of PMNs, PBMCs and the serum. sBAFF concentrations in the supernatants of PMNs of patients before and after treatment were higher than those in the supernatants of the control cells (0.29+0.09 ng/ml and 0.32+0.11 ng/ml respectively *versus* 0.12+0.04 ng/ml). Similarly to PMNs, the concentrations of sBAFF in the supernatants of PBMCs of patients before and after treatment were higher than those in the control cells (0.33+0.13 ng/ml and 0.41+0.19 ng/ml respectively *versus* 0.15+0.05 ng/ml). No significant differences in the concentrations of sBAFF between PMNs and PBMCs supernatants were observed.

In the serum of patients before and after treatment, sBAFF concentrations also increased in comparison to the control (2.25+0.97 ng/ml and 2.75+1.1 ng/ml respectively *versus* 1.01+0.34 ng/ml) (Table 2).

Discussion

The data concerning the role of PMNs in anti-tumor response are controversial [4,21,22,23]. In spite of phagocytosis or ADCC, the neutrophils are known to produce cytotoxic

mediators, including reactive oxygen species, peptides and cytokines that injure and eliminate tumor cells [4,21]. On the other hand, available data indicate that neutrophils may also promote tumor growth and metastasis [23].

The results of the present study, carried out on oral cavity cancer patients, appear to confirm the unfavorable role of neutrophils and mononuclear cells associated with high expression and secretion of tumor-promoting BAFF molecule.

Changes in BAFF expression in neutrophils and mononuclear cells of peripheral blood were associated with the activation of transcription factor NF-κB. The increased expression of BAFF at the mRNA and protein levels were accompanied by the increased expression of phospho-IκB protein level.

It is important to note that overexpression and secretion of BAFF by examined leukocytes were observed not only in patients before treatment, but also in patients after surgical procedure. The results described above suggest that changes in the expression of BAFF in PMNs and PBMCs are associated not only with the presence of tumor cells, but may also be triggered by the activity of different mediators, such as IFN-γ or G-CSF, responsible for expression mRNA of BAFF in human neutrophils [5]. However, cytokine analysis in patients with HNSCC, involving oral cavity cancer, revealed considerably decreased levels of IFN-γ [24]. In contrast, the constitutive expression of G-CSF, as demonstrated in HNSCC, may be responsible for increased expression of BAFF in examined leukocytes [25].

The overexpression of BAFF observed at mRNA and protein levels in PMNs and PBMCs, as well as the secretion of soluble form sBAFF by these cells, accompanied the increased concentrations of sBAFF in the serum of patients. The presence of considerable number of BAFF molecules can promote tumor development through selective activation of NF-κB. Constitutive activation of NF-κB was revealed in a wide variety of tumor types, including squamous cell carcinoma [26]. NF-κB regulates a number of genes involved in cancer promotion, clonal expansion, angiogenesis, adhesion, extravasation or degradation of extracellular matrix. NF-κB has also been shown to play a role in cancer cell apoptosis. Varied activation of NF-κB pathways may trigger changes in the expression of pro-apoptotic proteins, such as Fas, c-myc, p53, or anti-apoptotic proteins, such TRAF2 and Bcl-2 family molecules [27].

It has been demonstrated that anti-apoptotic effect of BAFF is associated with decreased levels of the pro-apoptotic protein Bax of the Bcl-2 family of proteins, participating in the regulation of mitochondrial signaling pathway [12,28]. Additionally, BAFF, through the activation of NF-κB or MAP kinases, may cause a strong up-regulation of the anti-apoptotic proteins, such as Mcl-1 and Bcl-2, i.e. other members of Bcl-2 family [28].

The unfavourable role of the examined leukocytes, associated with overexpression and secretion of BAFF, as demonstrated herein, may be enhanced by the presence of APRIL molecule, which, similarly to BAFF, acts as a tumor-promoting factor [29]. Mhawech et al. showed that tumor-infiltrating neutrophils in patients with squamous cell carcinoma (SCC) of the

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* – statistical differences with control (p<0.05)

oral cavity are a significant source of APRIL [6]. Furthermore, Toruner et al. have shown increased gene expression of APRIL in oral squamous cell carcinoma [29]. Simultaneously, the deficit of TRAIL, observed in previous studies, may facilitate tumor growth [8,11].

Concluding, the analysis of BAFF expression in neutrophils and mononuclear cells of patients with oral cavity cancer suggests a tumor-promoting rather than tumor-inhibiting activity of those cells, associated with overexpression and high secretion of BAFF. However, prospective studies on patients with potential premalignant disorders of oral cavity may confirm the role of this protein as a tumor-promoting factor in this location.

The changes to the secretion of TNF family proteins, together with changes to their serum concentrations, suggest the benefits of therapy based on the increased and/or decreased amount of those molecules in patients with oral cavity cancer. Available data from preliminary study demonstrate a therapeutic effect of recombinant human TRAIL (rhTRAIL) molecule in patients with the head and neck squamous carcinoma, involving the oral cavity cancer group. It has been confirmed that the application of rhTRAIL in combination with cisplatin resulted in a synergistic induction of death in tumor cells [30].

Current observations suggest future implications for immunotherapy in oral cavity cancer patients, based not only on the application of rhTRAIL, but also the modulation of BAFF molecule expression and secretion by leukocytes, and the levels thereof in the serum.

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