

## RESEARCH ARTICLE

# A proliferation-inducing ligand (APRIL) in neutrophils of patients with oral cavity squamous cell carcinoma

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**ABSTRACT.** Available data indicating a role for neutrophils in the tumor-host reactions are controversial. In 37 patients with oral cavity squamous cell carcinoma (OSCC), we investigated the expression of a tumor-promoting, proliferation-inducing ligand (APRIL) molecule by peripheral blood neutrophils isolated from blood samples collected at presentation and three weeks after surgery, and the serum levels of TGF- $\beta$  in the same samples. Additionally, we investigated the consequences of TLR4 activation by LPS for the synthesis of APRIL by those cells. The levels of mRNA for APRIL and TLR4 were measured using a real-time PCR method. Western blot analysis was used to assay the expressions of APRIL and ERK1/2 in cell lysates. The results of the present study revealed the unfavorable features of the detection, in the blood, of neutrophils displaying an enhanced expression of the tumor-promoting APRIL molecule. The increased expression and release of APRIL accompanying advanced stages of disease demonstrated by these cells, combined with the increased number of neutrophils, may be an important marker of disease progression in the patient group examined. Simultaneously, an increased level of circulating TGF- $\beta$  in the serum of these patients appeared to be associated with the overexpression of APRIL in their neutrophils. In contrast to the healthy controls, TLR4 expression and the ERK1/2 signaling pathway appear to play only minor roles in APRIL induction in the cells of patients with cancer. The changes presented in the current study suggest that modulation of the expression of tumor-promoting APRIL, in addition to TRAIL and BAFF, might be taken into account in the development of new strategies for supportive immunotherapy of OSCC disease and possibly for other types of neoplasm as well.

**Key words:** oral cavity squamous cell carcinoma (OCSCC), a proliferation-inducing ligand (APRIL), polymorphonuclear neutrophils (PMNs), Toll-like receptor 4 (TLR4), the extracellular signal-regulated kinases 1/2 (ERK1/2), transforming growth factor  $\beta$  (TGF- $\beta$ )

There is a good deal of evidence suggesting that chronic inflammation is involved in tumor promotion and progression [1]. Available data indicating a role for tumor-infiltrating, inflammatory cells, such as polymorphonuclear neutrophils (PMNs), in the tumor-host reactions are controversial [2, 3]. Conflicting results were also observed in patients with oral cavity squamous cell carcinoma (OCSCC), a type of head and neck squamous cell carcinoma (HNSCC), which is the sixth most frequent cancer in the world. A favourable anti-tumor aspect of neutrophil activity, associated e.g. with cytotoxic effects of human neutrophil peptide-1 (HNP1), has been demonstrated [4]. Unfavourable effects may be caused by oxidative stress or secretion of proangiogenic factors by these cells [5, 6].

Important mediators in the relationship between inflammation and cancer include cytokines produced by activated immune cells, as well as by cancer cells themselves [1].

Neutrophils are the first cells recruited from peripheral blood to tumor, and are a source of inflammatory cytokines, modulating the tumor microenvironment. It is known that these cells also have the ability to synthesize cytokines that can play a direct role in the proliferation, growth and survival of tumor cells in the local and systemic compartments [7, 8].

The circulating cancer cells observed in patients with OCSCC are in direct contact with neutrophils in the peripheral blood, as is the case within the tumor [9]. Our previous study, carried out in patients with OCSCC, revealed unfavorable features of peripheral blood neutrophils, associated with changes in the release of some proteins belonging to the tumor necrosis factor (TNF) superfamily. The high secretion of soluble TNFRp75 receptor that can limit the availability of TNF- $\alpha$  and deficit of tumor-inhibiting TNF-related apoptosis-inducing ligand (TRAIL), in addition to overexpression

of tumor-promoting B cell-activating factor (BAFF/Blys) in these cells, may facilitate tumor development in patients with OSCC [10-12].

Studies by Mhawech *et al.* [13] in patients with OCSCC, suggested that tumor-infiltrating neutrophils are the main source of another TNF-superfamily ligand, *i.e.* a proliferation-inducing ligand (APRIL), exhibiting tumor-promoting activity.

APRIL, also known as TNFSF13 or TALL-2, is a unique member of the TNF family. APRIL exists mainly as a secreted, soluble ligand and can also be expressed as a cell surface fusion protein with TWEAK, called TWEPRIL. APRIL is closely related to BAFF/Blys. The two ligands share two receptors: B cell maturation antigen (BCMA) and transmembrane activator and calcium signal-modulating cyclophilin ligand interactor (TACI) [14, 15]. However, neither receptor appears crucial for the tumor-promoting effects of APRIL. It has been demonstrated that the special receptors or binding partners for APRIL are heparan sulfate proteoglycans (HSPG), which play a role in the APRIL-mediated tumor-promoting action [16].

It has been shown that synthesis and expression of some TNF superfamily proteins in neutrophils can be modulated by exogenous and endogenous factors, including Toll-like receptor 4 (TLR4) ligands [17].

Toll-like receptor 4 (TLR4) is a member of the pattern recognition receptor family (PRRs), recognizing mainly the Gram-negative component (LPS) and *C. albicans*, respiratory syncytial virus (RSV), as well as endogenous agonists, such as heat shock proteins [18, 19]. TLR4 plays an important role in the initiation of signaling events that trigger the inflammatory response, which can influence tumor growth [18, 20]. Results from our laboratory demonstrated the role of LPS stimulation in the enhanced secretion of pro-inflammatory IL-1 $\beta$  and TNF- $\alpha$ , and increased secretion of nitric oxide (NO) by neutrophils in patients with OSCC [6, 12].

Our previous observations also indicated the involvement of TLR4 ligation by LPS in the induction of the APRIL molecule in human peripheral blood neutrophils. Furthermore, our results suggest that LPS encourages neutrophils to express APRIL through the ERK1/2 signaling pathway [21].

Among the endogenous factors, an important role in the biology of neutrophils is played by transforming growth factor  $\beta$  (TGF- $\beta$ ), which seems to be a major, proximal cytokine within tumors that defines the tumor-associated neutrophils (TAN) phenotype and skews differentiation toward the "N2" protumorigenic phenotype. Friendler *et al.* [22] suggested that "N2" phenotype cells are the majority of tumor-associated neutrophils (TANs), which may contribute to tumor growth and immunosuppression. In patients with OSCC, we investigated the activity of peripheral blood neutrophils associated with the expression of the APRIL molecule, in relation to the serum levels of TGF- $\beta$ . We also examined the effect of TLR4 ligation by LPS on the regulation of induction of this molecule.

The results obtained indicated that the enhanced expression and secretion of APRIL by neutrophils, and consequently its serum concentrations, accompanied tumor progression in patients with OSCC. Furthermore, the data presented

suggest that TGF- $\beta$  might be one of the factors responsible for the changes in APRIL expression, presumably associated with the polarization of neutrophils to the "N2" phenotype. In contrast to the healthy controls, TLR4 expression and the extracellular signal-regulated kinases 1/2 (ERK1/2) signaling pathway appear to play only minor roles in APRIL induction in the cells of cancer patients. The results obtained confirm a pro-tumorigenic activity of neutrophils, associated with the ability of these cells to synthesize some of the ligands belonging to the TNF superfamily.

## DONORS AND METHODS

### *Patients*

We examined 37 patients with squamous cell carcinoma of oral cavity (aged 45 to 59), treated in the Department of Maxillofacial and Plastic Surgery at the Medical University of Białystok. Study results were analyzed taking into account the clinical stage of the disease according to TNM classification (*table 1*). Examinations were carried out on patients at presentation and three weeks after the surgical removal of the tumor mass. For one week after surgery, patients received morphine sulphate pentahydrate (10 mg every 6 h for three days, Polfa Tarchomin S.A, Poland), paracetamol (1,000 mg every 12 h for seven days, Perfalgan; Bristol-Myers Squibb Pharmaceuticals, UK) for postoperative pain, and the antibiotic cephalosporin (1g every 12 hours for seven days, Tarfazolin, Polfa Tarchomin S.A). Eighty percent of patients were long-time tobacco users. Control subjects ( $n=15$ ) were non-smoking, healthy volunteers aged from 30 to 60 years (mean  $\pm$  SD: 42.5  $\pm$  15.3 years). None of the patients or control subject had concomitant diseases such as diabetes mellitus, liver disease, or rheumatoid arthritis.

**Table 1**  
Patient characteristics.

<b>All patients</b>	<b>37</b>
Male	29
Female	8
<b>Tumor localization</b>	
Oral cavity fundus	10
Tongue	5
Tongue + oral cavity fundus	5
Cheek mucosa	6
Inferior gingival	5
Lower lip	6
<b>TNM classification</b>	
T1/2	13
T3/4	24
<b>PMN count (%)</b>	
T1/2	51.3
T3/4	71.9

Three weeks following surgery, no clinical signs of infection were observed in patients. Patients had no significantly increased leukocytosis.

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

### **Isolation and culture of PMNs**

Peripheral blood neutrophils were isolated from blood samples collected at presentation and three weeks after surgery. The cells were isolated from peripheral blood treated with EDTA by way of density centrifugation, using Polymorphprep (Axis-Shield, Oslo, Norway) (density: 1.113 g/mL). After washing in PBS without  $\text{CaCl}_2$  and  $\text{MgCl}_2$  (GIBCO, Great Britain), the PMNs were separated by magnetic selection.

PMNs were separated by positive selection using a 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 rinsed with MACS buffer. Isolated PMNs were suspended in MACS buffer (up to  $5 \times 10^7$  total cells) and incubated with CD16 MicroBeads for 30 min at 4–8°C. After washing, the cells were suspended in MACS buffer. The purity of the isolated PMNs, determined by May-Grunewald-Giemsa-staining, was 99%.

The separated PMNs were suspended in the culture medium (RPMI-1640) to provide  $5 \times 10^6$  cells/mL and incubated in flat-bottomed, 96-well plates (Microtest III-Falcon, Franklin Lakes, USA) for 4 h at 37°C in a humidified incubator with 5%  $\text{CO}_2$  (NUAIRE®). LPS (10 ng/mL, Sigma) was tested to stimulate the expression of TLR4 and APRIL in PMNs. The viability of PMN measured after incubation was 94%.

In cells from the same blood samples, we investigated the role of ERK1/2 kinase in APRIL protein induction in PMNs at the protein level. For this purpose, p42/p44 MAPK inhibitor PD98059 (2'-amino-3'-methoxyflavone), a peptide that specifically inhibits ERK1/2 activation, was used. After isolation, the cells were treated with PD98059 (Calbiochem, Bad Soden, Germany) (40  $\mu\text{M}$ ) for 1 h before and during LPS incubation. The presence of inhibitor did not affect cell viability.

### **RNA isolation and cDNA synthesis**

For real-time PCR, total RNA was isolated from  $10^7$  untreated and stimulated neutrophils. An RNeasy Mini Kit, Qiagen, Germany, was used to isolate total RNA from PMNs, 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, identified by ethidium bromide staining, and an  $\text{OD}_{260/280}$  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 specifications in a 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\_TLR4\_2\_SG Assay (Qiagen), QuantiTec Hs\_TNFSF13\_2\_SG Assay (Qiagen) and QuantiTec Hs\_PRS18\_1\_SG Assay (s18) (Qiagen) as normalizer.

Real-time PCR was performed in duplicate in 20  $\mu\text{l}$  using the QuantiTect SYBR Green PCR Master Mix (Qiagen) following the manufacturer's instruction, and carried out in the Chromo4 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 s, 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 series 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 *TLR4* and *TNFSF13* transcripts were calculated after normalization of *TLR4* and *TNFSF13* products to s18. The value of  $C_T$  was determined by the first cycle number at which fluorescence was greater than the set threshold value. To calculate our data, we used the comparative  $C_T$  method for relative quantification ( $\Delta C_T$  method).

### **Western blot analysis**

Cytoplasmic protein fractions of PMNs were analyzed using western blotting for the presence of the APRIL protein and the activation of ERK1/2 kinases, by determining the phosphorylation status of ERK1/2 (p-ERK1/2). Cells were lysed directly in the presence of protease inhibitor cocktail (Sigma-Aldrich, CHEMIE GmbH P.O. Steinheim, Germany) by sonication, using a Vibra-Cell Ultrasonic Processor (Sonics&Materials, Inc., USA). Protein fractions were suspended in Laemmli buffer (Bio-Rad Laboratories, Hercules CA, USA), and then electrophoresed on SDS-PAGE. The resolved protein was transferred onto 0.45  $\mu\text{m}$  pore-sized nitrocellulose (Bio-Rad Laboratories, Hercules CA, USA). The nitrocellulose was incubated with the primary polyclonal antibody anti-APRIL (R&D Systems) and anti-phospho ERK1/2 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany). After washing in 0.1% TBS-T, the membrane was incubated 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). Bands intensity was quantified using the ImageJ method.

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.

### **Cytokine measurement by ELISA assays**

Soluble APRIL concentrations in the culture supernatants of PMNs and the serum were assessed using ELISA kits by R&D Systems (Minneapolis, USA).

The serum concentrations of TGF- $\beta$  were measured using an ELISA kit from BioSource Int. (California, USA).

### Statistical analysis

Statistical analysis was performed with a statistics package - Statistica 6.0 software. The 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. p-values below 0.05 were considered statistically significant.

## RESULTS

### Expression of APRIL-mRNA and TLR4-mRNA analyzed by real-time PCR

Real-time PCR analysis revealed differences in the expression of APRIL-mRNA between neutrophils in OSCC patients in early stages of disease (T1/2) and patients in advanced stages (T3/4) (figure 1). Unchanged expression of APRIL-mRNA levels in unstimulated neutrophils from patients in early phases of disease at presentation was observed. Increased expression of this molecule in cells stimulated by LPS was also observed. In contrast, in patients in advanced stages of disease, we found an increased expression of APRIL-mRNA in unstimulated neutrophils. No changes were observed after LPS stimulation (figure 1). Furthermore, levels of APRIL-mRNA in neutrophils from patients in stages T3/4 were higher than those in the cells from patients in stages T1/2.

It is important to note that the expression of APRIL-mRNA in the cells of all patients (stages T1/2 and T3/4) following surgical treatment remained at the same levels as before treatment (figure 1).

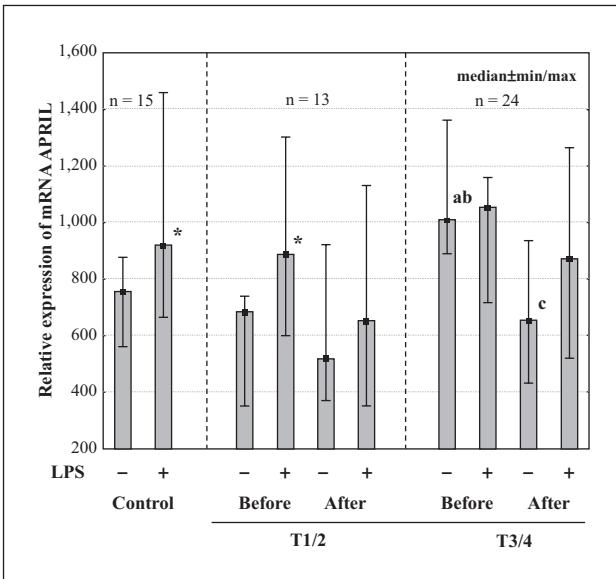


Figure 1

Expression of APRIL-mRNA in neutrophils from patients with OSCC before and after treatment. RT-PCR was performed on human neutrophils and mononuclear cells from peripheral blood, n = the number of persons studied. APRIL-mRNA level was normalized to house-keeping gene - s18. \* Statistically significant differences between unstimulated and LPS-stimulated cells ( $p < 0.05$ ), <sup>a</sup> statistically significant differences with control ( $p < 0.05$ ), <sup>b</sup> statistically differences between T1/2 and T3/4, <sup>c</sup> statistically significant differences between before and after treatment. Each value represents the median  $\pm$  minimum and maximum.

\* Statistically significant differences between unstimulated and LPS-stimulated cells ( $p < 0.05$ ), <sup>a</sup> statistically significant differences with control ( $p < 0.05$ ), <sup>b</sup> statistically differences between T1/2 and T3/4, <sup>c</sup> statistically significant differences between before and after treatment. Each value represents the median  $\pm$  minimum and maximum.

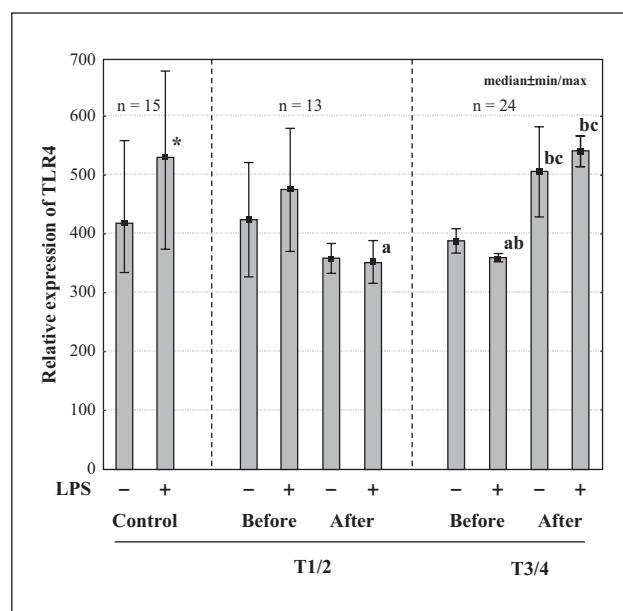


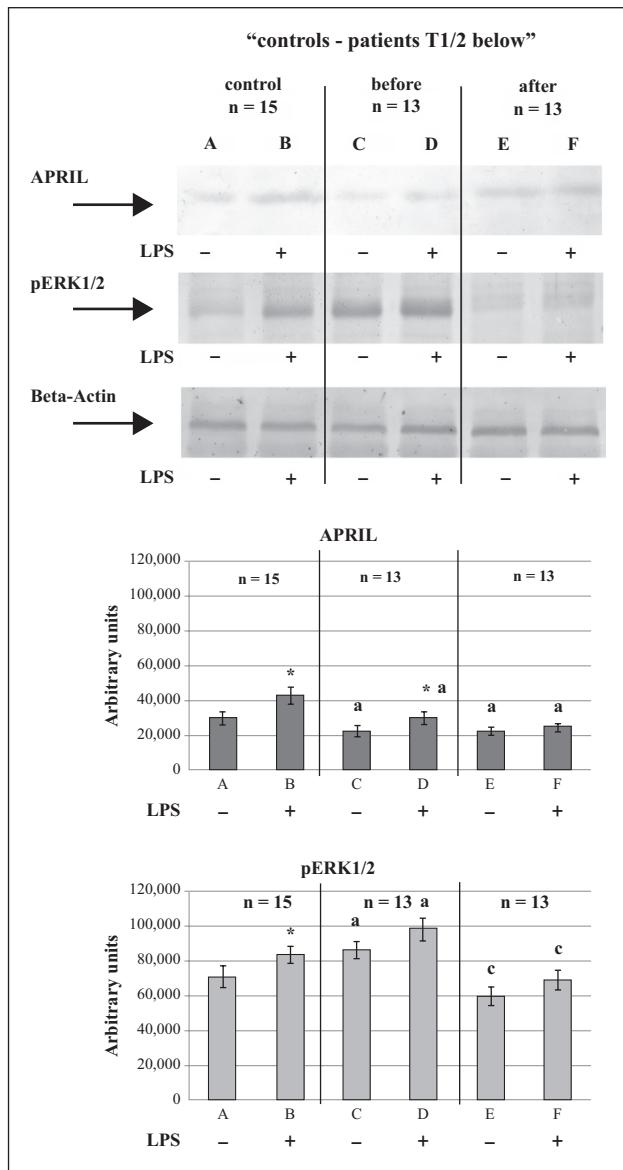
Figure 2

Expression of TLR4-mRNA in neutrophils of patients with OSCC before and after treatment. RT-PCR was performed on human neutrophils and mononuclear cells from peripheral blood, n = the number of persons studied. The TLR4-mRNA level was normalized to house-keeping gene - s18. \* Statistically significant differences between unstimulated and LPS-stimulated cells ( $p < 0.05$ ), <sup>a</sup> statistically significant differences with control ( $p < 0.05$ ), <sup>b</sup> statistically differences between T1/2 and T3/4, <sup>c</sup> statistically significant differences between before and after treatment. Each value represents the median  $\pm$  minimum and maximum.

Similarly to APRIL expression, levels of TLR4-mRNA were unchanged in unstimulated neutrophils from all patients before treatment (figure 2). TLR4 expression in the cells of patients in advanced stages, was very similar to that found in patients in early stages of disease. Furthermore, LPS-stimulation did not have a significant effect on TLR4 expression in the cells. After treatment, there were no significant changes in the expression of TLR4-mRNA in neutrophils from patients in early stages, as compared to results obtained in the cells of patients before treatment. Surprisingly, the expression of TLR4-mRNA in patients in advanced stages was higher than in the cells of patients before treatment.

### Expressions of APRIL and phospho-ERK1/2 in PMNs in cancer patients, assessed using the Western blot method

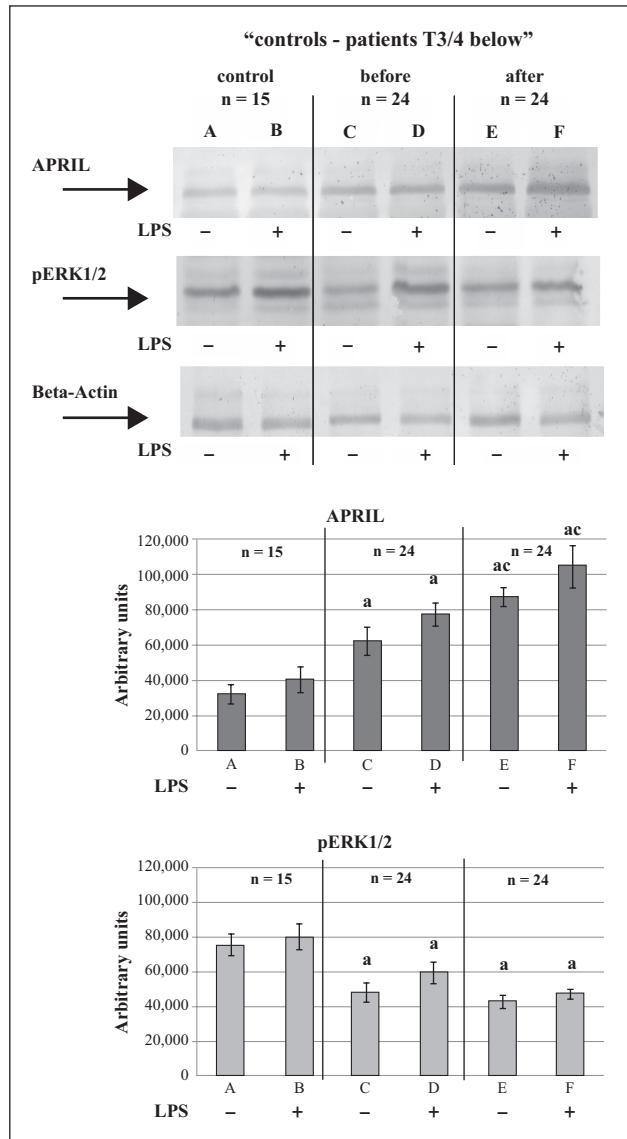
The presence of APRIL protein (27 kDa) and phospho-ERK1/2 (44/42 kDa) was shown in the neutrophils examined (figure 3). PMNs from patients at stages T1/2 before and after treatment demonstrated decreased in the expression of APRIL protein in comparison with the cells of the control group. In contrast to APRIL expression, significantly increased expression of phospho-ERK1/2 kinases in comparison to the controls was found in the lysates of PMNs from patients before treatment. ERK1/2 expression in the cells of patients after treatment was lower than that seen in cells of patients before treatment. LPS stimulation led to increased expression of APRIL and phospho-ERK1/2 in cells of patients before treatment.

**Figure 3**

Western blot analysis of APRIL and phospho-ERK1/2 protein expression in neutrophils of patients at stage T1/2, before and after treatment. **A)** unstimulated PMNs from controls, **B)** LPS-stimulated PMNs from controls, **C)** unstimulated PMNs from patients before treatment, **D)** LPS-stimulated PMNs from patients before treatment, **E)** unstimulated PMNs from patients after treatment, **F)** LPS-stimulated PMNs from patients after treatment, n: the number of persons studied, \* statistically significant differences between unstimulated and LPS-stimulated cells ( $p<0.05$ ), <sup>a</sup> statistically significant differences compared to controls ( $p<0.05$ ), <sup>c</sup> statistically significant differences between before and after treatment.

In patients with advanced stages of disease (T3/4), the expression of APRIL protein in neutrophils was increased in comparison with controls and cells from patients in early stages of disease. In contrast, expression of phospho-ERK1/2 in neutrophils was lower than that found in the controls (figure 4). LPS stimulation did not lead to an increase in the expressions of APRIL and ERK1/2 proteins in the cells of this patient group.

APRIL protein expression in PMNs was higher in patients after treatment than in patients before treatment. No significant changes in the expression of ERK1/2 protein levels in the patients were found after treatment. The above observations suggest a largely insignificant role of ERK1/2 kinases in APRIL induction in neutrophils of OSCC patients.

**Figure 4**

Western blot analysis of APRIL and phospho-ERK1/2 protein expression in neutrophils of patients at stage T3/4, before and after treatment. **A)** unstimulated PMNs from controls, **B)** LPS-stimulated PMNs from controls, **C)** unstimulated PMNs from patients before treatment, **D)** LPS-stimulated PMNs from patients before treatment, **E)** unstimulated PMNs from patients after treatment, **F)** LPS-stimulated PMNs from patients after treatment, n: the number of persons studied, <sup>a</sup> statistically significant differences compared to controls ( $p<0.05$ ), <sup>ac</sup> statistically significant differences between before and after treatment.

#### APRIL concentrations in supernatants of cells and serum

The ELISA data showed that the release of APRIL by PMNs in cancer patients in early stages, before and after surgical treatment, remained unchanged in comparison to its release by cells in the control group (table 2).

In patients with advanced stage disease, before treatment, the secretion of APRIL by PMNs was higher than that seen in the control cells and cells of patients in early stages of disease. After treatment, significantly decreased secretion of APRIL by cells was observed.

Next, we measured the serum concentrations of APRIL in all patients. We found increased concentrations of APRIL in patients at stages T3/4 before treatment, in comparison to controls and patients at stages T1/2 (table 2). Examinations

**Table 2**  
APRIL concentrations in supernatants of PMNs, and the serum and TGF- $\beta$  concentrations in the serum of patients with OSCC.

APRIL (ng/mL)					
Control n = 15	Patients before treatment		Patients after treatment		
	T1/2 n = 13	T3/4 n = 24	T1/2 n = 13	T3/4 n = 24	
PMNs	2.45 ± 1.12	2.1 ± 0.7	4.7 <sup>ab</sup> ± 1.43	1.72 ± 0.9	3.6 <sup>ab</sup> ± 1.22
Serum	2.1 ± 0.8	4.76 <sup>a</sup> ± 2.6	7.47 <sup>ab</sup> ± 2.76	5.0 <sup>a</sup> ± 2.61	6.45 <sup>a</sup> ± 3.33
TGF- $\beta$ (ng/mL)					
Serum	2.47 ± 0.76	8.88 <sup>a</sup> ± 5.32	12.21 <sup>ab</sup> ± 4.86	8.71 <sup>a</sup> ± 5.30	13.85 <sup>ab</sup> ± 6.08

<sup>a</sup> significant differences with control (p<0.05)

<sup>b</sup> significant differences with T1/2 (p<0.05)

n: number of persons studied

after treatment showed that the serum levels of APRIL in all patients were at the same levels as those in patients before treatment (table 2).

#### TGF- $\beta$ serum concentrations

The ELISA data also showed increased concentrations of TGF- $\beta$  in the serum of patients with early and advanced stages of disease, as compared to healthy controls (table 2). The concentrations of TGF- $\beta$  in the serum of patients at stages T3/4 were higher than those in patients at T1/2 stages. There were no significant changes in TGF- $\beta$  concentrations in the serum of patients after treatment.

#### DISCUSSION

A number of clinical and experimental studies have focused on the measurement of cytokine expression as parameters of the immune potential of cancer patients. Available data, including our own observations, indicate that the production of cytokines by human neutrophils may have different effects on tumor development [7, 8, 10, 11]. The results of the present study, carried out on oral cavity cancer patients, revealed unfavorable features of neutrophils associated with the changed ability to produce the tumor-promoting APRIL molecule. The demonstrated increased expression and release of APRIL by these cells accompanying advanced stages of disease, combined with the increased number of neutrophils, may be an important marker of disease progression in the patient group examined.

Results involving neutrophil counts in patients with OCSCC are in agreement with those demonstrated by Trelakakis *et al.* [23], who also observed systemic differences in the PMN compartment associated with tumor size in HNSCC patients with oral cavity disease. Increased circulating peripheral blood neutrophil numbers have also been identified as a poor prognostic factor in other cancer diseases, *e.g.* renal cell carcinoma [24].

Increased expression and secretion of APRIL by neutrophils, together with an increase in serum concentrations of APRIL in patients with advanced OCSCC, observed in the present study, may directly and/or indirectly influence tumor development via interaction with HSPG on tumor cells [13]. The direct effect associated with APRIL-induced tumor proliferation was confirmed by the

inhibition of APRIL binding to HSPG, which prevented the induction of tumor cell proliferation [16]. The indirect effect of APRIL-HSPG interactions resulting from binding of HSPG to the extracellular matrix (ECM) and/or different soluble ligands, may lead to the modulation of tumor cell adhesion, their migration and invasion [16, 25].

Another aspect of the tumor-promoting action of APRIL may be dependent upon the protection of the tumor cells from apoptosis. It was demonstrated that APRIL, through the activation of NF- $\kappa$ B or MAPK kinases, leads to a strong up-regulation of anti-apoptotic proteins, such as Mcl-1 and Bcl-2 belonging to Bcl-2 family of proteins. High expression of Mcl-1 and Bcl-2 may significantly inhibit the mitochondrial pathway of apoptosis in cancer cells [26]. Immunochemistry investigation in patients with oral squamous cell carcinoma showed different expressions of Bcl-2 protein within the tumor [27, 28]. However, it has been suggested that patients with greater Bcl-2 expression have a worse prognosis; its low level seeming to be associated with higher survival rate [28, 29].

It is interesting to note that, in contrast to neutrophils from healthy control, changes in the expression of APRIL in cells of patients in advanced stages of disease, before and after treatment, was accompanied by diverse changes in expression of the TLR4 receptor. The relationship between these proteins suggest the presence of distinct mechanisms responsible for their activation in the neutrophils of the tumor-bearing host than those in control cells.

The above suggestion appears to be confirmed by the differences in the expression of APRIL and pERK1/2 kinases, observed in neutrophils from the patients examined. Relationships between these proteins suggest that ERK1/2 does not play the role of critical messenger in the induction of APRIL in these cells.

It is not clear whether this is a reflection of the modification of neutrophil function caused by tumor cells or the reason for the changes associated with tumor progression. One of the possible explanations of the distinct mechanism of APRIL induction in neutrophils of cancer patients may be the presence of circulating mediators, such as immuno-suppressing TGF- $\beta$ . Recent studies by Jang *et al.* [30] on mouse macrophages showed that TGF- $\beta$  is capable of stimulating APRIL expression in these cells. The source of TGF- $\beta$  in patients with OSCC may be the neutrophils, as well as Treg, whose count significantly increased in the circulation [31, 32]. The presence of large amounts of TGF- $\beta$  may induce the formation of protumorigenic "N2"

phenotype neutrophils, analogous to the “N2” cells presented in the microenvironment of tumor [22]. The increased concentrations of TGF- $\beta$  observed in the serum of patients with OSCC according to tumor progression, appear to confirm the role of this cytokine in the modulation of APRIL expression in neutrophils and may be one of markers of the “N2” phenotype. Simultaneously, the demonstrated, unaltered expression of TLR4 in these cells suggests that TGF- $\beta$  does not have impact on the activity of neutrophils mediated by this receptor in OSCC patients.

In conclusion, the results obtained prove that the tumor-promoting properties of neutrophils in patients with oral cavity cancer may be caused not only by enhanced inflammatory activity, but also by the changes in the synthesis and secretion of the APRIL molecule. The data presented herein, together with previously reported alterations in expression of other TNF superfamily ligands, appear to be an obvious indication of the unfavorable role of these cells in patients with OSCC and other types of neoplasms. This might be confirmed by our studies carried out in patients with B-cell chronic lymphocytic leukemia (B-CLL) that revealed similar relationships between APRIL, BAFF and TRAIL secretion by neutrophils [33]. Based on the above observations, it can be assumed that the alterations in TNF superfamily secretion by these cells are independent of the type of neoplasm. Further investigation will be helpful to explain fully the effective role of neutrophils in tumor-host reactions.

However, the changes presented in the current study suggest that modulation of the expression of tumor-promoting APRIL, in addition to TRAIL and BAFF, might be taken into account in the development of new strategies for supportive immunotherapy of OSCC disease and possibly for other types of neoplasm as well.

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