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IL-17 family cytokines in neutrophils of patients with oral epithelial squamous cell carcinoma

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Epithelial squamous cell carcinoma is the predominant histological type among cancers of the head and neck. It is characterised by high growth dynamics and a low survival rate of patients. The usefulness of various markers and prognostic factors is assessed to improve treatment results in patients with epithelial squamous cell carcinoma.

The involvement of neutrophils (PMNs) in the neoplastic process and scarce evidence for the role of interleukin 17 (IL-17) family cytokines in these reactions encouraged us to undertake a study in this field. We assessed the expression and capability of neutrophils in patients with oral epithelial squamous cell carcinoma to release IL-17E and IL-17A in relation to their serum levels and expression of the specific receptors, i.e. IL-17R and IL-17BR. For the sake of comparison, the expressions of the proteins examined were assessed in autologous peripheral blood mononuclear cells (PBMCs). The levels were determined in the patients prior to treatment, taking into consideration the stage of the disease according to TNM classification.

Western blot analysis revealed no differences in the expressions of IL-17E and IL-17A either in PMNs or PBMCs of the patients as compared to the healthy subjects. However, the expressions of IL-17BR and IL-17R were found to be higher in both groups of cells in cancer patients as compared to the control. The use of ELISA method revealed that the levels of IL-17E and IL-17A were higher in cell supernatants and blood serum of the patients than of the healthy subjects. No differences were noted in the protein expression in the cells or concentration in supernatants of the patients with different stages of the disease.

Our findings as well as observations reported by other authors seem to indicate some new aspects of the biological role of IL-17 family cytokines, not only as markers of the inflammatory process but also as indicators of leukocyte activity in IL-17A and IL-17E-dependent reactions in patients with oral epithelial squamous cell carcinoma.

Key words: IL-17, neutrophils, oral epithelial squamous cell carcinoma

The majority of oral epithelial squamous cell carcinoma cases are diagnosed in a late stage of the disease, resulting in a low survival rate observed for a number of years [1]. The development of the neoplastic process in these patients is conditioned, among others, by interactions between the immune system and tumor cells [2].

Cell-type reactions are the major component of the immune response of the body to the neoplastic process. Histological investigations of the tumor cell composition have provided evidence indicating the involvement of neutrophils in early stages of tumor growth. Neutrophils as an essential component of a nonspecific response undergo rapid recruitment and easily recognise neoplastic cells, leading to their eradication. These cells are capable to release a number of cytokines that may affect the course of the neoplastic process [3, 4].

Our earlier findings showed, among others, altered secretion of IL-1β [5], IL-6, TNF-a and their natural regulators [6], as well as release of VEGF or IL-18 by neutrophils in oral carcinoma patients [7].

In recent years, the role of certain cytokines of the interleukin 17 family in the neoplastic process has been suggested [8, 9, 10].

It has been demonstrated that human neutrophils synthesize IL-17A [11] and possess their own membrane receptor,

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i.e. IL-17R [12]. Our earlier findings also indicated the presence of IL-17E and IL-17BR in human neutrophils [data not published].

The long discussed role of neutrophils in the neoplastic process and insufficient evidence for the involvement of IL-17 family cytokines in these reactions made us perform a study in this field. We assessed the expression and capabilities of neutrophils of patients with oral epithelial squamous cell carcinoma to release IL-17E and IL-17A in confrontation with their serum levels and expression of the specific receptors, i.e. IL-17R and IL-17BR. For the sake of comparison, the expressions of the proteins examined were evaluated in autologous peripheral blood mononuclear cells.

Materials and methods

The study involved a group of 32 patients with oral epithelial squamous cell carcinoma, aged 42-73 years, hospitalized in the Department of Maxillofacial Surgery, Medical University of Bialystok. Assays were performed in patients before the treatment. Study results were analyzed taking into account a clinical stage of the disease according to TNM classification.

In all patients leukocytosis was ranging from 4.65 to 8.35 x 10^3/ml consisting predominantly of PMNs.

The control group constituted 15 healthy people, aged from 20 to 60 years, volunteer blood donors.

Cells were isolated from whole blood collected with heparin (10 IU/ml-Heparin, Polfa-Łódź, Poland) using Gradisol G (1.115g/ml, Polfa-Łódź, Poland) according to *Zeman et al* [13]. Sera were obtained from blood samples collected without anticoagulant agents.

The two leukocyte fractions obtained, i.e. polynuclear cells and mononuclear cells, were suspended in a culture medium containing RPMI-1640 and Hanks' fluid (BIOMED-Lublin, Poland), subjects' own serum, 100U/ml penicillin and 50ng streptomycin (Polfa Trachomin S.A., Poland), reaching the concentration of $5x10^6$ cells/ml. Then, the cells were incubated in microplatelets (Microtest III-Falcon, BD Biosciences, Bedford, USA) at 37°C, in an incubator with a flow of 5% CO_2 (NUAIRE™).

After 20 hours, the supernatant was collected from each well and stored at - 20°C.

Informed written consent was obtained from all participants and the Ethics Committee of the Medical University of Bialystok approved the study.

Expressions of proteins IL-17E, IL-17A both receptors IL-17BR and IL-17R assessed by the Western blot method. PMN and PBMC were subjected to lysis by means of sonification in the presence of protease inhibitors (Sigma-Aldrich, Steinheim, Germany). The lysate was suspended in Laemli's buffer (Bio-Rad Laboratories, Herkules CA, USA). The cytoplasmic fraction of proteins underwent electrophoresis on SDS-PAGE (Bio-Rad Laboratories, Herkules CA, USA). The protein fractions were transferred onto nitrocellulose (Bio-Rad Laboratories, Herkules CA, USA). Then, nitrocellulose was incubated with suitable polyclonal anti-IL-17E, anti-IL-17A, anti-IL-17BR and anti-IL-17R antibodies (R&D Systems, Minneapolis, USA). After rinsing with 0.1% TBS-T, nitrocellulose was incubated with alkaline phosphatase-labelled antibody against IgG (Victor Laboratories, Burlingame, CA, USA). Immunoreactive protein bands were obtained by adding the BCIP/NBT Liquid Substrate System (Sigma-Aldrich, Steinheim, Germany).

IL-17E and IL-17A levels assessed using ELISA method. IL-17E concentrations in blood serum and cell supernatants were determined by ELISA method using a PeproTech kit (Rocky Hill, USA) according to the instructions enclosed. Human recombinant IL-17E was used as a standard.

IL-17A levels in the serum and cell supernatants were measured by ELISA method using R&D Systems kits (Minneapolis, USA) according to the instructions given. Human recombinant IL-17A was used as a standard.

Statistical analysis .Results were elaborated statistically using the t-Student test. $P<0.05$ was considered statistically significant.

Results

1. The expressions of IL-17E and IL-17A in PMNs and PBMCs of healthy subjects and patients with oral epithelial squamous cell carcinoma assessed by Western blot. The cell lysates of PMNs and PBMCs of healthy subjects and patients with oral epithelial squamous cell carcinoma showed the presence of IL-17E, 34 kDa, and IL-17A, 35 kDa (Fig.1).

No significant differences were found in the expressions of IL-17E and IL-17A either in PMNs or PBMCs in the group of patients, as compared to healthy subjects (Fig.1).

No significant differences were also observed in the expressions of IL-17E and IL-17A between PMNs and PBMCs of cancer patients (Fig.1).

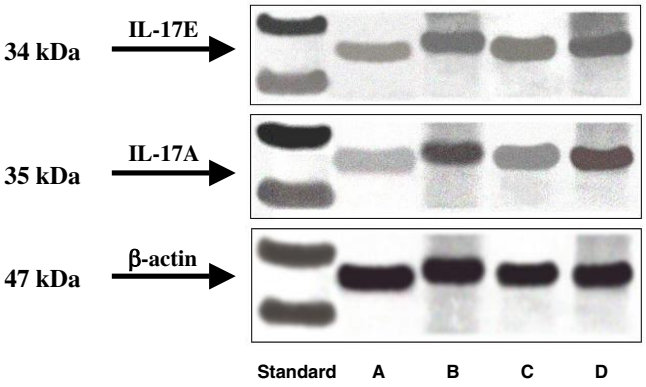
There were also no differences in the expressions of the cytokines studied in the cells of patients according to the stage of tumor advancement (Fig.1).

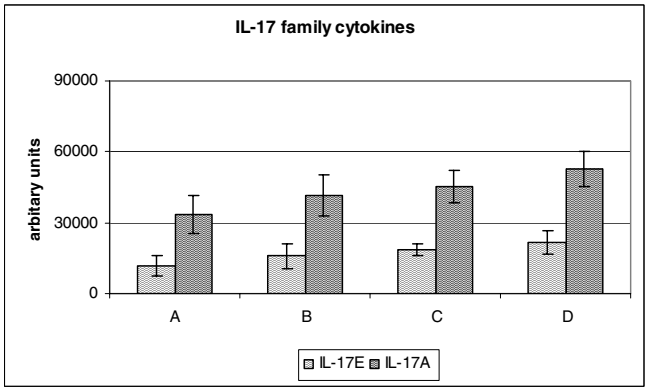
2. The expressions of IL-17BR and IL-17R in PMNs and PBMCs of healthy subjects and patients with oral epithelial squamous cell carcinoma assessed using Western blot. The cell lysates of PMNs and PBMCs of healthy subjects and patients with oral epithelial squamous cell carcinoma showed the presence of IL-17BR, 56 kDa, and IL-17R, 110 kDa (Fig.2).

The expressions of IL-17BR and IL-17R in PMNs and PBMCs were significantly higher in cancer patients than in healthy subjects (Fig.2).

No significant differences were observed in the receptor expressions between PMNs and PBMCs of cancer patients (Fig.2).

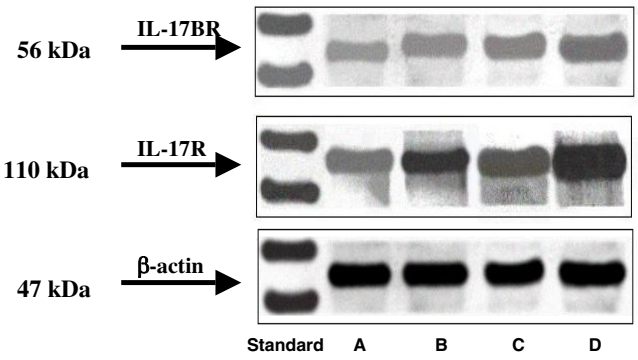
Moreover, no changes were noted in the expressions of IL-17BR and IL-17R in the cells of patients in relation to the stage of tumor advancement (Fig.2).

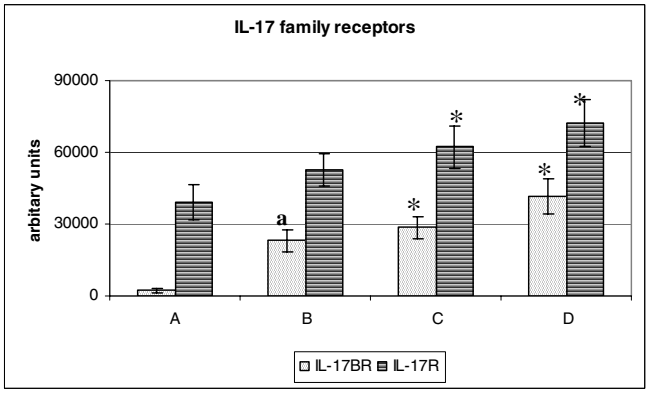




A – PMN of healthy subject
B – PBMC of healthy subject
C – PMN of patients (Fig. 1 C&D)
D – PBMC of patients (Fig. 1 C&D)

Figure 1. Expressions of IL-17E and IL-17A in PMN and PBMC in healthy subjects and patients assessed using the Western blot method.





* – a statistically significant difference between the cells of healthy subjects and patients (p<0.05)
a – a statistically significant difference between PMN and PBMC of healthy subjects (p<0.05)

A – PMN of healthy subject
B – PBMC of healthy subject
C – PMN of patients (Fig. 2 C&D)
D – PBMC of patients (Fig. 2 C&D)

Figure 2. Expressions of IL-17BR and IL-17R in PMN and PBMC in healthy subjects and patients assessed using the Western blot method.

3. The concentrations of IL-17E and IL-17A in blood serum and cell supernatants of healthy subjects and patients with oral epithelial squamous cell carcinoma assessed by ELISA. PMNs and PBMCs of cancer patients showed higher potential to release IL-17E and IL-17A as compared to the control group (Table 1).

No significant differences were found in the levels of IL-17E and IL-17A in cell supernatants from cancer patients in relation to staging (Table 1).

Moreover, no differences were revealed in the levels of IL-17E and IL-17A between PMN and PBMC supernatants in the patients (Table 1).

Table 1. IL-17E and IL-17A concentrations in blood serum and cell supernatants of PMN and PBMC in healthy subjects and patients using ELISA method.

	IL-17E pg/ml			IL-17A pg/ml		
	PMN ‘x ± SD	PBMC ‘x ± SD	serum ‘x ± SD	PMN ‘x ± SD	PBMC ‘x ± SD	serum ‘x ± SD
Control	8.04 ± 1.64	9.03 ± 1.47	10.03 ± 1.29	9.18 ± 2.97	10.52 ± 3.26	15.27 ± 4.32
Patients in stage I, n=8	13.41* ± 1.21	14.96* ± 1.42	17.04* ± 1.32	25.89* ± 1.61	26.34* ± 1.71	31.34* ± 2.83
Patients in stage II, n=8	14.32* ± 0.54	15.13* ± 0.91	17.83* ± 2.16	26.34* ± 3.52	30.80* ± 3.91	35.22* ± 4.10
Patients in stage III, n=8	15.10* ± 0.91	17.19* ± 2.36	17.99* ± 2.53	25.61* ± 0.65	28.56* ± 0.53	29.07* ± 3.44
Patients in stage IV, n=8	17.85* ± 2.49	18.01* ± 1.66	18.61* ± 1.27	25.00* ± 0.63	26.21* ± 2.71	27.02* ± 3.44

* –– a statistically significant difference between healthy subject and patients (p<0.05)

The analysis of serum IL-17E and IL-17A levels in patients with oral epithelial squamous cell carcinoma showed significantly higher values as compared to the control group (Table 1).

No differences were noted in the levels of the cytokines examined in the serum of patients with various stages of tumor advancement (Table 1).

No correlation was observed between the levels of IL-17E and IL-17A in cell supernatants and serum (Table 1).

Discussion

Neutrophils perform a number of various functions responsible for eradication of cancer cells, e.g. through the oxygen-dependent and independent intracellular killing, antibody-dependent cytotoxicity (ADCC) and synthesis and release of cytokines [14, 15].

Our study showed an enhanced ability of PMN cells to secrete IL-17E and IL-17A in patients with oral epithelial squamous cell carcinoma and thus confirmed the involvement of these cells in the reactions that accompany the neoplastic process.

There is evidence for both inhibitory and stimulatory, directly or indirectly, effect of IL-17A on tumor growth. Enhanced secretion of IL-17A by the leukocytes examined can increase the secretion of IL-1β, IL-6 and TNF-α by macrophages, thus stimulating recruitment and function of lymphocytes, including cancer-specific T cells (CTL) [16, 17, 18]. Moreover, NF-κB activation in stromal cells by IL-17A, by inducing the production of GM-CSF [19] and chemokines, such as CXC, stimulates granulopoiesis and recruitment of neutrophils [20]. The antineoplastic action of IL-17A can become enhanced through stimulation of maturation of dendritic cells due to increased expression of class II MHC and co-stimulatory molecules [21]. Its direct inhibitory effect on the growth of tumor cells has been described. *Benchetrit et al* revealed an inhibitory effect of IL-17A on the growth of mastocytoma and plasmocytoma cell lines [16].

On the other hand, the increased release of IL-17A by the immune system cells that infiltrate a tumor may promote progression of the neoplastic process. The IL-17A has been shown to stimulate cancer cells to produce vasculitic factors, e.g. VEGF [10]. Moreover, IL-17A is likely to cause a rise in the expressions of adhesion molecules (ICAM-1, ICAM-3 and E-selectin) on endothelial cells, thus promoting cell migration and formation of secondary foci [11].

Data concerning the role of IL-17E in the neoplastic process are rare. The increase in the production of IL-17E by leukocytes observed in the group of patients may counterbalance the effects induced by IL-17A over-expression and inhibit cellular response, e.g. by stimulating the production of suppressive IL-10 [9]. The secretion of IL-17E may also inhibit the proliferation of human progenitor bone marrow cells of the granulocyte-macrophage series (CFU-GM), thus reducing the number of mature leukocytes [22]. Apart from being a positive tumor growth regulator, IL-17E can also increase recruitment of B cells to the tumor microenvironment and enhance production of specific antibodies [9, 23].

Thus, the ultimate effect of IL-17A and IL-17E seems to result from a balance between them, cooperation of other cytokines and the presence of membrane receptors on target cells. Our study revealed high expressions of the specific IL-17R and IL-17BR, which indicated elevated sensitivity of PMNs and PBMCs of patients to the action of IL-17A and IL-17E. The direct effect of the cytokines examined on PMNs and PBMCs is manifested, among others, by IL-17A dependent modulation of apoptosis in neutrophils [24]. It has been also found that IL-17E activates Th2 lymphocytes to the production of IL-4 and IL-5 [23].

High levels of IL-17A and IL-17E observed in the sera of patients with oral epithelial squamous cell carcinoma are likely to affect these mechanisms. The high serum concentrations of these cytokines can be caused by the increased ability of the leukocytes examined to synthesize and their release. However, lack of correlation between the cytokine levels in supernatants and blood serum suggests that also other cells can be their important source.

Natural expression of IL-17A has been detected in some cancer cells. The presence of mRNA and IL-17A protein has been found in the cells of granuloma sarcomatodes *in vitro.* The expression and secretion of IL-17A by neoplastic T cells have been detected in half of the biopsies investigated [25]. An increased level of IL-17A has also been noted in ovarian and endometrial specimens of cancer patients [26]. However, the elevated expression of IL-17E has been observed only in specimens of cancerous human prostate [8].

Our findings as well as observations reported by other authors seem to indicate new aspects of the biological role of IL-17 family cytokines not only as inflammatory markers but also as indicators of leukocyte activity in IL-17A and IL-17E-dependent reactions in oral carcinoma patients. Lack of differences in cytokine secretion and expression of IL-17 family receptors between PMNs and PBMCs suggests equal involvement of both groups of cells in cytokine-dependent reactions in the study group of patients. Further studies on the activity of IL-17 family molecules and their mutual interactions, with the involvement of neutrophils, may contribute to broadening the knowledge of their functions in cancer-burdened conditions.

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