Effect of IL-18 on leukocyte expression of iNOS and phospho-IκB in patients with squamous cell carcinoma of the oral cavity

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The inducible synthase of nitric oxide (iNOS) is responsible for the synthesis of nitric oxide (NO) in neutrophils (PMN) and in peripheral blood mononuclear cells (PBMC). This enzyme is controlled by a number of cytokines which accomplish their biological effect via e.g. activation of NF-κB pathway.

The aim of the present study was to assess the expression of iNOS and production of NO by PMN and PBMC in patients with oral cavity squamous cell carcinoma (SCC), and to examine the role of the NF-κB pathway in the IL-18-stimulated activation of this enzyme.

The production of NO and iNOS expression in PMN were reduced, while iNOS expression in PBMC was increased but NO production by these cells remain unchanged. Patients after treatment showed lower intensity of iNOS expression compared to that observed before treatment. Moreover, both before and after treatment iNOS expression was inversely correlated with phospho-IκB expression in PMN and in PBMC.

Significantly higher levels of total NO were observed in the serum of Stage IV patients before and after treatment as compared to the control group.

Altered expression of iNOS and NO generation by PMN and PBMC may have an unfavorable effect on the course of antineoplastic response in patients with squamous cell carcinoma of the oral cavity. Intensification of iNOS expression and NO production in Stage IV patients, induced by rhIL-18, suggests its beneficial effect on the activity of leukocytes in patients with squamous cell carcinoma of the oral cavity.

Key words: neutrophils, peripheral blood mononuclear cells, squamous cell oral cancer, nitric oxide, inducible synthase of nitric oxide, NF-κB

Neutrophils, an essential component of the non-specific response, in the state of activation are a major group of cells involved in the early phase of tumor growth. This is due to the fact that neutrophils are quickly recruited and easily recognize cancer cells [1–3].

Activated neutrophils are able to destroy neoplastic cells by exerting a direct cytotoxic effect via release of such reactive oxygen species as superoxide anion radical, hydroxy radical, hydrogen peroxide and nitric oxide [1, 4, 5].

Studies have shown that the role of nitric oxide in the neoplastic process depends on its production and concentration [4, 6]. The inducible nitric oxide synthase (iNOS) is one of the enzymes responsible for NO synthesis in neutrophils. In a two-stage reaction and in the presence of NADPH, this enzyme introduces oxygen to L-arginine, converting it into L-citruline and nitric oxide [7].

The inducible NOS, 130 kDa, unlike its constitutive forms (eNOS and nNOS), appears in cells a few hours after induction and generates nanomolar amounts of NO [8, 9]. Its activity is independent of Ca^{2+} and calmodulin, although its structure contains sites that bind these two factors. NOS is produced by macrophages, neutrophils, lymphocytes and hepatocytes. Moreover, iNOS is part of cytosole fraction of many pathological cells, including cancerous sqamous cells of the oral cavity [7, 10].

The expression of iNOS is cytokine-controlled. The studies so far have shown that in human neutrophils iNOS is activated by such cytokines as IL-β1, TNF-α, IFN-γ and IL-15 [6, 9, 11]. There are reports on the induction of the synthase by IL-18 in chondrocytes and NK cells [12, 13]. IL-18 is a broad-spectrum antineoplastic cytokine, exerting its biological effect via the activation of NF-κB pathway, which can be one of the mRNA iNOS induction modes [14, 15].

NF-κB is a dimer and occurs in the cell cytoplasm in the in-

active form of protein complex. Due to cell stimulation, IκB is phosphorylated and the released NF-κB is translocated to the cell nucleus where it initiates transcription of the respective genes [14].

The aim of the study was to assess the expression of iNOS and NO production by neutrophils in patients with squamous cell carcinoma and to examine the involvement of NF-κB pathway in the IL-18-stimulated activation of this enzyme. As the presence of phospho-IκB is a sign of NF-κB activation, its expression may confirm or exclude the involvement of NF-κB pathway in the activation of iNOS in neutrophils.

Patients and methods

We examined 24 patients with squamous cell carcinoma of oral cavity treated in the Department of Oral and Maxillofacial Surgery, Medical Academy of Bialystok. Assays were performed before the treatment and 3 weeks after surgical removal of the tumor mass. Study results were analyzed taking into account a clinical stage of the disease according to TNM classification. There were no clinical signs of infection observed in patients. Patients were not administered any drugs.

Control subjects (n=15) were healthy people aged from 21 to 40 years.

Cells were isolated from heparinized (10U/ml) whole blood by Gradisol G gradient 1.115 g/ml (Polfa) by ZEMMAN et al [26]. This method enables simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMC), containing 95% lymphocytes and polymorphonuclear cells (PMNs) containing 94% PMNs. The purity of isolated PMNs and PBMCs was determined by May-Grunewald-Giemsa-staining. The cells were suspended in the culture medium (HBSS) to provide $5x10^6$ cells/ml and were incubated in flat-bottomed 96-well plates (Microtest III-Falcon) for 4 h at 37 °C in a humidified incubator with 5% CO_2 (NUAIRE^{TM}). LPS (10 μg/ml; Difco, Detroit M) and/or rhIL-15 (50 ng/ml; R&D Systems) were tested to stimulate secretion by PMN and PBMC.

Western blot analysis. The cells were lysed by sonications. Cytoplasmic protein fractions of PMN and PBMC were suspended in Lamli buffer (Bio-Rad Laboratories) and than were electrophoresed on SDS-PAGE. The resolved protein was transferred onto 0.2 μm pore-sized nitrocellulose (Bio-Rad Laboratories). The nitrocellulose was incubated at +4 °C for 18 h with the primary monoclonal antibody anti-iNOS or anti-fosfo-IκB (R&D Systems). After washing with 0.1% TBS-T, the membrane was incubated at room temperature for 1 h with alkaline phosphatase anti-mouse IgG Abs (Vector Laboratories). Immunoreactive protein bands were visualized following the addition of AP Conjugate Substrate Kit (Bio-Rad Laboratories). The illustrations show typical picture of iNOS and phospho-IκB expression for each patient group in various progression stages of the disease.

Determination of total nitric oxide (NO_3^-/NO_2^-) concentration in cell cultures and serum. Nitric oxide produced in cells in the presence of superoxide anion-radical is rapidly converted to nitrate (V) and nitrate (III) (NO_3^-, NO_2^-). Nitrate (V) and nitrate (III) are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrate (V) and nitrate (III) concentrations. NO production by PMN and PBMC was determined using an indirect method based on measurement of NO_2^- ion concentration in culture supernatants and serum according to Griess's reaction. In the samples analyzed, nitrates (V) were reduced to nitrates (III) in the presence of cadmium, and then converted to nitric acid III that gave a colour reaction with Griess's reagent [7]. NO_2^- ion concentrations were determined by spectrophotometric analysis at λ=540 nm with reference to a standard curve.

Statistical evaluation. The results obtained were analyzed statistically using Microsoft Excel spreadsheet and Statistica 5.1 suite. Data were presented as mean ± standard deviation (SD). Data distribution normality was determined using Kolmogorov-Smirnov test. Since the data were not normally distributed, for comparison of variations between assayed groups, U-Mann-Whitney nonparametric tests were applied to unrelated results, and Wilcoxon test was applied to related results. For analysis of correlation between parameters tested, Pearson's linear correlation was used, and its significance was assessed using Student's t-test for correlation coefficient. A statistical significance level of $p<0.05$ was assumed.

Results

Expression of iNOS in PMN and PBMC. In patients with squamous cell carcinoma of the oral cavity and in the control group, PMN and PMBC expressed iNOS protein of approximate molecular mass 130 kDa (Fig. 1, 3, 5).

Prior to treatment, the expression of iNOS in PMN in Stage II and III patients was found to be less pronounced compared to the control. In PBMC, however, it was more intensified than in the control group and higher than in PMN of patients (Fig. 1, 3).

After treatment, PMN and PBMC in Stage II and III patients exhibited lower iNOS expression as compared to that observed before treatment (Fig. 1, 3).

Enhanced expression of iNOS in PMN and PBMC was observed in Stage IV patients before and after treatment, compared to control. The expression was higher before than after treatment. In the presence of LPS and rhIL-18, iNOS expression was increased in PMN and PBMC in these patients in comparison to unstimulated cells (Fig. 5).

Expression of phospho-IκB in PMN and PBMC. The expression of phospho-IκB protein, app. 37 kDa, was found in PMN and PBMC of patients with squamous cell carcinoma of the oral cavity (Fig. 1, 3, 5).

The expression of phospho-IκB in PMN and PBMC in Stage II and III patients before and after treatment was intensified compared to the control (Fig. 2, 4).

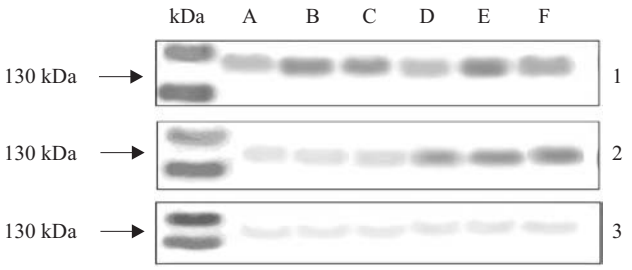

Figure 1. Western blot analysis of iNOS protein expression in human PMN and PBMC in Stage II patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage II patients before treatment, 3 – Stage II patients after treatment.

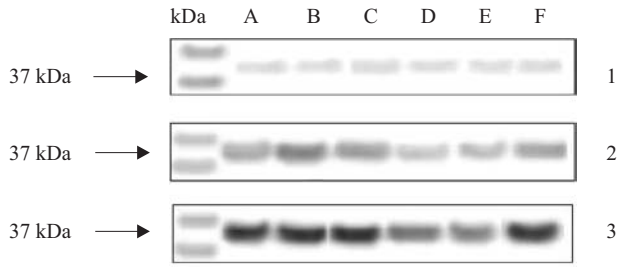

Figure 4. Western blot analysis of fosfo-IκB protein expression in human PMN and PBMC in Stage III patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage III patients before treatment, 3 – Stage III patients after treatment.

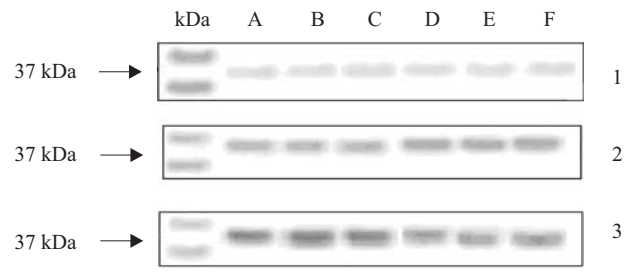

Figure 2. Western blot analysis of fosfo-IκB protein expression in human PMN and PBMC in Stage II patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage II patients before treatment, 3 – Stage II patients after treatment.

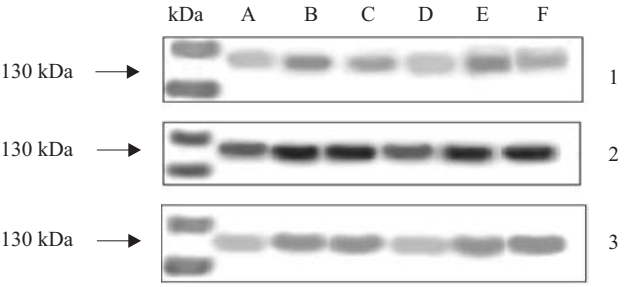

Figure 5. Western blot analysis of iNOS protein expression in human PMN and PBMC in Stage IV patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage IV patients before treatment, 3 – Stage IV patients after treatment.

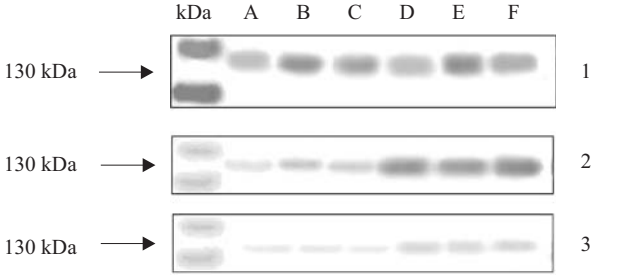

Figure 3. Western blot analysis of iNOS protein expression in human PMN and PBMC in Stage III patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage III patients before treatment, 3 – Stage III patients after treatment.

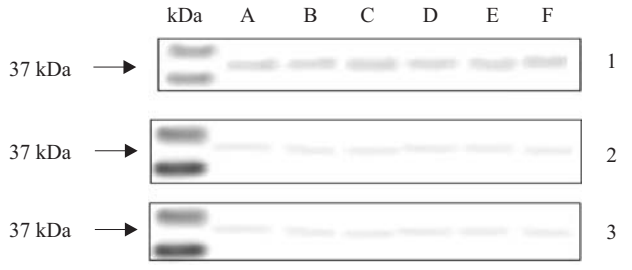

Figure 6. Western blot analysis of fosfo-IκB protein expression in human PMN and PBMC in Stage IV patients before and after treatment and controls subject.
A – PMN, B – PMN+LPS, C – PMN+rhIL-18, D – PBMC, E – PBMC+LPS, F – PBMC+rhIL-18; 1 – control subject, 2 – Stage IV patients before treatment, 3 – Stage IV patients after treatment.

In Stage III patients before treatment, LPS-stimulated PMN and rhIL-18-stimulated PBMC expressed an increase in phospho-IκB in comparison to unstimulated cells (Fig. 4).

After treatment, the phospho-IκB expression in unstimulated and LPS-stimulated PMN in Stages II and III was more enhanced compared to that before treatment and higher than in PBMC (Fig. 2, 4).

In Stage IV patients, before and after treatment,

phospho-IκB expression in PMN and PBMC was less pronounced as compared to the control cells (Fig. 6).

Concentration of total NO (NO_3^-/NO_2^-) in PMN and PBMC supernatants. In Stage II and III patients before treatment, both unstimulated and stimulated PMN were significantly less capable to release NO than in healthy subjects (Tab. 1). In contrast, PBMC of these patients have unchanged ability to NO production.

After treatment, the concentrations of total NO in unstimulated PMN supernatants were lower in comparison to those obtained prior to treatment. In the supernatants of unstimulated PMN and PBMC obtained from Stage IV patients, before and after treatment, total NO concentrations were comparable to those of control (Tab. 1). Incubation of these cells in the presence of LPS and rhIL-18 caused a significant increase in the production of NO compared to unstimulated cells.

Concentrations of total NO (NO_3^-/NO_2^-) in blood serum. Significantly higher values of total NO concentration were noted in the blood serum of Stage IV patients before and after treatment as compared to the control (Fig. 7). The concentration of total NO in Stage IV patients before treatment was higher than in Stages II and III (Fig. 7).

In Stage IV patients after treatment serum total NO levels were lower than before treatment.

Discussion

Many clinical and experimental studies have demonstrated that low NO level may promote cancer growth, while high concentrations have a cytotoxic effect on neoplastic cells [4, 6].

Thus, the impaired NO production by PMN observed in patients with Stage II and III squamous cell carcinoma of the oral cavity before treatment may be connected with promotion of neoplastic growth in these patients. However, unaltered NO release and higher iNOS expression in mononuclear cells are likely to equalize, to a certain degree, the defect of this enzyme in neutrophils.

Different relations between NO production and iNOS expression in PMN and PBMC in patients with squamous cell carcinoma of the oral cavity suggest the involvement of various mechanisms affecting NO synthesis in these cells.

The presence of intracellular inhibitor of NO synthases in PMN may result in poor ability of these cells to generate NO in Stages II and III [17]. Reduced release of NO by PMN can also be due to the presence of natural cytoplasmic agents that exhibit the activity of NO synthase inhibitors, including dimetylo-α-arginin (ADMA) [18–20].

Further decrease in iNOS expression observed in Stages II and III after treatment may indicate intensification of permanent neutrophil defect in the enzyme expression, with the resulting low NO production.

Changes in iNOS expression may be caused by activation of various intracellular signal pathways. Inverse correlations between iNOS expression and phospho-IκB expression observed in patients and control subjects seem to suggest that iNOS induction in PMN and PBMC is not directly dependent on NF-κB pathway, and may occur with the involvement of other transcription factors, such as IFR-1, NF-IL-6, AP-1 or STAT1α, which undergo activation under the influence of various mediators, and even cytokines [8, 6, 9].

We assessed the role of rhIL-18 in the induction of iNOS expression and NO production by PMN and PBMC. Lack of rhIL-18 effect on iNOS expression and on NO production by PMN and PBMC, observed in Stage II and III patients, may be the result of a reduced expression of the cytokine membrane receptor (IL-18R). This seems to be confirmed by KOBASHI et al, who observed a markedly decreased level of mRNA IL-18R in mononuclear cells in cancer patients as compared to healthy control subjects [21].

However, in Stage IV patients both iNOS expression and NO production were increased in the presence of rhIL-18, which may be caused by an effect of high concentrations of other iNOS-activating cytokines, such as IL-1β, IL-6 or TNF-α. A study of JABLONSKA et al has demonstrated that concentrations of these cytokines in the serum of patients with squamous cell carcinoma of the oral cavity increase with the progression of the neoplastic process [22].

NO produced *in vivo* by PMN and PBMC and released to blood may exert a systemic effect on a number of systems and organs. In contrast to NO production by cells examined, we observed the highest serum concentrations of total NO in patients with advanced neoplastic process. The results are in

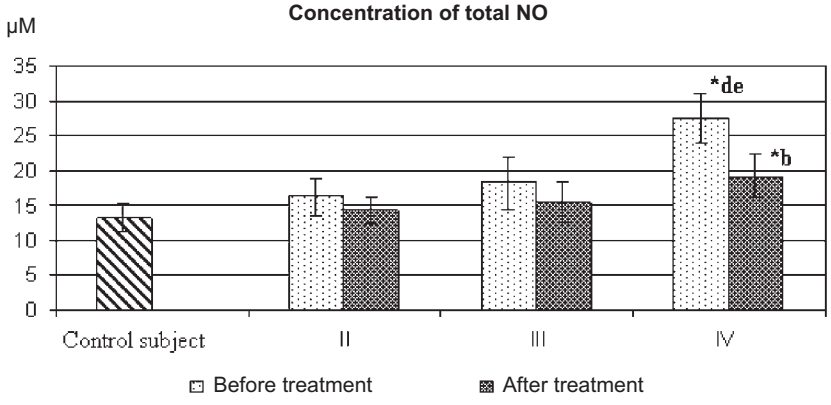

Figure 7. Concentrations of total NO (NO_3^-/NO_2^-) in blood serum of patients in different stages of disease.
[*]statistical differences with control subject ($p<0.05$), [b]statistical differences between patients before and after treatment ($p<0.05$), [d]statistical differences between patients in stage II and patients in stage IV stage ($p<0.05$), [e]statistical differences between patients in stage III and patients in stage IV stage ($p<0.05$).

Table 1. Concentrations of total NO (NO_3^-/NO_2^-) in PMN and PBMC supernatants lysates of PMN and PBMC of patients in different stages of disease.

Cells	Concentrations of total NO (NO_3^-/NO_2^-) ($mM/5x10^6$ kom/ml)													
	Control subject		Before treatment						After treatment					
			Patients in stage II		Patients in stage III		Patients in stage IV		Patients in stage II		Patients in stage III		Patients in stage IV	
	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD	PMN $\bar{x}$ ±SD	PBMC $\bar{x}$ ±SD
Unstimulated	20,87 ±3,05	19,68 ±3,51	16,73* ±4,3	17,78 ±3,8	13,44* ±2,25	17,93 ±4,95	19,64[e] ±4,72	19,03 ±3,86	13,02*[b] ±1,78	13,42*[b] ±2,15	12,08* ±2,56	14,3*[b] ±4,9	22,26d[e] ±3,9	18,63[d] ±3,1
LPS – stimulated	30,01[a] ±5,25	26[a] ±5,57	16,9* ±4,44	19,13* ±6,2	15,92* ±4,22	19,04* ±6,75	28,43[ade] ±3,34	27,75[a] ±4,65	13,26* ±3,31	16,37* ±4,29	13,58* ±2,56	15,01*[b] ±3,77	27,06[ade] ±4,73	26,3[ade] ±6,4
rhIL-18 – stimulated	26,45[a] ±3,44	22,5[a] ±4,32	17,58* ±2,96	17,82* ±2,01	14,45* ±2,7	18,17* ±4,32	32,66[ade] ±4,74	25,06[ade] ±2,28	16,88* ±2,61	15,12* ±3,07	13,85* ±3,5	16,78* ±3,99	25,15[ade] ±1,9	25,68[ade] ±4,82

*statistical differences with control subject ($p<0.05$), [a]statistical differences between unstimulated and stimulated cells ($p<0.05$), [b]statistical differences between patients before and after treatment ($p<0.05$), [c]statistical differences between PMN and PBMC ($p<0.05$), [d]statistical differences between patients in stage II and patients in stage IV stage ($p<0.05$), [e]statistical differences between patients in stage III and patients in stage IV stage ($p<0.05$).

agreement with data presented by BEEVI et al, who also observed high NO concentrations in the serum of patients with Stage III/IV squamous cell carcinoma of the oral cavity [23].

A lack of direct correlation between the concentrations of total NO in cell supernatants and in blood serum in the study group of patients may suggest even another possibility apart from PMN and PBMC, source of this mediator. It may be the neoplastic cells, as suggested by our own results obtained from patients after surgical resection of tumor mass, who had reduced total NO level in blood serum.

Also, GAVILANES et al showed a considerable increase in the expression and activity of iNOS in the mucous membrane of patients with squamous cell carcinoma of the oral cavity as compared to slight or lacking activity in healthy subjects [24].

Concluding, changed iNOS expression and NO production by neutrophils and mononuclear cells may have unfavorable effect on the course of antineoplastic treatment response in patients with squamous cell carcinoma of the oral cavity. Enhanced expression of iNOS and increased production of nitric oxide by the studied cells in Stage IV due to rhIL-18 effect suggest its beneficial effect on the activity of leukocytes in oral carcinoma patients. However, increase of NO secretion may be responsible for overexpression of cGMP leading to uncontrolled signal transduction [5, 8]. Therefore, larger prospective studies, are needed to explain a role of NO in patients witch squamous cell carcinoma of the oral cavity.

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