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iNOS expression and NO production by neutrophils in cancer patients

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Introduction:

The tumor-polymorphonuclear neutrophil (PMN) relationship can be altered by the release of toxic molecules, such as nitric oxide (NO). The aim of the present study was to examine the expression of the inducible synthase of NO (iNOS) and NO production by human neutrophils of patients with oral cavity cancer. For comparison we performed similar examinations in autologous peripheral blood mononuclear cells (PBMCs).

Materials and Methods:

PMNs and PBMCs were isolated from the whole blood of 27 patients with squamous cell carcinoma of the oral cavity. iNOS protein expression in these cells was detected by Western blot. Total nitrite as an indicator of NO concentrations in the culture supernatants and the serum of patients was measured using a colorimetric assay.

Results:

The PMNs of oral cavity cancer patients showed a significantly lower intensity of iNOS expression than those of healthy controls. The PBMCs of patients showed a more intensive expression of iNOS than the PMNs, but a lower intensity than the PBMCs of the controls. The expression of iNOS in rhIL-6 and rhIL-15-stimulated PMNs and PBMCs of patients increased in comparison with unstimulated cells. We observed lower productions of NO by PMNs and PBMCs of patients than those of the control group.

Conclusions:

The results revealed that altered iNOS expression and NO production are more characteristic of PMNs than of PBMCs of patients with oral cavity cancer. Additionally, this study provided new information about IL-6 and IL-15 activity in a tumor-bearing host.

Key words:

neutrophils • peripheral blood mononuclear cells • squamous oral cavity cancer • nitric oxide • inducible synthase of nitric oxide

Abbreviations:

PMN – neutrophil, **PBMC** – peripheral blood mononuclear cell, **NO** – nitric oxide, **iNOS** – inducible nitric oxide synthase.

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INTRODUCTION

Human polymorphonuclear neutrophils (PMNs), which comprise 50–70% of circulating leukocytes, have been postulated to have both beneficial and detrimental roles in the immune response to tumor^{4, 18}. During their growth phase, tumors are infiltrated first by PMNs, then by macrophages, and lastly by T lymphocytes¹⁶. Furthermore, PMNs can play a role in cytokine-induced tumor rejection, often in cooperation with CD8⁺ T lymphocytes⁴. On the other hand, interactions between PMNs and tumor cells may support tumor progression, since tumor cells can exploit PMNs and alter their function to increase their metastatic ability¹⁸.

The tumor-PMN relationship can be altered by the release of a toxic molecule, such as nitric oxide (NO)^{7, 19}. It has been well established that NO plays a dual role in tumor growth¹⁰. The anti-tumor activity of NO involves inhibition of tumor cell proliferation, differentiation, and metastatic spread as well as immunosuppression^{3, 10}. The tumoricidal effect of NO is associated with the induction of DNA-dependent protein kinase catalytic subunit expression, which is the key enzyme in DNA repair¹⁰. Furthermore, NO reacts with the superoxide anion to form a peroxynitrite anion, a highly toxic molecule which causes DNA damage and protein modification³. However, NO has also been demonstrated to be a positive factor in permitting tumor growth, including mutagenicity, angiogenesis, and metastasis⁵.

NO is produced by the metabolic conversions of L-arginine into L-citrulline by the activity of NO synthase (NOS)^{7, 19}. In the synthesis of NO, all isoforms depend on the substrate L-arginine and on cofactors such as NADPH, BH₄, FAD, FMN, O₂, and protoporphyrin IX¹⁰. PMNs were shown to express an inducible isoform of NOS (iNOS) which is independent of calcium and calmodulin^{7, 19}. Several examinations demonstrated that iNOS expression is controlled by an increasing number of agonists, especially by pro-inflammatory mediators. The most prominent cytokines involved in iNOS stimulation are IFN- γ , TNF- α , IL-1 β , IL-6, and IL-15^{10, 17}. IL-6 and IL-15 are cytokines with a wide range of effects on PMN function in normal and pathological conditions, involving malignancy⁸. There are data indicating the influence of IL-6 and IL-15 on iNOS expression in different cells, but not in PMNs^{7, 20}.

The aim of this study was to examine the expression of iNOS and NO production by human neutrophils of patients with oral cavity cancer after rhIL-6 and rhIL-15 stimulation. For comparative purposes we

performed similar examinations in autologous peripheral blood mononuclear cells (PBMCs). The key question was whether the expression of iNOS and NO production are more characteristic of PMNs or PBMCs in patients with oral cavity cancer. Studies of this function of PMNs and comparison with that of PBMCs may extend our knowledge of the role of these cells in NO-mediated reactions in tumor-bearing hosts.

MATERIALS AND METHODS

We examined 27 patients between 45 and 49 years of age with squamous cell carcinoma of the oral cavity treated in the Department of Oral and Maxillofacial Surgery of the Medical University of Białystok. The patients had received no treatment or medication before the examination. Patients with active infection and other diseases were excluded from the study. Control subjects (n=15) were healthy people aged from 30 to 53 years (mean 41.5 years). The study was approved by the local ethics committee and all patients gave written informed consent.

Cells were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient 1.115 g/ml (Polfa, Poland) as described by Zeman et al.²¹. This method enables simultaneous separation of two highly purified leukocyte fractions: one of mononuclear cells (PBMCs), containing 95% lymphocytes, and one of polymorphonuclear cells (PMNs), containing 94% PMNs. The purity of the isolated PMNs and PBMCs was determined by May-Grunewald-Giemsa staining. The cells were suspended in culture medium (RPMI-1640) to produce 5×10⁶ cells/ml and then 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% CO₂ (NUAIRETM). LPS (10 μ g/ml; Difco, Detroit, MI, USA) and/or rhIL-15 (50 ng/ml; R&D Systems, Minneapolis, USA) and rhIL-6 (50 ng/ml; R&D Systems, Minneapolis, USA) were tested to stimulate secretion by PMNs and PBMCs.

Western blot analysis

The cells were incubated for 4 h and then lysed directly by sonification. Cytoplasmic protein fractions of PMNs and PBMCs were suspended in Lamli buffer (Bio-Rad Laboratories, Hercules, CA, USA) and then were electrophoresed on SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). The resolved protein was transferred onto nitrocellulose with a pore size of 0.2 μ m (Bio-Rad Laboratories, Hercules, CA, USA). The nitrocellulose was incubated at +4°C for 18 h with the primary monoclonal

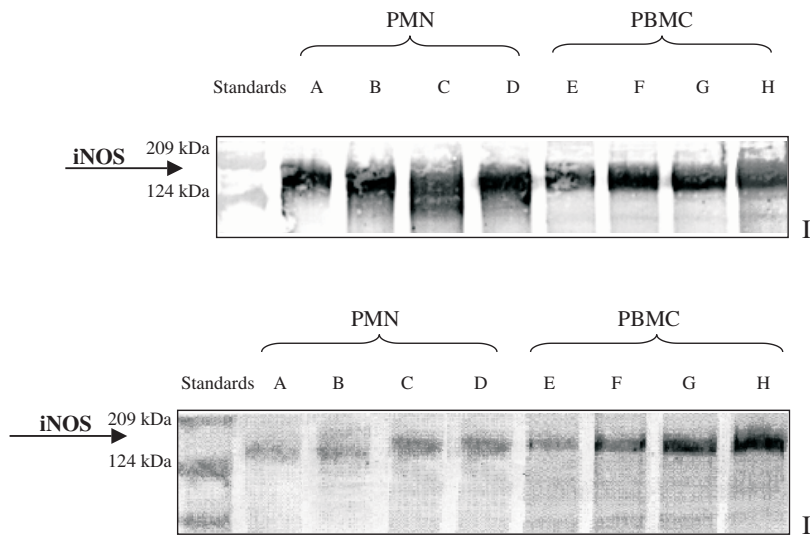


Figure 1. Western blot analysis of iNOS protein expression in human PMNs and PBMCs. Shown is the detection of iNOS proteins from human PMNs: unstimulated (lane A), LPS stimulated (lane B), rhIL-15 stimulated (lane C), rhIL-6 stimulated (lane D) and PBMC unstimulated (lane E), LPS stimulated (lane F), rhIL-15 stimulated (lane G), and rhIL-6 stimulated (lane H). Whole-cell lysates were obtained from fresh PMNs and PBMCs of healthy donors (I) and patients with squamous cell carcinoma (II). One hundred micrograms of total protein from the cells were loaded onto a 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with 1:1000 dilution of monoclonal antibody anti-iNOS. Sizes of the protein standards are given in kilodaltons. One representative Western blot of a few independent experiments is shown.

antibody anti-iNOS (R&D Systems, Minneapolis, USA). After washing with 0.1% TBS-T, the membrane was incubated at room temperature for 1 h with alkaline phosphatase anti-mouse IgG antibodies (Vector Laboratories, Burlingame, CA, USA). Immunoreactive protein bands were visualized following the addition of AP Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Determination of NO concentrations

Total nitrite as an indicator of NO concentration was measured using a colorimetric, non-enzymatic assay (OXIS International Inc., USA). NO is rapidly converted to nitrite and nitrate in typical oxygenated aqueous solutions. This assay provides for chemical reduction of nitrite by granulated cadmium, followed by spectrophotometric analysis of total nitrite using Griess's reagent. Absorbance was determined at 540 nm. The concentration of nitrite in culture supernatant or serum was determined with reference to a sodium nitrite standard curve. Results were expressed as μM of nitrite per 5×10^6 cells.

Statistics

Statistical analysis was assessed by Student's *t*-test, and $p < 0.05$ was taken as statistically significant.

RESULTS

iNOS protein expression in human PMNs and PBMCs detected by Western blot

We used a specific monoclonal antibody directed against the intracellular form of iNOS in PMNs and, for comparison, in PBMCs. The antibody in unstimu-

lated and stimulated human PMNs and PBMCs identified bands of 130 kDa from each donor of the control group.

Western blot analysis showed that the samples of unstimulated PMNs and PBMCs of the control group contained a 130 kDa protein that was stained by anti-iNOS monoclonal antibody (Fig. 1). LPS- and IL-6-stimulated cells expressed a slight increase in iNOS protein in comparison with unstimulated cells. After rhIL-15 stimulation, control PMNs and PBMCs also expressed a slight increase in iNOS protein, but lower than in LPS- or IL-6-stimulated cells. Comparison of iNOS bands in control PMNs and PBMCs indicated that the expression of iNOS in these cells is on the same level.

The samples of PMNs of oral cavity cancer patients showed a substantially lower intensity of iNOS expression than those of the controls. The PBMCs of patients expressed iNOS more intensively than did PMNs, but less intensively than the PBMCs of the controls. Patient PMNs and PBMCs stimulated by rhIL-15 and rhIL-6 showed a substantially higher expression of iNOS protein than did unstimulated cells.

NO production by PMNs and PBMCs

In this study we examined total nitrite as an indicator of NO concentrations in the culture supernatants of PMNs and PBMCs of controls and oral cavity cancer patients (Table 1). Unstimulated and LPS-, rhIL-15-, and rhIL-6-stimulated PMNs of patients secreted lower amounts of NO than those of the control group. Unstimulated, rhIL-15- and rhIL-6-stimulated PBMCs of patients and controls produced similar

Table 1. Total nitrite production by PMNs and PBMCs of controls and patients with oral cavity cancer

	Nitrite ($\mu\text{M}/5 \times 10^6$ cells/ml) X $\pm$ SD			
	controls (n=15)		patients (n=27)	
	PMN	PBMC	PMN	PBMC
Unstimulated	20.87 $\pm$ 3.05	19.68 $\pm$ 3.51	15.43 ^a $\pm$ 4.68	17.79 $\pm$ 4.28
LPS stimulated	30.01 ^c $\pm$ 5.25	26.0 ^{ce} $\pm$ 5.57	16.59 ^a $\pm$ 4.31	19.37 ^b $\pm$ 5.70
rhIL-6 stimulated	25.00 ^d $\pm$ 5.30	27.7 ^{ce} $\pm$ 14.01	20.33 ^{cb} $\pm$ 5.64	26.55 ^c $\pm$ 16.97
rhIL-15 stimulated	24.19 ^d $\pm$ 3.16	23.07 ^d $\pm$ 4.12	18.07 ^a $\pm$ 4.89	24.26 ^c $\pm$ 14.24

^a Significant difference between cells of patients and control group ($p < 0.001$).

^b Significant difference between cell of patients and control group ($p < 0.05$).

^c Significant difference between unstimulated and stimulated cells ($p < 0.001$).

^d Significant difference between unstimulated and stimulated cells ($p < 0.05$).

^e Significant difference between PMNs and PBMCs ($p < 0.05$).

amounts of NO. RhIL-6 and rhIL-15 exerted significant effects on the production of NO by PMNs and PBMCs of the patient group in comparison with unstimulated cells (Table 1).

Total nitrite in the serum

The concentrations of total nitrite in the serum of patients were insignificantly lower than in the serum of control group ($14.26 \pm 1.57 \mu\text{M}$ and $16.06 \pm 4.02 \mu\text{M}$, respectively).

DISCUSSION

In this study we observed substantially lower iNOS expression associated with a lower production of NO by PMNs in patients with oral cavity cancer in comparison with a control group of healthy persons. NO production by unstimulated PBMCs, in contrast to that by PMNs, of patients and of the control group were on the same level. Taking into account that low concentrations of NO act favorably in tumor progression, the low production of NO by the examined cells may have unfavorable effects on local defense mechanisms in patients with oral cavity cancer. Additionally, nitric oxide was reported to exert angiogenic properties in various tumor models. It was demonstrated that the synthesis and function of vascular endothelial growth factor and basic fibroblast growth factor are stimulated by NO^{5,10}. On the other hand, angiostatin and thrombospondin-1, the two main inhibitors of angiogenesis, are suppressed by NO¹⁰.

In this study we also observed that rhIL-6 and rhIL-15 were important stimulators of NO production by PBMCs as well as by PMNs of patients. The effects of rhIL-6 on NO production by PMNs are similar to the data of Goode et al.⁷, who demonstrated that the release of NO by these cells increased by exposure to

IL-6. They also found that LPS is a significant stimulator of NO production by PMNs. Moreover, Takeichi et al.¹⁷ reported that stimulation by LPS induces the secretion of NO by human PMNs.

In contrast to the control group, we did not observe a significant effect of LPS on NO secretion by PMNs of cancer patients. This is in agreement with data of Yan et al.¹⁹, who also demonstrated that human PMNs produce a significantly low level of NO in response to LPS and may lack the ability to secrete this molecule in detectable amounts.

The inducible effect of rhIL-15 on the production of NO by control PMNs appears to confirm other authors' data indicating a role of IL-15 in the enhancement of phagocytosis, nuclear factor- κ B activation, IL-8 production, or in a delay of apoptosis in these cells^{2,6,11,14}.

In patients with oral cavity cancer we found no influence of rhIL-15 on NO secretion by PMNs. These results are similar to our previous observations in oral cavity cancer patients. The PMNs of these patients were not sensitive to rhIL-15 stimulation, but we found a priming effect of rhIL-15 on the production of IL-1 β by LPS-stimulated cells⁸. Musso et al.¹² also demonstrated a priming effect of IL-15 on the oxidative burst elicited by fMLP.

Changes in NO production by PMNs from the control and patient groups are reflected in iNOS expression in these cells. This confirms a significant role of iNOS in NO secretion by neutrophils. However, there are data indicating the presence of constitutive NOS (cNOS) in PMNs that requires calcium and calmodulin for activation⁹. Since cNOS produces only small amounts of NO, it is probable that cNOS did not have an effect on NO production in the patients with oral cavity cancer^{9,19}. Similarly, a novel

non-enzymatic pathway for NO synthesis in PMNs involving hydrogen peroxide and D- or L-arginine also did not influence the production of this mediator¹³.

Similar relations were observed between NO production and iNOS expression in autologous PBMCs. This may suggest that the same molecules presented on tumor cells or secreted by them are responsible for the activity of PMNs and PBMCs in patients with oral cavity cancer. For example, the presence of iNOS expression in tumor cells may cause the lack of a relationship between the production of NO by PMNs and PBMCs and NO concentration in the serum of the examined patients. Rosbe et al.¹⁵ indicated that iNOS activity is present in head and neck squamous cell carcinoma, suggesting that iNOS can play a significant role in tumor growth. Brennan et

al.¹ observed a significant relationship between iNOS expression and lymph node metastasis in these patients. Chen et al.³ demonstrated that iNOS expression correlated with cervical lymph node metastasis in oral squamous cell carcinomas.

In conclusion, the obtained results have revealed that altered iNOS expression and NO production are more characteristic of the PMNs than of the PBMCs of patients with oral cavity cancer. Additionally, this study provides new information about IL-6 and IL-15 activity in tumor-bearing hosts.

Further studies, involving the examination of iNOS and NO production according to the clinical course of patients after treatment, are required to investigate their potential implications and prognostic importance in this patient group.

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