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VEGF- and EGF-mediated cooperation between eosinophilic granulocytes and tumor cells in gastric and colon cancer

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ABSTRACT

Aim of the research was to analyze the secretion of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) by blood eosinophilic granulocytes in vitro, as well as expression of receptors VEGFR and EGFR in tumor tissue in gastric and colon cancer with tumor-associated tissue eosinophilia (TATE).

Materials and methods. A total of 52 patients with gastric cancer and 50 patients with colon cancer were examined. The material of the research included supernatants of eosinophil cultures and samples of malignant tumors tissues of the stomach and colon. Enzyme-linked immunosorbent assay (ELISA) was used to determine the contents of VEGF and EGF in the eosinophil culture supernatants in vitro. The expression of VEGFR and EGFR in tumor tissue was evaluated by immunohistochemistry. The results were analyzed by statistical methods.

Results. An increase in basal and r-IL-5-induced VEGF secretion by blood eosinophils *in vitro* in gastric cancer patients with TATE was identified. The concentration of EGF in the culture of blood eosinophils stimulated with r-IL-5 in vitro in patients with TATE was increased, regardless of the localization of pathological process both in patients with gastric cancer and colon cancer. TATE in gastric cancer and colon cancer was associated with decreased expression of EGFR by tumor cells, while VEGFR expression was not dependent on the eosinophilic infiltration of the tumor.

Conclusion. Hypersecretion of VEGF and EGF (upon stimulation with r-IL-5) by blood eosinophils in vitro in patients with gastric and colon cancer with tissue eosinophilia indicates an increase in the activity of eosinophilic granulocytes. Deficiency of VEGF and EGFR expression in tumor might underlie a disturbance of cooperative interaction between eosinophils and tumor cells in malignant tumors of the stomach and colon.

Key words: eosinophil, growth factors, growth factor receptors, gastric cancer, colon cancer.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

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VEGF- и EGF-опосредованная кооперация эозинофильных гранулоцитов и опухолевых клеток при раке желудка и толстого кишечника

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РЕЗЮМЕ

Цель исследования – проанализировать секрецию сосудисто-эндотелиального фактора роста (VEGF) и эпидермального ростового фактора (EGF) эозинофильными гранулоцитами крови *in vitro* и экспрессию рецепторов VEGFR и EGFR в опухолевой ткани при раке желудка и толстого кишечника в ассоциации с тканевой эозинофилией.

Материалы и методы. Обследовано 52 пациента с раком желудка и 50 пациентов с раком толстого кишечника. Материалом исследования служили супернатанты суспензионной культуры эозинофильных гранулоцитов и образцы тканей злокачественных новообразований желудка и толстого кишечника. Методом иммуноферментного анализа определяли содержание VEGF и EGF в супернатантах культуры эозинофильных гранулоцитов *in vitro*. Экспрессию VEGFR и EGFR в опухолевой ткани оценивали методом иммуногистохимии. Полученные результаты анализировали статистическими методами.

Результаты. Установлено увеличение базальной и индуцированной рекомбинантным интерлейкином (r-IL) 5 секреции VEGF эозинофильными гранулоцитами крови *in vitro* у больных раком желудка, сопровождающимся тканевой эозинофилией. Концентрация EGF в культуре эозинофилов крови *in vitro* при добавлении r-IL-5 повышалась у больных с эозинофильной инфильтрацией опухолевой ткани вне зависимости от локализации патологического процесса как у больных с раком желудка, так и с раком толстого кишечника. Эозинофильная инфильтрация опухолевой ткани при раке желудка и раке толстого кишечника сочеталась с гипоэкспрессией опухолевыми клетками EGFR; экспрессия рецептора VEGFR не зависела от присутствия эозинофильных гранулоцитов в ткани опухолей.

Заключение. Гиперсекреция сосудисто-эндотелиального фактора роста VEGF и эпидермального ростового фактора EGF (при индукции r-IL-5) эозинофилами крови *in vitro* у больных раком желудка и толстого кишечника с тканевой эозинофилией свидетельствует о повышении активности этих клеток. Дефицит экспрессии в опухолевой ткани рецепторов VEGFR и EGFR обусловливает нарушение кооперативного взаимодействия эозинофильных гранулоцитов и опухолевых клеток при злокачественных новообразованиях желудка и толстого кишечника.

Ключевые слова: эозинофил, ростовые факторы, рецепторы, рак желудка, рак толстого кишечника.

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INTRODUCTION

The growth and progression of malignant tumors are largely influenced by the activity of surrounding immune and stromal cells, which are collectively called tumor microenvironment (TME) [1]. The influence of different elements of TME is known to affect the drug and immune resistance of tumor cells, their ability to undergo senescence, and various other processes essential for malignant growth [2]. However, the role of some of the participants of TME, such as eosinophils, still remains ambiguous. Eosinophil granulocytes are mostly known for their capability to elicit cytotoxicity against tumor cells [3-5]. At the same time, mounting evidence suggests that eosinophils might be involved in tumor development via expression of numerous humoral factors and receptors [6].

Eosinophil granulocytes secrete various mediators, including vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Similar to hormones, these pleiotropic factors act on various cells and produce different receptor-mediated effects, being involved in cell proliferation and maturation, chemotaxis and other cellular processes. The role of growth factors in the pathogenesis of tumor progression is well studied [7-8]. Thus, an interaction between EGF and its cognate receptor on cancer cells activates protein synthesis and cell proliferation [9]. VEGF is a pivotal angiogenic factor which stimulates formation of vascular and lymphatic vasculature of the tumor [10]. In addition, elevated expression of VEGF and EGF receptors (VEGFR and EGFR) in the tumor tissue is observed in malignant tumors of different histological types and localizations, and is considered to be a negative prognostic criterion [11].

It is speculated that eosinophilic granulocytes infiltrating gastric and colon tumors are able to influence the activity of transformed cells by producing growth polypeptides, thereby stimulating self-sustenance, hyperproliferation and invasiveness of the tumor, as well as enhancing the formation of tumor neovasculature. The study of mechanisms responsible for cooperation between eosinophils and tumor cells will considerably expand our knowledge about the role of tissue eosinophilia in the pathogenesis of gastric and colon

cancer.

Aim of the research was to study the features of VEFG and EGF secretion by blood eosinophils *in vitro* and analyze the expression of corresponding cognate receptors in the tumor tissue in patients with gastric and colon cancer with or without TATE.

MATERIALS AND METHODS

The study was carried out in the laboratory of clinical and experimental pathophysiology of Pathophysiology Division, SSMU (Head of the Division – DMedSc, Professor, Corresponding Member of RAS Urazova O.I.) and the pathological anatomy division of Tomsk Regional Oncologic Dispensary (Head of the Division – DMedSc Purlik I.L.) A total of 52 patients with gastric cancer and 50 patients with colon cancer (C18-C20) undergoing treatment in Tomsk Regional Oncologic Dispensary (Chief Doctor – S.V. Mazeina) participated in the research. All patients were examined and operated on prior to the use of radio- and chemotherapy. The diagnosis of gastric and colon cancer was based on clinical examination and morphological data. All patients were divided into groups with regard to the clinical form of malignancy and the presence of TATE. As a result, 25 gastric cancer patients with TATE (average age 65.3±4.7; 27 gastric cancer patients without TATE (average age 62.9±5.2); 23 colon cancer patients with TATE (average age 63.0±7.3); and 32 colon cancer patients (average age 61.3±6.0) without TATE were included in the study.

The exclusion criteria were as follows: preoperative radiotherapy and chemotherapy, malignancies of other localizations, chronic allergic, autoimmune, and infectious diseases. The control group included 36 healthy volunteers (22 men and 14 women, average age 57.1±3.3). Tissue samples of gastric and colon cancer and supernatants of suspension culture of eosinophilic granulocytes were used as the materials of the study.

To analyze intra-tumoral expression of VEFGR and EGFR, gastric and colon cancer samples were immunohistochemically stained via an automatic immunohistostainer Bond-maX (Leica Biosystems, Germany) using murine anti-VEFGR antibodies, clone KLT9, working concentration 1:100 («Novocastra», Leica Biosystems, Germany), and murine anti-EGFR antibodies, clone EGFR.25, RTU. The expression level was judged based on the relative number of tumor cell positive for VEGFR and EGFR [13,14]. Minimum of 300 cells within the areas of maximal receptor expression were calculated.

Eosinophilic granulocytes were isolated from the whole blood (20 mL, collected from the cubital vein on an empty stomach) using Ficoll-Paque density gradient (ρ = 1.077 g/mL) with subsequent immunomagnetic separation using «Eosinophil isolation kit» (Miltenyi Biotec GmbH, Germany) according to manufacturer's protocol. To stimulate cytokine production by eosinophils, 10^{-8} g/mL of recombinant IL-5 (r-IL-5) («Biosource», Belgium) was added to the samples.

Concentration of VEGF and EGF in supernatants of suspension cultures of eosinophils was evaluated by ELISA according to manufacturer's protocols (RayBio, US). Optical density was measured on a photometric analyzer Multiscan EX (Finland).

Statistical analysis of the result was done using «Statistica for Windows» Version 8.0 («StatSoft Inc.», US). Shapiro-Wilk test was used to assess the normality of the samples. Quantative parameters in the comparison groups were presented as a mean (M) and standard deviation (σ), as well as median (Me), upper (75%) and lower (25%) quartiles (Me (Q_1-Q_3)). The significance of difference among independent samples with non-gaussian distribution was assessed using non-parametric Mann-Whitney U test. To compare means of studied samples, one-way analysis of variance (F-test) was applied. Differences were considered significant at $p < 0.05$.

RESULTS

A crosstalk between malignant cells and elements of tumor microenvironment is considered a major factor in the pathogenesis of oncological diseases. One of the mechanisms underlying this cooperation is the interaction between growth factors secreted within the tumor site and their cognate receptors. Among them, VEGF and EGF have been shown to play a significant role in the development of gastric and colon cancer [10, 15, 16].

Different cells within TME are capable of secreting growth factors [9, 11, 17]. According to R. Shamri et al. (2010), tissue eosinophils produce VEGF in the areas of tumor-associated necrosis [18]. High serum levels of VEGF were observed in colon, breast and kidney cancers [16, 19]. Also, upregulated intra-tumoral expression of VEGF is considered a negative prognostic factor in patients with lung and prostate cancers [20].

In the present study we analyzed basal and r-IL-5-induced secretion of VEGF by eosinophils *in vitro* in gastric and colon cancer patients. An increase in eosinophil-derived VEFG secretion *in vitro*, both intact and induced, was demonstrated only in gastric cancer patients with TATE. At the same time, r-IL-5-induced VEFG secretion did not differ significantly from the basal levels ($p>0.05$), which might indicate a depletion of functional reserves, and as a result, decreased reactivity of the cells (Tab 1).

In gastric cancer with TATE, as well as in colon cancer with or without TATE, basal and stimulated VEGF secretion by eosinophils was comparable to the control group values (Tab. 1).

Таблица 1
Table 1

Секреция ростовых факторов в *in vitro* культуре эозинофильных гранулоцитов у больных раком желудка и толстого кишечника, пг/мл, *Me* (Q_1–Q_3)

Secretion of growth factors *in vitro* in eosinophil culture in gastric and colon cancer patients, pg/ml, *Me* (Q_1–Q_3)

Группы обследованных лиц Groups the examined persons		VEGF		EGF	
		Базальная секреция Basal secretion	Секреция, индуцированная r-IL-5 Secretion, induced by r-IL-5	Базальная секреция Basal secretion	Секреция, индуцированная r-IL-5 Secretion, induced by r-IL-5
Здоровые доноры Healthy donors		1,83 (0,40–2,35)	3,76 (1,81–5,10)	0,52 (0,20–0,93)	0,19 (0,08–0,21)
Больные раком желудка Patients with gastric cancer	с эозинофильной инфильтрацией ткани опухоли, $n = 19$ with eosinophilic infiltration of tumor tissue, $n = 19$	26,23 (15,52—33,03) $p_1 < 0,05$	15,88 (11,42—21,23) $p_1 < 0,05$	0,65 (0,54—1,23)	1,13 (0,80—2,72) $p_1 < 0,05$
	без эозинофильной инфильтрации ткани опухоли, $n = 21$ without eosinophilic infiltration of tumor tissue, $n = 21$)	3,66 (2,18—5,09) $p_2 < 0,05$	1,80 (1,16—2,98) $p_2 < 0,05$	0,79 (0,37—1,95)	0,31 (0,15—2,94)
Больные раком толстого кишечника Patients with colon cancer	с эозинофильной инфильтрацией ткани опухоли, $n = 23$ with eosinophilic infiltration of tumor tissue, $n = 23$	3,43 (1,35—5,74)	4,01 (1,35—6,46)	0,78 (0,43—1,09)	0,88 (0,65—1,33) $p_1 < 0,05$
	без эозинофильной инфильтрации ткани опухоли, $n = 20$ without eosinophilic infiltration of tumor tissue, $n = 20$	3,83 (2,62—5,04)	2,12 (1,22—3,70)	0,68 (0,58—1,21)	0,86 (0,45—1,35)

Примечание. Уровень статистической значимости различий по сравнению с аналогичными параметрами у здоровых доноров – p_1; у больных с тканевой эозинофилией – p_2; r-IL-5 – рекомбинантный IL-5.
Note. The level of statistically significant differences in compare to similar parameters in healthy donors – p_1; in patients with tissue eosinophilia – p_2; r-IL-5 – recombinant IL-5.

Eosinophilic granulocytes via production of VEGF can modulate proliferation of cells which express complementary receptors on their surface [7]. VEGF receptors are most abundant on the membrane of endothelial cells, while also expressed in various malignancies, including thyroid, cervical and prostate cancer, and others [21, 22]. Thereby, VEGF can both directly and indirectly influence proliferation of tumor cells.

Таблица 2
Table 2

Экспрессия рецепторов ростовых факторов VEGFR и EGFR в опухолевой ткани при раке желудка и толстого кишечника, %, $M \pm \sigma$
Expression of growth factor receptors VEGFR and EGFR in tumor tissue in gastric and colon cancer, %, $M \pm \sigma$

Показатель Characteristic	Локализация опухоли Tumor localization			
	Рак желудка, $n = 52$ Gastric cancer, $n = 52$		Рак толстого кишечника, $n = 55$ Colon cancer, $n = 55$	
	с эозинофильной инфильтрацией ткани опухоли, $n = 25$ with eosinophilic infiltration of tumor tissue, $n = 25$	без эозинофильной инфильтрации ткани опухоли, $n = 27$ without eosinophilic infiltration of tumor tissue, $n = 27$	с эозинофильной инфильтрацией ткани опухоли, $n = 23$ with eosinophilic infiltration of tumor tissue, $n = 23$	без эозинофильной инфильтрации ткани опухоли, $n = 32$ without eosinophilic infiltration of tumor tissue, $n = 32$
VEGFR	13,20 ± 8,47	12,81 ± 10,43	15,22 ± 9,34	14,87 ± 12,37
	$F = 0,02$; $p > 0,05$		$F = 0,01$; $p > 0,05$	
EGFR	9,16 ± 4,62	23,15 ± 12,72	12,65 ± 9,92	20,19 ± 11,42
	$F = 26,69$; $p < 0,05$		$F = 6,49$; $p < 0,05$	

Примечание. Уровень статистической значимости различий между показателями у пациентов с тканевой эозинофилией и без тканевой эозинофилии – p; F – значение F-статистики по результатам дисперсионного анализа.
Note. The level of statistically significant differences between indicators in patients with and without tissue eosinophilia – p; F – value of F-statistic based on the results of variance analysis.

Immunohistochemical staining showed the presence of VEGFR-expressing cancer cells in all examined tissue samples of gastric and colon cancer. The relative content of intra-tumoral VEGFR-positive cells in patients with gastric and colon cancer was comparable between samples with and without TATE. (Tab. 2).

We did not observe any association between VEGFR expression and the presence of VEGF-producing eosinophils in gastric cancer patients with TATE; VEGFR was predominantly expressed on the membranes of endotheliocytes. It is known that VEGF increases the permeability of vessel wall with its subsequent disorganization, which enables tumor cell intravasation and metastasis [23]. VEGF is also capable of stimulating angiogenesis via recruiting bone marrow hematopoietic and endothelial precursor cells [24].

EGF is another growth factor that enhances proliferation of endothelial and cancer cells [25-26]. EGF is expressed in platelets, leukocytes, and other cell types, and can be found in urine, blood plasma, saliva, etc. [14, 21]. Binding of EGF to its receptor EGFR stimulates DNA replication, synthesis of oncogenic proteins and contributes to uncontrolled cell proliferation [10].

Excessive EGF secretion as a result of mutations and enhanced gene expression has been shown to underlie the formation and progression of a variety of epithelial malignancies [25].

In the study, the basal eosinophil-derived secretion of EGF in patients with gastric and colon cancer did not differ significantly from the control group, regardless of the presence of tumor-associated tissue eosinophilia (Tab. 1). On the other hand, stimulation of cells with r-IL-5, a key eosinophil activator, was associated with an increased concentration of EGF in eosinophil culture in patients with gastric cancer and patients with colon cancer with TATE (Tab. 1). This data indicates that under additional stimulation, eosinophils are capable of mobilizing their functional reserves and upregulating EGF production.

The evaluation of intra-tumoral expression of EGFR showed that in patients with gastric and colon cancer with TATE, percentage of EGFR-positive tumor cells was 2.5 and 1.5 times lower, respectively, compared to patients without TATE (Tab. 2). Hypo-expression of EGFR in cancer cells significantly affects their proliferative capacity due to an imbalance of reception and signal transduction mechanisms [27]. Low expression of EGFR in tumor tissue is a positive prognostic factor and is associated with the absence of regional metastasis in breast, prostate and gastric cancer [28, 29]. In colon cancer, EGFR expression level was shown to correlate with the risk of relapse and general survival rate after surgery [30].

CONCLUSION

Malignant tumors of gastrointestinal tract with tumor-associated tissue eosinophilia are associated with an increased *in vitro* secretion of VEGF (in case of gastric cancer) and EGF (in case of gastric and colon cancer upon stimulation with r-IL-5) by blood eosinophils. At the same time, insufficient intratumoral expression of VEFGR and EGFR in gastric and colon cancer, apparently, affects the ability of eosinophils to regulate tumor growth and progression via secretion of growth factors. The indirect effect of eosinophilic granulocytes on the tumor neoangiogenesis, which enables survival and dissemination of malignant cells, should also be considered.

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