The Role of Vascular Endothelial Growth Factor Receptor (VEGFR-3) in Uterine Cervix Carcinogenesis

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Abstract

In spite of the huge achievements made in the early detection of uterine cervix cancer, this disease remains one of the most widespread human malignancies throughout the world. Nowadays, there is a tendency of cervical cancer to affect the youth population. Early metastasizing is a critical point in evolution of this disease. Lymphatic vessels (LV) represents the primary route of cancer cells spreading towards the distant sites. That is why understanding of new lymphatics recruitment by the tumor, at molecular level, could be a potent support in solving of metastasizing. VEGFR-3 is a tyrosine-kinase receptor, specific for lymphatic endothelial cells, that being activated provides their proliferation, surviving and migration. It has been demonstrated that VEGF-R3 mediates invasion activity of tumor cells into surrounding stroma and vascular structures. *The aim* of this research was to study the importance of VEGFR-3 in cervical carcinogenesis. *Material and methods:* We studied biological material, taken by targeted biopsy and conization, from women with detectable cervical lesions. Detection VEGFR-3 was made using monoclonal antibody anti VEGF-R3 through Avidin-Biotin working system, LSAB+ technique. Lymphatic microvascular density was assessed by podoplanin labeling, using anti D2-40. A statistical analysis was made with SPSS 13.0. *Results:* We obtained VEGFR-3 expression in LV, blood vessels, tumor mass and stromal cells. The highest density of VEGFR-3 + LV was in CIN III with gradually decreasing in invasive stages. In invasive carcinoma we obtained statistically significant correlation between tumor VEGFR-3 expression and vascular invasion. *Conclusions:* VEGFR-3 is not specific for lymphatic endothelium. It can not be used for alone lymphatic microvascular density assessing. It is actively involved in vascular invasion.

Key words: lymphatic vessels, cervical cancer, lymphatic endothelial cells, vascular invasion, VEGFR-3, D2-40.

Роль рецептора сосудистого эндотелиального фактора роста (VEGFR-3) в процессе канцерогенеза шейки матки

Несмотря на огромный успех, достигнутый в выявление рака шейки матки (РШМ) на ранних стадиях развития, данное заболевание остается одной из самых часто встречающихся злокачественных новообразований во всем мире. На сегодняшний день наблюдается тенденция омоложения РШМ. Раннее метастазирование является ключевым моментом в эволюции заболевания. Лимфатические сосуды представляют собой первичный путь распространения опухолевых клеток. В связи с этим, понимание механизмов образования лимфатических сосудов на молекулярном уровне, может помочь решить эту проблему. VEGFR-3 является тирозин-киназным рецептором. Он находится на поверхности лимфатических эндотелиальных клеток, при активации которого происходит их пролиферация, миграция и выживание. Было доказано, что VEGFR-3 регулирует инвазию раковых клеток в строму и сосуды. *Цель работы:* изучить роль VEGFR-3 в канцерогенезе РШМ. *Материал и методы:* был изучен биологический материал, полученный посредством прицельных биопсий и конизаций у пациенток с макроскопически выявленным поражением шейки матки. Для определения VEGFR-3 была использована система Avidin-Biotin, техника LSAB+, с применением первичного антитела анти VEGFR-3. Для определения лимфатических сосудов были использованы моноклональные антитела анти D2-40. Статистический анализ данных был произведен при помощи SPSS 13.0. *Результаты:* мы выявили экспрессию VEGFR-3 не только в лимфатических сосудах, а также в кровеносных сосудах, в опухолевой массе и клетках стромы. Наибольшая плотность лимфатических сосудов была определена на стадии CIN III (интраэпителиальная цервикальная неоплазия), с последующим прогрессивным уменьшением. На стадии инвазивного рака мы получили статистически значимую корреляцию между интенсивностью экспрессии VEGFR-3 опухолью и сосудистой инвазией, а также между интенсивностью экспрессии VEGFR-3 опухолью и внутрисосудистыми опухолевыми эмболами. *Выводы:* VEGFR-3 не является специфическим маркером для лимфатического эндотелия. Данный маркер не может использоваться самостоятельно для определения лимфатической микрососудистой плотности. VEGFR-3 активно вовлечен в процесс сосудистой инвазии.

Ключевые слова: лимфатические сосуды, рак шейки матки, лимфатические эндотелиальные клетки, сосудистая инвазия, VEGFR-3, D2-40.

Introduction

The role of HPV in cervical carcinogenesis is well-established and documented. This fact allowed the researchers to perform different strategies focused mainly on the prevention of this type of neoplasia, in contrast with other tumor diseases. As a result, both the morbidity and the mortality drastically decreased. Despite the facts named-above, cervical cancer remains one of the most frequent tumors, especially in those countries where the screening methods are not set enough.

There is a critical moment, related to its size, till which the newly-grown tumor can develop without any blood supply support. Overcoming this moment does not disturb the continuing development of the neoplasm. It happens due to a secretion of a wide variety of active substances (growth factors, cytokines), by the tumor cells and the stromal elements, which lead to the development of new blood vessels from the preexisting ones. This phenomenon, called angiogenesis (AG), not only provides the tumor with necessary nutrients and gasses, but also represents a gate for the neoplastic cells spread to the distant sites. A lot of varieties of human tumors, in parallel with blood vessels, co-opt the new lymphatic vessels, also, through lymphangiogenesis (LAG). The lymphatic vasculature, as well as the blood network, plays a crucial role in the tumor cells spreading. The lymphatic vessels represent the main ways which through the regional lymph nodes are metastaticaly affected. The importance of tumoral LAG beco-

mes clear in the light of the effects which consequently appear after metastatic damage of the regional lymph nodes. Their affection hardly influences clinico-pathologic parameters, such as: tumor grading, therapeutic management, surviving.

The most effective mitogens for endothelial cells are represented by a family of glycoproteins called VEGF (Vascular Endothelial Growth Factor), which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF *(Platelet Derived Growth Factor)*. These growth factors are key regulators of vasculogenesis, AG and LAG, and promote their specific effect via transmembrane tyrosin-kinase receptors: VEGFR-1, VEGFR-2 and VEGFR-3. VEGF-A and VEGF-B are specific ligands for VEGFR-2 and VEGFR-1, and promote AG. VEGF-C and VEGF-D are ligands for VEGFR-3 and promote LAG. VEGFR-2 can bind not only proangiogenic mediators, but also VEGF-C (in it's fully enzyme processed variant), whereas VEGFR-3 has affinity only for growth factors which induce lymphangiogenic signals.

During the early embryonic development VEGFR-3 is expressed in blood endothelial cells (BEC), and represents a key regulator of primary capillary plexus formation [5]. Contact with vascular smooth muscle cells (vSMC) downregulates the VEGFR-3 in endothelial cells [17]. Once the LAG begins, the expression of VEGFR-3 gradually decreases in blood vessels, and eventually becomes restricted to the lymphatic vessels and fenestrated blood capillaries. VEGFR-3 is responsible for the proliferation, migration and organization of new lymphatics. Continues ligand-induced activation of newly-formed lymphatic endothelial cells (LEC) by VEGFR-3 provides their surviving during embryogenesis and postnatal period (first two weeks), after which lymphatic vessels become independent of VEGFR-3 [1]. In adults, VEGFR-3 remains expressed in lymphatic vessels devoid of vascular smooth muscle cells. This finding suggests that VEGFR-3 is important in nascent blood vessels and is redundant when vessels are mature. VEGFR-3 gene-targeted mice have severe heart failure with embryonic mortality at E9. 5 (E-embryonic day) due to catastrophic defects in remodeling of the primary venous plexus. Early embryonic lethality of the VEGFR-3 null-mice hampered the assessment of the importance of this growth factor receptor in LAG, because these mice die before the emergence of the lymphatic vessels [2]. However, the identification of missense mutations in VEGFR-3 in patients with hereditary lymphedema has provided the support of this gene in lymphatic development [4].

It is widely accepted that VEGF-C/VEGFR-3 axis plays the crucial role in LAG. Skobe M. et al. [12] described a correlation between VEGF-C levels and VEGFR-3 amount in intratumoral area of breast cancer. Moreover, they showed dependence between VEGFR-3 phosphorylation activity, induced by VEGF-C overexpression, and regional lymph nodes metastases. In animal models, the induction of LAG by VEGF-C/VEGFR-3 axis increased tumor metastases via the lymphatic system [13].

In addition to its expression on LEC there are a lot of papers which describe the expression of VEGFR-3 in vascular tumors [10], lymphangiomas [9], Kaposhi sarcoma, myoepithelial cells in mammary glands [3], neoplastic cells in a wide variety of solid human tumors, monocytes, macrophages, dendritic cells, blood endothelial cells (BEC). These data have shown that VEGFR-3 expression is not restricted to endothelial cells. There is a growing body of evidences that VEGFR-3 expression and function correlate with metastatic activity of cancer cells in solid tumors.

The aim of this study was to elucidate the features of VEGFR-3 expression in all stages of the uterine cervix neoplasia progression.

Material and methods

We studied biological material taken by targeted biopsy and conization, from women with macroscopically detectable lesions of the uterine cervix area, at the Institute of Oncology from 2006 to 2009. For each case we made routine histological staining with hematoxilin and eosin, in order to establish the morphological diagnosis and tumor grading, as well. As a result, we obtained following lesions: squamous cell metaplasia – 12 cases (n = 12), CIN I (n = 8), CIN II (CIN II - 6), CIN III (n = 24), microinvasive carcinoma (n = 16) and invasive squamous cell carcinoma (n = 26). Additional 5 μm thick sections, for each case, we stained with anti VEGFR-3. Immunohistochemical staining was performed in accordance with LSAB+ technique, using Avidin-Biotin working system. As a primary monoclonal antibody we used anti VEGFR-3 (Santa Cruz, US Carpinteria), 1:200. After antigen retrieval, performed by heating with microwave oven in retrieval solution pH6 (Dako Cytomation), we incubated the specimens with primary antibody for 30 minutes. Incubation time for both, secondary and tertiary antibodies was 15 minutes. After applying each antibody, we rinsed specimens carefully with PBS buffer phosphate solution pH 7.2. Nuclei counterstaining we made with Lille modified hematoxiline. For lymphatic microvascular density assessment, we used D2-40 immunolabeling. Staining with D2-40 (clone D2-40, Dako Cytomation, Denmark) was made on additional sections, for each case, using the same technique and working system. The entire immunohistochemical technique was performed by autostainer (Dako Cytomation). Images were taken with Nikon Eclipse (E600) photo camera. Counting of immunolabeled lymphatic vessels was done using the method proposed by Van der Auwera [15]. Evaluation of the intensity expression of VEGFR-3 was made using a scale with 4 values: 0 – absence of expression; 1- low intensity; 2 – intermediate expression; 3 – high intensity. Statistical analyses of obtained data we made with SPSS 13.0, using Paired-Samples T Test and bivariate correlation, where $p < 0.05$ was considered as statistically significant.

Results

Squamous metaplasia: D2-40 positive lymphatic vessels (LV) were large, with wide lumen, placed in deep stroma. We found VEGFR-3+ LV and blood vessels (fig.1). In all cases, the squamous stratified epithelium was negative for VEGFR-3, whereas the basal layer was positive, and considered as inner positive control. VEGFR-3 positive cells were not present in stroma.

CIN I and II: The shape, localization and size of LV were not significantly different in comparison with squamous metaplasia. On the other hand, in CIN II stage, small vascular structures appeared with VEGFR-3 positive endothelium. Among these vessels not only LV but also blood vessels were present, deduction made on the presence of erythrocytes into their lumen. The intensity of VEGFR-3 expression by the neoplastic epithelium ranged between 0 and 2. We also noticed the presence of few cellular elements in stroma, positive for VEGFR-3.

CIN III: LV were present not only in deep stroma, but also in lamina propria, just in front of the epithelial mass (fig.2). Almost all vascular structures from superficial lamina were small, flattened, elongated, with narrow lumen. Many of VEGFR-3+ vessels were blood vascular structures. We found a discontinuous pattern of D2-40 expression by the basal layer of stratified epithelium. Epithelial expression of VEGFR-3 was present in 71.2% (19 cases). The mean value of the expression intensity was 2.

Microinvasive carcinoma: The density of LV was lower than in CIN III. Morphological features of LV were approximately the same like in severe intraepithelial neoplasia. A lot of vessels were placed in immediate vicinity of invasive front. We noticed that mainly small vessels express VEGFR-3. Inside the tumor mass expression was heterogeneous. Usually, the central part of the tumor had more intensive expression than the invasive front (fig.3). The number of the stromal cells, positive to VEGFR-3 was significantly higher than in previous stages of uterine neoplasia progression.

Invasive carcinoma: We found LV not only in the stroma, but also inside the tumor mass. Both vascular densities, detected with D2-40 and VEGFR-3 (fig.4), were much higher in peritumoral area than in intratumoral. LV from the peritumoral stroma were larger and perfusable, in comparison with those, placed intratumorally. Vascular invasion was detected in 15 of 26 cases (57.7%) of invasive carcinoma. Intratumoral LV were detected in 25 cases (96.1%). In 3 cases (11.5%) we found intratumoral LV with emboli (fig.5), and in all these specimens peritumoral LV were also invaded. Peritumoral invaded LV were large, with well-distinguished lumen (fig.6). VEGFR-3 expression in the tumor mass had the same features with expression in microinvasive carcinoma (fig.7). In 5 cases – 19.23% - we detected diffuse, strong VEGFR-3 expression in tumor mass (fig.8). The intensity of VEGFR-3 expression by the vascular emboli was always higher than the expression by the tumor mass. We also found, statistically significant correlation ($p = 0.037$) between tumor expression of VEGFR-3 and vascular invasion. Numerous stromall cells were positive for VEGFR-3.

Table 1

Marker	Squamous metaplasia	CIN I	CIN II	CIN III	Microinvasive carcinoma	Invasive ccarcinoma
D2-40	9.2	10.5	13.8	18.71	16	8.5
VEGFR-3 (LV)	3	3.6	4.2	8.2	8	4.6
VEGFR-3 (general vascular density)	1.6	7	9.4	21	17.3	12.6

Discussions

In this study, using monoclonal antibody for VEGFR-3, we demonstrated that this molecular agent is necessary not only for LV formation, but also has a wider spectrum of activity in cervical carcinogenesis.

Initially, it was described as a tyrosine-kinase receptor that binds specifically vascular endothelial growth factors, which enhance the mitogenic activity of lymphatic endothelial cells, VEGF-C and VEGF-D [7]. Further investigations showed that VEGFR-3 is acquired for blood vessels formation, as well [6]. Moreover, using this marker for tumor-induced lymphangiogenesis studying, a lot of researchers revealed its affinity for malignant cells in wide spectrum of solid human cancers. Many consequent molecular investigations showed that intensity of VEGFR-3 expression correlates with aggressive metastatic behavior of the tumor.

Podoplanin is a transmembranar glycoprotein, expressed in podocytes, fibroblasts, striated muscles, alveolar cells type I, lymphatic endothelial cells. Commercially available marker for podoplanin – D2-40 – allows identifying of fixation resistant epitope in formalin fixed and paraffin embedded sections, and seems to be the most specific lymphatic marker [14].

Each tumor produces a wide variety of molecular factors that provide the formation of own tumor vascular supply. Gradually, during the tumor progression, in this process stromal cellular elements are actively involved. As a result, the density of newly-formed blood and lymphatic vessels increases. In this study, we obtained a linear increasing of D2-40+ LV till CIN III stage. Beginning with microinvasive carcinoma stage, LV density progressively decreased. Our data are consistent with results of other authors [16]. This phenomenon could be explained by the fact that tumor, in its invasive stage of progression, destroys vessels rather than induces formation of others. It is well known that VEGF-C overexpression activates VEGFR-3. We have reported that intensity of VEGF-C expression correlates with the stage of uterine cervix neoplasia progression, being the highest in the invasive carcinoma stage [8]. In this case appears one question: if VEGF-C overexpression is the highest in invasive carcinoma, why the density of VEGFR-3 LV is so low in the same stage? We think that there is nothing surprising in this situation, and explain this high discrepancy between VEGFR-3 and VEGF-C expression by the fact that receptor is hidden by its ligand.

Expression of VEGFR-3 in blood endothelial cells was often previously reported. Moreover, it has been suggested that this lymphatic specific receptor, is necessary for tumor-induced angiogenesis. It has been shown that VEGFR-3 has a crucial role in early blood vessels formation. It has been suggested that tumoral angiogenesis repeat the same stages, and each stage is regulated by the same molecular players, embryonic angiogenesis passes through. In this research, almost all blood vessels were small, an indication that these vessels were young. However, we can not explain the positive reaction of endothelial cells from large blood vessels. Expression of VEGFR-3 in blood endothelial cells makes it improperly for lymphatic microvascullar density assessing.

At the moment there are some strong debates about the expression of VEGFR-3 in tumor mass. Tatiana Petrova et al. [11], consider that this receptor's expression is restricted in solid tumors to lymphatic and blood endothelium. On the other hand, there is an increasing body of evidences about its expression not only by the tumor mass, but also by the stromal cells. Our data are consistent with the second opinion. Tumoral expression of VEGFR-3 correlated with vascular invasion, in our study, whereas it has been proved that VEGFR-3 together with VEGF-C, increase the motility of neoplastic cells and facilitate vascular penetration.

Concluding remarks

During the cervical carcinogenesis, VEGFR-3 plays a multifactor role. It is involved in formation of blood and lymphatic tumor-derived vasculature. This molecular agent has a highly-expressed heterotopic affinity. Hiding of the receptor by its ligand and concomitant expression by the lymphatic and blood endothelial cells makes impossible the studying of lymphatic microvascular density, using only VRGFR-3. It is needed to duplicate the procedure with D2-40. Positive immunoreactivity of all intravascular emboli, corroborated with statistically significant correlation between tumor expression of the marker and vascular invasion, strongly indicates the crucial importance of VEGFR-3 in metastasizing. Taken together, all these findings show that targeted anti VEGFR-3 blocking could a potent therapeutic strategy against tumor progression, especially vascular invasion and metastases, during the cervical carcinogenesis.

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