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Macrophage Density Correlates with Severity of Uterine Cervix Neoplasia

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Abstract

Despite all recent efforts, cancer of the uterine cervix still remains one of the most frequent malignancies among women. Lymphatic vessels represent the primary route of tumor cells dissemination in cervical cancer. It has been demonstrated that cervical neoplasia actively participates in the recruitment of new blood and lymphatic vessels. Macrophages are extremely versatile cells which have a significant contribution to tumor progression. **The aim:** 1) To establish the correlation between tumor-associated macrophages (TAM) and the grade of the uterine cervix neoplasia; 2) To evaluate the distribution of TAM within both intratumoral and peritumoral areas. **Material and Methods:** Ninety-six cases were studied. The specimens were fixed in buffered formalin and paraffin embedded. Step sections, 5μm thick, were performed for each case. Initial sections were stained with haematoxylin-eosin, for the pathological diagnosis and grading of the tumor. Lesions were classified as follows: squamous cell metaplasia (n = 12), CIN I (n = 8), CIN II (n = 6), CIN III (n = 24), microinvasive carcinoma (n = 16) and invasive squamous cell carcinoma (n = 26). Additional sections for each case were stained for CD68 antibody, in order to highlight the macrophages. Quantification of macrophage population has been made based on hot-spot technique. The arithmetic media of 3 (× 200) fields represented the final result. **Results:** We found a statistical correlation between peritumoral macrophages (PTM) and intratumoral macrophages in all stages of cervical neoplasia, macrophage density and tumor stage ($p = 0.01$). In 16 cases we found vascular invasion. Almost in all these cases (87.5%) intravascular tumor emboli were embedded with CD68+ cells. **Conclusions:** based on these findings, we consider that macrophages are key regulators of cervical cancer progression. TAM targeted management could be an essential therapeutic strategy, not only in order to suppress the progression of cervical neoplasia, but also to inhibit macrophage-mediated vascular invasion.

Key words: uterine cervix cancer, macrophages, cellular density, tumor progression, CD68.

Корреляция плотности макрофагов с тяжестью неоплазии шейки матки

Рак шейки матки остается одной из самых часто встречающихся злокачественных заболеваний женского населения. Лимфатические сосуды являются первичным путем метастазирования при данном заболевании. Было доказано, что клетки цервикальной неоплазии активно участвуют в образовании новых лимфатических сосудов. Макрофаги – многофункциональные клетки, оказывающие большое влияние

на прогрессирование опухоли. **Цель:** 1). Выявление корреляции между макрофагами и стадией прогрессии неоплазии шейки матки; 2). Определение особенностей распределения макрофагов внутри опухолевой массы и вокруг нее. **Материал и методы**. Было изучено 96 случаев. Материал фиксировали в формалине с последующим заключением в парафин. Для каждого случая производили срезы, толщиной в 5 мкм. Изначально, срезы окрашивали гематоксилин-эозином для определения гистопатологического диагноза. Были получены следующие группы поражений: плоскоклеточная метаплазия (n = 12), CIN I (n = 8), CIN II (n = 6), CIN III (n = 24), микрокарцинома (n = 16), инвазивный рак (n = 26). Для выявления макрофагов, производили иммуногистохимическое исследование с использованием маркера CD68. Подсчет популяции макрофагов производили по методике hot-spot. **Результаты.** Мы получили статистически значимую корреляцию между внутритуморальными и перитуморальными макрофагами во всех стадиях прогрессии неоплазии шейки матки, между плотностью макрофагов и стадией опухоли (p = 0,01). В 16 случаях выявили сосудистые эмболы. Почти во всех случаях (87,5%) внутрисосудистые эмболы содержали в себе CD68+ клетки. **Выводы**: Основываясь на полученных результатах, мы считаем, что макрофаги вовлечены в прогрессию рака шейки матки.

Ключевые слова: рак шейки матки, макрофаги, клеточная плотность, опухолевая прогрессия, CD68.

Introduction

The crucial importance of the HPV infection in the development of cervical cancer was well established in the middle of the '90s. Based on this evidence, strategies were developed, mainly focused on the prevention of this disease, which led to the dramatic decreasing of its incidence. Despite on the facts named above, cervical cancer still remains one of the most frequent neoplasia for women.

Cancer progression is a complex biological phenomenon, characterized by a multitude of intrinsic and extrinsic events, such as: the blocking of negative signals, the enhancing of positive signals, the over-expression of membrane receptors for pro-tumor growth factors, the promotion of cellular motility, and the recruitment of new blood vessels and new LV.

The ability of cancer cells to migrate from the primary tumor and to give rise to new cellular colonies at the distant sites influences tumor grading, therapeutic management, patient's survival. The lymphatic way of metastasizing involves the regional lymph nodes (RLN), and represents an important criterion of the poor prognosis.

Tumor progression can not be supported only by the tumor cells' related molecular factors. A great importance in cancer development is played by the cells from tumor microenvironment (fibroblasts, myofibroblasts, mast cells, macrophages) [1]. Nowadays the mutual inducing mechanisms between tumor cells and stromal cells are well known. The fact that tumor inflammatory infiltrate (TII) correlates with cancer's progression is widely accepted [2]. Macrophages are extremely versatile and are one the most numerous cell populations in the TII. In addition to a large number of pro-tumor factors, synthesized by the tumor-associated macrophages (TAM), it has been established that these cells produce significant amounts of VEGF-C, which is one important lymphangiogenic factor, as well as VEGF-D.

The pro-tumoral role of TAM in human cancers is supported by many clinical studies that found a correlation between high macrophage density and poor patient prognosis. Many macrophage products released in the tumor stroma can directly stimulate the growth and promote the tumor cell migration and metastasis. Among the molecular factors that mediate these effects are epidermal growth factor (EGF), transforming growth factor β (TGF β), vascular endothelial growth factor (VEGF), cytokines, chemokines.

The aim was: 1) To establish the role of TAM in lymphangiogenesis; 2) To evaluate correlation between TAM density and the stage of cervical neoplasia progression; 3) To assess the involvement of TAM in vascular invasion.

Material and methods

Targeted biopsies taken from conization were investigated, at Institute of Oncology from Republic of Moldova between June 2008 and May 2009, in patients with macroscopically detectable cervical lesions. The specimens were fixed in buffered formalin and paraffin embedded. Step sections, 5μm thick, were performed for each case. Initial sections were stained with haematoxylin-eosin, for the pathological diagnosis and grading of the tumor. Lesions were classified as follows: squamous cell metaplasia (n = 12), CIN I (n = 8), CIN II (n = 6), CIN III (n = 24) 17 CIN III and 7 CIS microinvasive carcinoma (n = 16) and invasive carcinoma (n = 26). Additional sections for each case were stained for CD68, with LSAB+ technique, using Avidin-Biotin working system. Antigen retrieval was done by microwave oven heating in retrieval solution pH6 (Dako Cytomation). Incubation of primary antibody was for 30 minutes. Identification of primary antibody we performed with DAB chromogen (Dako Denmark). Quantification of macrophage population has been made in accordance with hot-spot technique. The arithmetic media of 3 (×200) fields represented the final result. For nuclei counterstaining, we used Lille's modified Hematoxylin. Images were taken using a Nikon Eclipse (E600) camera. The entire immunohistochemical analysis was performed on autostainer (Dako Cytomation).

Results

Squamous metaplasia

Morphologically, macrophages were small with cytoplasmatic pattern of immunostaining. The density of PTM ranged between 73 and 90, with an average of 85.2. The values of ITM ranged between 16 and 23, with an average of 21.2.

CIN I and CIN II.

PTM were placed mainly in lamina propria. ITM were bigger, placed in all layers of the epithelium, with higher density in the basal, parabasal and intermediate layers. Statistically significant correlation was found between stromal macrophage and intraepithelial macrophage densities (p = 0.044).

CIN III.

PTM ranged between 149 and 312, with an average of 124.4. Intratumoral macrophages were distributed in all

layers, with a slight increased density in the basal $^{1}/_{3}$ of the squamous epithelium (fig. 1).

The lowest density of ITM was 51, the highest – 144, the mean – 103.7. A statistically significant correlation was found between peritumoral macrophages and intratumoral macrophages (0.015).

Microinvasive and invasive cancer

PTM ranged between 165 and 416, with an average of 298.6. ITM were big with an evident tendency to form clusters and with less intensity of the CD68 expression in comparison with PTM. They were ranged between 109 and 310, with an average of 200. Correlation between PTM and ITM was significant (p = 0.001).

In invasive carcinoma, PTM were placed diffuse in peritumoral stroma, mainly around vessels and were organized in groups (fig. 2).

PTM ranged between 219 and 617, with an average of 413.6. ITM were also diffused inside the whole tumor mass (fig. 3).

We observed a tendency of macrophages to fuse and form big multinucleated cells. This phenomenon was found by us only in invasive carcinoma within epithelial tumor mass (fig.3).

Cells were significantly bigger than PTM, while the intensity of expression often was lower. The highest ITM density was 522, the lowest -189, the mean – 322.8. Significant correlation was found between PTM and ITM (p = 0.012). We have found no correlation between both PTM and ITM densities and vascular invasion.

Vascular invasion

We have found vascular invasion in 16 of 26 cases of invasive carcinoma. Almost all emboli from invaded vessels contained CD68+ macrophages (fig. 5).

In 22 cases (84.61%) we detected intercalated CD68+ cells among the endothelial cells of vessels (fig. 6).

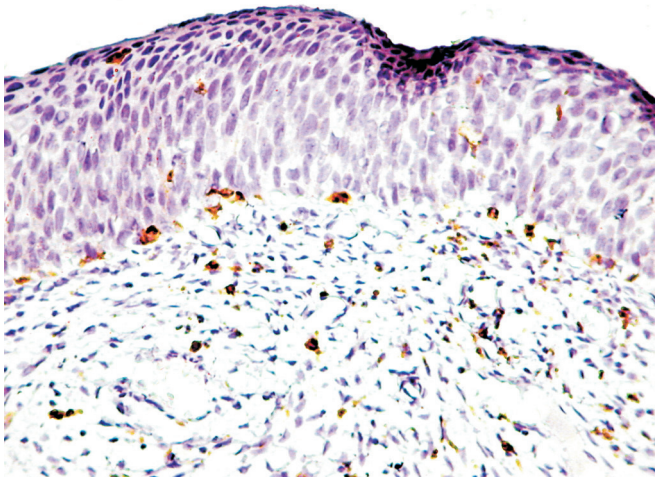

Fig. 1. Cervical Intraepithelial Neoplasia III (x100). Macrophages are located in all layers of stratified squamous epithelium, with a slight increasing of their density in basal and parabasal layers.

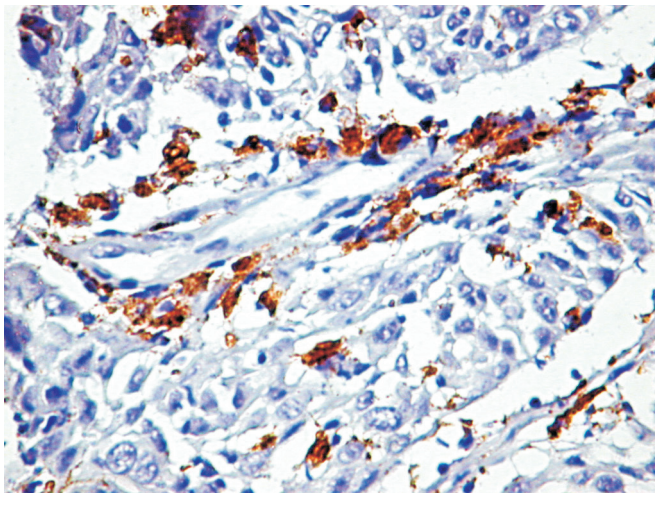

Fig. 2. Invasive carcinoma (x200). Peritumoral macrophages placed around vascular structure.

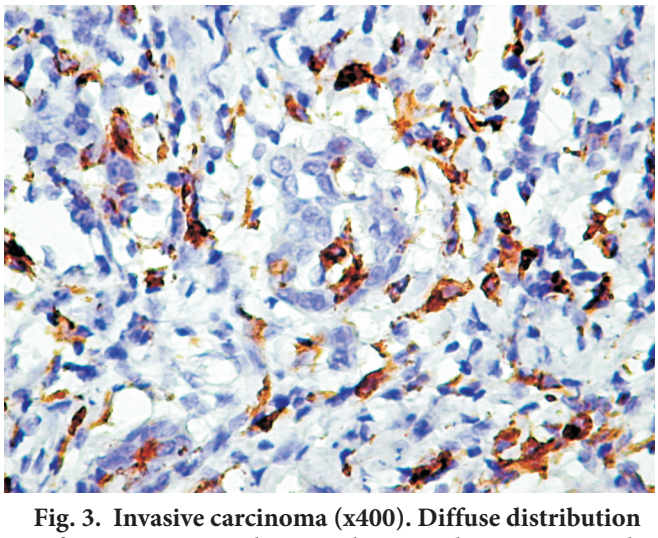

Fig. 3. Invasive carcinoma (x400). Diffuse distribution of tumor associated macrophages within peritumoral stroma.

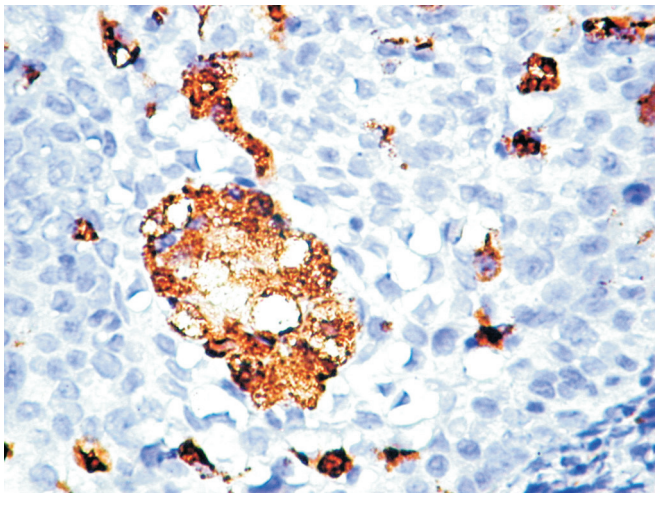

Fig. 4. Invasive carcinoma (x400). Multinucleated CD68+ cluster inside the tumor mass.

Table 1

Type of cervical lesion	MS (n = 12)	CIN I (n = 8)	CIN II (n = 6)	CIN III (n = 24)	Microinvasive Carcinoma (n = 16)	Invasive Carcinoma (n = 26)
PT M	85.2	106.38	118.14	124.4	298.6	413.6
IT M	21.2	56.88	84	103.7	200	322.8

Discussions

It is well known that macrophages are one the most versatile cells [3]. There is an increasing body of evidence that proves that macrophages represent a key regulator in progression of different human solid tumors. Linear increasing of both, ITM and PTM densities, from pre-invasive to invasive cervical lesions, detected in our study, strongly indicates on catalytic function of these cells in cervical carcinogenesis. The same results have been reported before [4]. It is well known that macrophages have the ability to proliferate. This phenomenon was described in detail in wound healing and glomerulonephritis [5], while, in recent literature there are few data about macrophage proliferation in tumors. It seems to be clear about the origin of PTM. Tumor cells produce a broad spectrum of cytokines and growth factors which are chemoatractants for macrophage precursor cells and lead to their accumulation into the stroma of peritumoral area, and further differentiation into adult macrophages. It is supposed that ITM have a dual origin: intratumoral migration of periepithelial macrophages and their local proliferation. Based on the fact that in all groups of lesions PTM density was higher than ITM, and on the statistical correlation found between them, we suggest that ITM population is mainly provided by the PTM invasion into the tumor mass.

There is a big amount of evidences (experimental and clinical) that proved without any doubts the TAM's role in cancer-cell spreading. This role is mediated by a variety of pathogenic chains, orchestrated by TAM. On one hand, macrophages determine the cancer cells mobility, through EGF secretion, and stromal invasion, by extracellular matrix remodeling. On the other hand, macrophages are actively involved in tumor-derived angiogenesis and lymphangiogenesis. As a result, detaching of neoplastic cell, from its primary locus, and vessel penetrating is much easier. Presence of CD68+ cells almost in all intravascular emboli, obtained by us, underpin these statements.

There is an increasing body of evidence that macrophages are actively involved in LAG [6]. There is a dual mechanism of LAG promoted by macrophages: macrophage transdifferentiation into the LEC, and synthesis of VEGF-C.

Cervical carcinogenesis consists of several well-distinguished stages: CIN, carcinoma in situ, microcarcinoma, invasive carcinoma. It has been reported that potent lymphangiogenic switch occurs in high-grade CIN stage [7]. LAG is dependent on LEC proliferation. VEGF-C (vascular endothelial growth factor C) is the main mitogenic factor which controls this proliferation. It has been demonstrated that macrophages represent an important source of VEGF-C. Macrophage

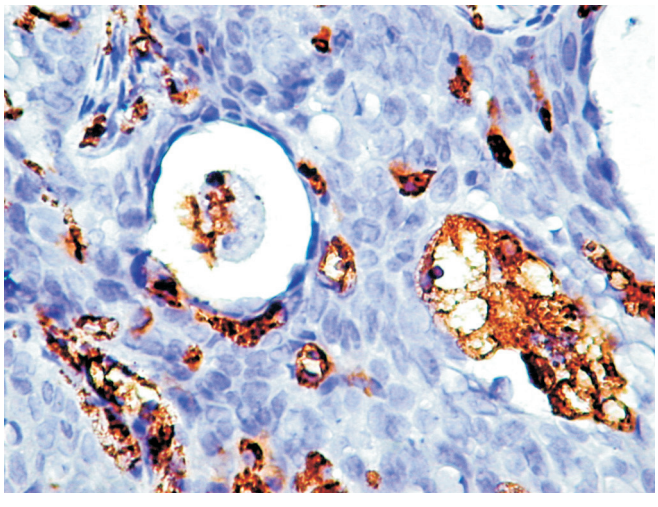

Fig. 5. Invasive carcinoma (x200). Intravascular tumor embolus embedded with macrophages.

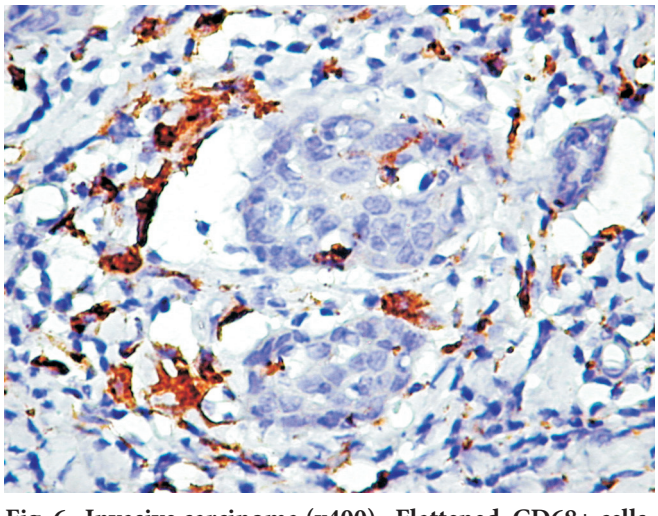

Fig. 6. Invasive carcinoma (x400). Flattened, CD68+ cells, intercalated between endothelial cells of invaded vessel.

precursors express VEGFR-3, transmembrane receptor which transduce VEGF-C signals. It has been shown that VEGF-C, produced by tumor cells, interacts with VEGFR-3, from membrane of macrophage precursors, and determines their recruitment [8]. Once these cells reach to the peritumoral area, they begin to secrete own VEGF-C, which enhance the macrophage recruitment and also interact with VEGFR-3 on LEC. On the other hand, macrophages are able to transdifferentiate into LEC and to integrate into sprouting LV [9]. In our study, we detected clusters of macrophages, located around small LV, in CIN III, microcarcinoma and invasive carcinoma. Moreover, in all stages named above, we observed CD68+ cells

intercalated between LEC. This macrophage intercalation was observed, predominantly, in small and flattened LV (hallmarks for young vascular structures) from peritumoral stroma, and also in large but invaded vessels. Our results are consistent with data presented by other authors, and support the hypothesis according which integration of macrophage precursors, transdifferentiated into LEC, occurs in newly-formed LV. We consider this mechanism crucial in the formation of new LV because proliferating LV not only enlarge their size and become able to support the migration of tumor emboli, but also increase the area for targeted action of VEGF-C, secreted both by the tumor and stromal cells, via increasing number of VEGFR-3 expressing cells in newly-formed LV.

Concluding remarks

Cancer progression represents an extremely sophisticate mutual interaction between a variety of molecular agents related both to tumor mass and tumor microenvironment. From this point of view, macrophages are one of the most important sources of a wide spectrum of biologically active substances that mediate the tumor progression.

Linear increasing of TAM density during the cervical neoplasia progression, their predominant location around vascular structures, integration of CD68+ cells into the endothelium of the vessels, demonstrate their crucial importance in uterine cervix neoplasia progression.

Based on these findings, we consider that macrophages are key regulators of cervical cancer progression. TAM targeted management could be an essential therapeutic strategy, not only in order to suppress the progression of cervical neoplasia, but also to inhibit macrophage-mediated vascular invasion.

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