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Macrophages and dendritic cells density correlates with depth of invasion in the prostate carcinoma

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Abstract

Background: Immune cells interact not only with tumor cells but also with stromal cells facilitating the progression of neoplasia. The ongoing battle between immune cells and the tumor is an important factor influencing the clinical course and outcome of treatment in various types of cancer. The aim of the study was to identify the prognostic value of dendritic cells and macrophages in prostate carcinoma.
Material and methods: This retrospective study analyzed 73 samples of prostate cancer. The macrophages and dendritic cells have been evaluated using the immunohistochemical methods with CD68 (macrophages) and S100 (dendritic cells). Macrophages were quantified as intratumoral and peritumoral, and dendritic cells – intraepithelial and stromal. The results were analyzed statistically.
Results: For evaluation of the prognostic impact of immune cells was accomplished a correlation between the total number of CD68+/S100+ cells and the Gleason score. Thus, statistically significant correlations were obtained both for CD68+ cells (intratumoral p=0.008, peritumoral p=0.001), and for S100+ cells (intraepithelial p=0.036, stromal p=0.042). In addition, a statistically significant positive linear correlation was observed between the density of intraepithelial S100+ cells and intratumoral CD68+ cells (p=0.018).
Conclusions: The increase in the density of S100+ and CD68+ cells, as well as the significant association of their density with the histological degree of the tumor allow us to consider these cells as predictive biomarkers in prostate carcinomas.
Key words: prostate cancer, dendritic cells.

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Introduction

Frequently, the prostate benign and malignant proliferations are accompanied by inflammatory processes. The link between inflammation and cancer, studied very intensely in the last years, was identified 150 years ago, when in 1863, Virchow, noticed that cancer tends to occur in sites of chronic inflammation. While recently, increased the number of researches which demonstrate that acute inflammation contributes to cancer regression [1], there are multiple epidemiological studies claiming that chronic inflammatory diseases are frequently associated with an increased risk of cancer [1-3]. Inflammatory foci are dominated by multiple immune cells, such as: macrophages, lymphocytes, plasma cells, mast cells, etc.

From this wide range of cells, macrophages represent an important component of the tumor inflammatory infiltrate [2, 4]. Macrophages, as well as other inflammatory cells generate a large amount of growth factors, cytokines and chemokines that can cause irreversible DNA damage. Moreover, the macrophages can act as pro-inflammatory or anti-inflammatory cells, depending on the type of stimulus received from the neighboring microenvironment. In general, two major macrophage phenotypes are identified: M1 and M2. The M1 macrophages, or classically activated macrophages, are aggressive cells, intensively involved in phagocytosis, and promotion of Th1 responses. Also, they are important inflammatory cytokine secreting cells, such as IL-12, IL-18 and IL-23. In tumors, classically activated macrophages play an important role in the recognition and destruction of cancer cells, and their presence in the tumor mass is usually related to a favorable disease prognosis. M2 macrophages or alternatively activated macrophages are anti-inflammatory cells, actively involved in the angiogenesis and tissue regeneration. They secrete increased amounts of IL-10 and other anti-inflammatory cytokines [5]. Depending on secreted molecules, are identified several subsets of M2 macrophages.

These are: 1) M2a macrophages – called profibrotic, which secrete IL-4 and IL-13; 2) M2b macrophages – contain IC or TLR/IL1-R ligands and are involved in the regulation of immunity and, often, are called regulatory cells; 3) M2c macrophages – secrete IL-10 and TGF-β, and sometimes are described as inactivated cells, which are involved in suppressing immunity, tissue regeneration and matrix re-

modeling; 4) M2d macrophages or tumor-associated macrophages (TAM) – increase tumor progression and growth by promoting the process of neo-angiogenesis [5].

The tumor microenvironment, significantly affects the polarization of macrophages. Growth factors, such as CSF-1 (colony stimulating factor 1) and VEGF (vascular epithelial growth factor), MCP-1 (chemotactic protein-1 monocyte), as well as several chemokines CCL can induce monocyte chemotaxis in the tumor microenvironment [6]. Neutralization of MCP-1 completely blocks the accumulation of macrophages in the tumors [7]. VEGF, in addition to its powerful angiogenic role, recruits monocytes into the tumor microenvironment. VEGF blockade leads not only to the reduction of vascular density, but also to the reduction of macrophage infiltrate [8]. Multiple studies have shown that the invading property of tumor cells depends largely on macrophages. The invasion of tumor cells, mainly, is accompanied by directed migration of macrophages [9].

Dendritic cells (DC) represent the most efficient antigen-presenting cells, which have the function of tissue sentinel. They belong to monocyte-macrophage cell lines that strongly express the protein S100 [10]. As immature cells, they take up the antigens from peripheral tissues, process them, and then expose antigen molecules to the membrane surface as molecules of the class I and II histocompatibility complex [11, 12]. During antigen processing, the dendritic cell undergoes the maturation process, which determines its migration into the secondary lymphoid organs, where it becomes a competent cell in presenting of antigen to T lymphocyte. Thus, dendritic cells initiate the special antigen-specific immune response [13]. Another functional characteristic of dendritic cells is their effective ability to increase the immunomodulatory and cytotoxic potential of NK cells, which essentially contribute to the removal of tumor cells [14-16]. Furthermore, DCs can also directly mediate tumor-targeted cytotoxicity [17].

The progression of prostate cancer is accompanied by a marked suppression of local immunity, which includes the apoptotic death of dendritic cells [18]. Tumor cells can significantly inhibit the monocyte conversion into the dendritic cells. It is also recognized that prostate cancer not only destroys mature dendritic cells, but also inhibits their genesis and maturation, leading to decreased production of antigen-presenting cells and inhibition of their functional activity [18-20]. The rapid-growing tumors are usually poorly infiltrated by DC and unable to trigger DC recruitment and activation that result in delayed or insufficient antitumor immune responses. However, the mechanisms regulating migration and homeostasis within the tumor are not well understood.

In this study, we examined the cell population composition of CD68+ macrophages and S100+ dendritic cells. We also examined the prognostic value of these cells and their correlation with the proven parameter of the biological aggressiveness of prostate cancer: degree of tumor differentiation. The obtained results could lead to improved therapeutic modalities in patients with prostate cancer.

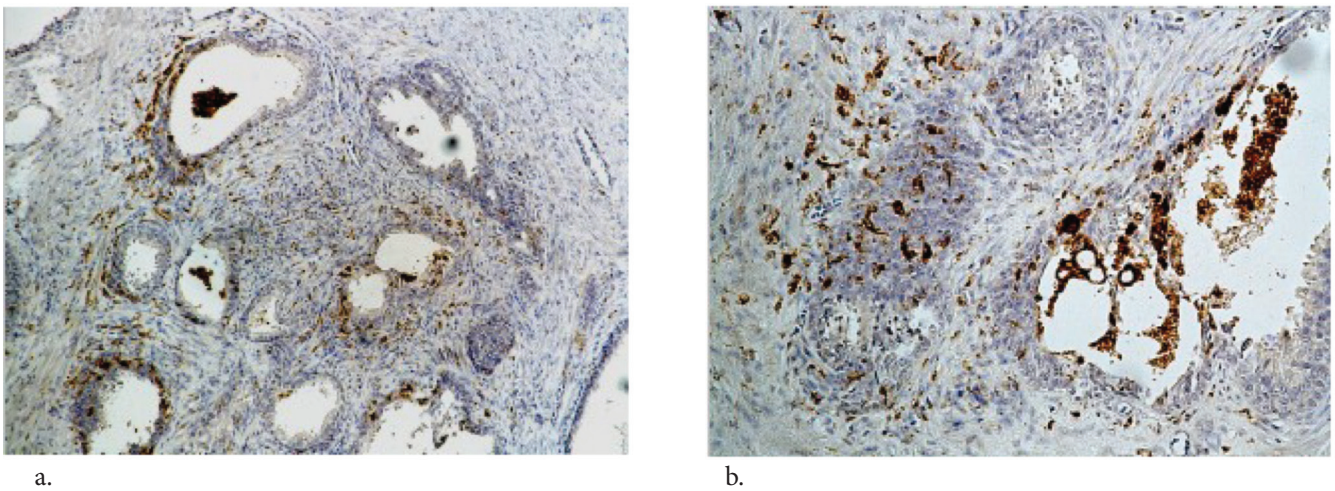
Material and methods

The study included 73 cases of prostate carcinoma. Histological grading of prostate carcinoma is an important step in defining the diagnosis and prognosis. Thus, the prostate cancer specimens were divided into 2 groups: acinar and non-acinar carcinomas. For the histological differentiation of acinar adenocarcinomas, the Gleason score was used. The adenocarcinoma specimens were grouped in: *well-differentiated (Gleason score 2-5), medium-differentiated (Gleason score 6-7) and poorly-differentiated (Gleason score 8- 10)*. Non-acınar carcinomas were considered poorly differentiated cancers. Due to early lysis of the study material, the histological pieces were rapidly harvested. The biopsy fragments, after fixation in 10% buffered formalin, were primarily processed following the standard procedures. Sections 5 µm thick were sliced off each block, which were mounted on histological and silanized slides. Histopathological profiling was performed on hematoxylin-eosin stained sections.

The immunohistochemical study included 2 monoclonal antibodies: anti-CD68 *(clone 514H12, predilute, Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK)* and anti-S100 *(polyclonal, predilute, Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK)*. The application of primary antibodies was preceded by the exposure to Bond Epitope Retrieval Solution 2 (20 minutes) for anti-CD68 and Bond Enzyme 1 (10 minutes) for anti-S100. Incubation with primary antibodies was 20 minutes, compatible working system was Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK) and 3.3" diaminobenzidine was the chromogen used. Counterstaining was performed with modified Lille's hematoxylin. The entire immunohistochemical technique was performed with DakoCytomation Autostainer. The final product of the reaction entailed staining cell brown. CD68+ macrophages were quantified in the peritumoral and intratumoral areas of the stroma, and S100+ dendritic cells were counted in both the stroma and intraepithelium. Microscopic examination was performed using the Nikon Eclipse E600 microscope.

Assessment of CD68+ macrophages was performed by the modified hot spot method. Initially, the highest cell density field was identified in the studied stromal areas, at the microscopic magnification x100, subsequently immunoreactive cells were counted in 3 fields, at the microscopic magnification x400. The average value of the three fields was used as data for analysis. CD68+ macrophages located near areas of necrosis or associated with inflammatory infiltrate were excluded from the evaluation.

The modified hot spot method was used for the quantitative evaluation of S100+ dendritic cells. S100 was also expressed by glial cells, and the presence of the chromogenic signal in nerve structures was considered as a positive internal control. Only S100+ dendritic cells, which had cytoplasmic extensions, were included in the evaluation. After identifying the field with the highest cell density in the studied areas, at x100 microscopic magnification, the immunoreactive cells were counted in 5 visual fields, at x400 microscopic magnification.



a. b.

Fig. 1. Distribution of CD68+ cells in prostate carcinoma, ×10; anti-CD68 immunoreaction, DAB

Statistical analysis was performed using SPSS13.0 and Microsoft Excel 2010 software. Images were taken and processed using Lucia G system.

Results

Immunohistochemical analysis revealed the non-homogeneous and heterogenic character of CD68+ and S100+ immune cells distribution. CD68+ cells were preferentially located: along the tumor invasion border, in the stroma of tumor foci, including in the lumen of transformed acini (fig. 1).

The numerical distribution of CD68+ cells varied in the studied areas, the highest density being for intratumoral areas (tab. 1). Except non-acinar carcinomas, where the great density of CD68+ cells was recorded in the peritumoral areas.

Table 1

The mean density of CD68+ and S100+ cells in the prostate carcinoma specimens and comparison of cellular density between the studied areas

n*	CD68+ macrophages		S100+dendritic cells	
	Intratumoral	Peritumoral	Stroma	Intraepithelial
73	**72.7±4.7**	44.7±5,0	16.0±1.1	**22.4±1.3**
	t = 4.456 p=<0.001		*t = 3.963 p=<0.001*	

*n – the number of cases included in the study, p – value obtained by Student test

In relation to the histological types of adenocarcinomas was observed a linear increase of CD68+ macrophages, both intratumoral and peritumoral (fig. 2). The density of CD68+macrophages was relatively uniform, both intratumoral and peritumoral, in most of well- and medium-differentiated adenocarcinomas. Except four cases (two well-differentiated and two medium-differentiated carcinomas), where the number of intratumoral macrophages ranged between 101-186 cells/field. In poorly-differentiated adenocarcinomas, the numerical distribution of immunoreactive macrophages was much more heterogeneous. It was observed that in five cases (17%) the density of CD68+ cells in both studied areas was identical.

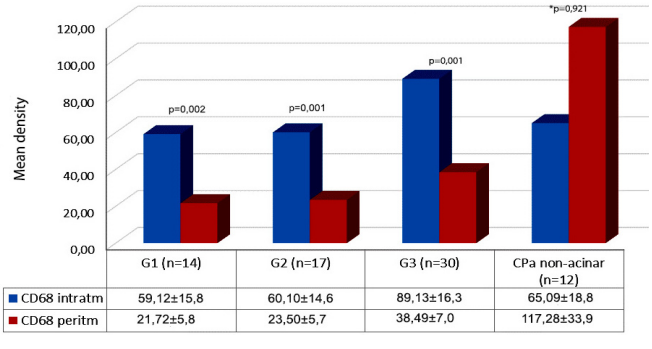


Fig. 2. The mean density of CD68+ cells in prostate carcinoma stroma, where:

G1 – well-differentiated adenocarcinoma, G2 – medium-differentiated adenocarcinoma, G3 – poorly- or undifferentiated adenocarcinoma, CPa – non-acinar carcinoma,

*no true differences were established between the compared groups

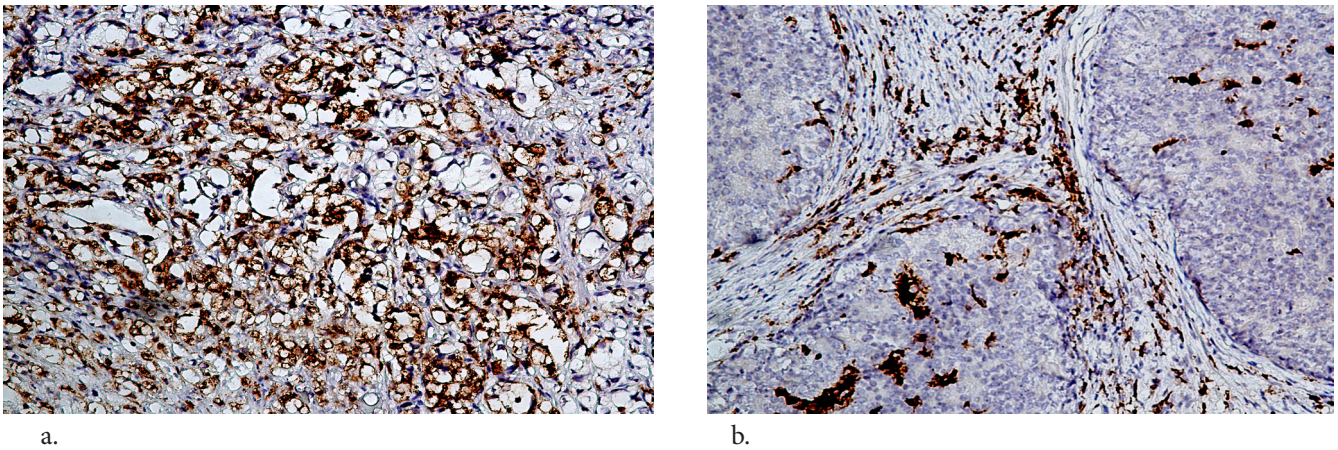
The stromal CD68+ macrophages of prostate adenocarcinomas showed morphological heterogeneity. Thus, two distinct cell populations were highlighted:

1. Small-sized cells (mostly) – intensely branched, with thin and long cytoplasmic extensions, intensely and weakly granulated cytoplasm, and round, or oval to elongated shape;

2. Large-sized cells – characterized by round shape, rare, short cytoplasmic extensions, sometimes polynucleated, and highly granular cytoplasm.

The distribution of small-sized CD68+ macrophages frequently had an infiltrative character, in contrast to large-sized cells, which were often located alone among stromal cells or formed small cell groups in the tumor mass (fig. 3).

Also, numerous CD68+ cells have been observed in: most of intravascular tumor emboli and peripheral areas



a. b.

Fig. 3. Heterogeneous character of CD68+ cell populations distribution in the stroma of malignant hyperplastic lesions: a) infiltrative features of small-sized CD68+ cells; b) comparative distribution of small-sized versus large-sized cells; x40; anti-CD68 immunoreaction, DAB

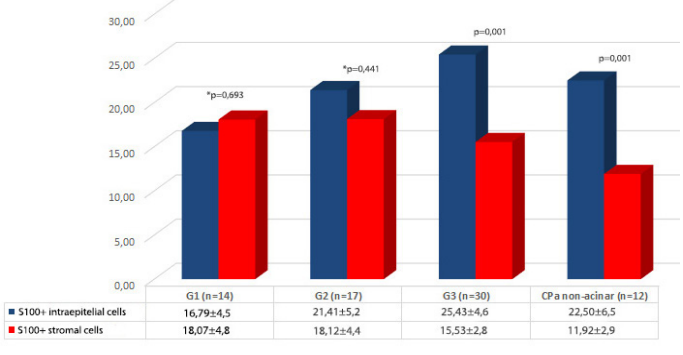
of inflammatory infiltrates. A particular aspect was the circumscription by the CD68+ cells of prostatic sympexions, indirectly, that suggest about the changes in the composition of the glandular secretion. In relation to blood vessels, CD68+ macrophages were located in the thickness of the vascular wall, less often among endothelial cells.

Comparing density of macrophages from peritumoral areas with intratumoral areas was obtained a partial correlation. In order to evaluate the prognostic impact of macrophages in adenocarcinomas, it was considered appropriate to achieve the correlation between the total number of CD68+ cells and the Gleason score, thus obtaining a statistically significant correlation (p=0.001). Splitting down the correlation on studied areas were obtained statistically significant correlations for both intratumoral – p=0.008, and peritumoral – p=0.001.

Distribution of S100+ cells has been studied both intraepithelial and stromal. Distribution of immunoreactive cells was non-homogeneous in both areas. However, in most of prostate carcinomas the high density was recorded in the intraepithelial areas (tab. 1). Evaluation of S100+ dendritic cells variation in carcinoma groups revealed the heterogeneous character for well-differentiated adenocarcinomas and the relatively homogeneous character for poorly differentiated adenocarcinomas and small cell carcinomas. It was observed that starting with the medium-differentiated carcinomas, the density of S100+ cells was raised for the intraepithelial areas (fig. 4).

However, in 13 cases of prostate carcinoma: 7 medium-differentiated adenocarcinomas (41.2%), 5 poorly-differentiated adenocarcinoma – 16.7%), one case of non-acinar carcinoma (8.3%)), high density of dendritic cells was recorded for the stromal areas. Also, we noticed that in three cases of the total samples with adenocarcinoma (4.1%), the density of S100+ cells in both studied areas was identical.

It was interesting to observe some S100+ tumor cells, especially in the tissue samples of poorly-differentiated and non-acinar carcinomas – 16.4% (n = 12), (fig. 5). An extremely important fact was the observation of S100+ tumor



	G1 (n=14)	G2 (n=17)	G3 (n=30)	CPa non-acinar (n=12)
S100+ intraepitelial cells	16,79±4,5	21,41±5,2	25,43±4,6	22,50±6,5
S100+ stromal cells	18,07±4,8	18,12±4,4	15,53±2,8	11,92±2,9

Fig. 4. Comparative aspect of intraepithelial and stromal positive S100+ dendritic cells distribution in prostate carcinomas, G1 – well-differentiated adenocarcinoma, G2 – medium-differentiated adenocarcinoma, G3 – poorly or undifferentiated adenocarcinoma, CPa – non-acinar carcinoma or small cell carcinoma, p – value obtained by Student test,

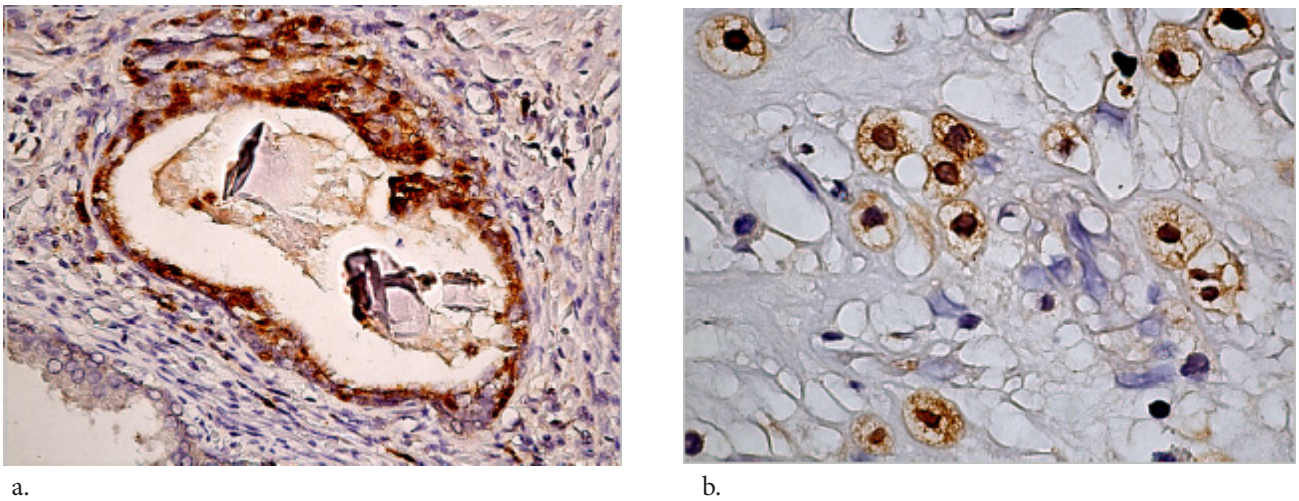
*no true differences were established between the compared groups

cells in the lumen of blood vessels, as well as at the site of tumor metastasis into vessel.

In most of specimens, S100+ cells formed the cell clusters, much rarely were located isolate. Frequently, the dendritic cell clusters were followed by polymorphonuclear cells infiltrate. A particular aspect observed was the presence of single dendritic cells in the lumen of glandular acini and ducts.

S100+ dendritic cells could be characterized as a heterogeneous cell population. Heterogeneity is determined either by the intensity of immunolabeling (thus, are cells that intensely express the marker (mostly) and cells with low-intensity) or by cell size (thus, are small and large- sized (dominant) cells) (fig. 6).

In order to evaluate the prognostic impact of dendritic cells in adenocarcinomas, it was considered appropriate to achieve the correlation between the total number of S100+ cells and the Gleason score, thus obtaining statistically significant correlations both intraepithelial (p=0.036) and stromal (p=0.042). In addition, to highlight the interrela-



a. b.

Fig. 5. Expression of the S100 marker of tumor cells, ×10, x40; anti-S100 immunoreaction, DAB.

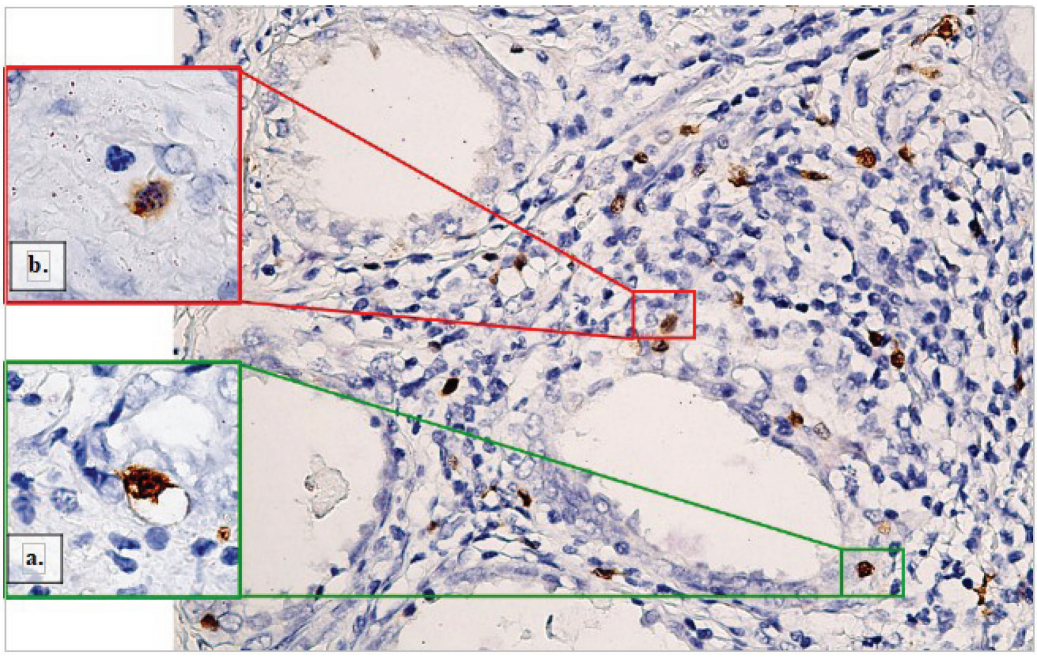


Fig. 6. Identification of the heterogeneous character of the immunolabeling expression.

a) cells that highly express the marker S100, b) cells with low-intensity of immunolabeling, ×40; anti-S100 immunoreaction, DAB

tionships between the immune cells in the adenocarcinoma stroma, there were correlated the densities of dendritic cells and macrophages, thus, was obtained a statistically significant correlation (p=0.018) between S100+ intraepithelial dendritic cells and CD68+ intratumoral macrophages.

Discussion

Tumor cells secrete several pro-inflammatory cytokines, which promote the infiltration of the tumor microenvironment with various immune cells, such as macrophages, neutrophils, NK cells, dendritic cells, mast cells, T and B-lymphocytes. Subsequently, the tumor microenvironment facilitates the angiogenesis, proliferation and invasion of carcinoma. According to our data, in prostate carcinoma the number of macrophages and dendritic cells is increased compared to normal prostate tissue. Distribution of macrophages CD68+, especially for intratumoral areas, was uneven, which indirectly demonstrates the active involvement of macrophages in the process of tumor initiation and progression. The high amount of macrophages was largely achieved due of intratumoral macrophages. In addition, a linear increase of CD68+ macrophages was noticed in relation to the histological grade of adenocarcinomas, in both intratumoral and peritumoral.

In the literature, there are studies that present similar data about the number of macrophages. Thus, Gollapudi et al. have demonstrated increased levels of TAM in prostatic intraepithelial neoplasia compared to those from benign le-

sions tissues [21]. At the same time, they have reported that patients with high Gleason score contain the higher number of TAM. Many clinical researches have shown that there are various correlations between TAM density and the tumor prognosis [22, 23]. It is also very important, that TAMs in different tumor compartments, apparently, have opposite effects on the progression of prostate cancer [22, 23].

Studying the morphological features of CD68+ cells in the prostate carcinoma samples, were observed two different cell populations. Some studies have described two different types of macrophages: M1 phenotype, which has an anti-tumor effect and M2 phenotype that promotes angiogenesis, tumor growth and metastasis. Analysis of various studies revealed that the density of the M1 macrophages could not be considered a prognostic factor, only the M1/M2 ratio could be a true and independent prognostic factor. Cellular and molecular interaction between the M1 and M2 population has an important determining role for prognosis in cancer patients. Tumor-associated macrophages (TAMs) mainly represent a variety of the M2 phenotype, although in some studies they have been described as a mix of M1 and M2 phenotype [24].

Being part of the tumor microenvironment, dendritic cells influence in positive or negative way the course of malignant disease. Several studies have pointed out the central role of the dendritic cells in antitumor immunity [25]. Our results have shown an increase of dendritic cells density in prostate carcinoma tissues compared to normal tissues. The numerical difference from the control group was significant. In this study, we noticed that the highest degree of infiltration with S100+ dendritic cells was associated with poorly-differentiated adenocarcinomas. Thus, our data demonstrate that the increase of the S100+ dendritic cells density in prostate adenocarcinoma is associated with an unfavourable prognosis (the evidence was to obtain a statistically significant correlation with the Gleason score).

Dendritic cells play a crucial role in many types of human cancers. Various studies have shown that the presence of high intratumoral DC density is associated with a favourable prognosis [26]. In addition, there is evidence related to the loss of T lymphocyte activation capacity after exposure to dendritic cells in contact with tumor cells [27] and/or related to the loss of their maturation capacity in the absence of migration into lymph nodes [28]. Thus, the increase of dendritic cells into tumor areas could be associated with an unfavorable prognosis. Our results are different from those reported about other tumors. So, in gastric and cervical carcinomas no significant associations were observed between the degree of dendritic cells infiltration and the degree of tumor differentiation [29, 30].

The correlation of increased S100+ dendritic cells density with a good prognosis has been proven in cases of colorectal, lung and esophageal cancers [31-33]. For other tumors (e.g. squamous cells laryngeal carcinoma) DC infiltration does not correlate with histological grade, tumor stage, or survival [34]. The prognostic value of S100+ cells for patients with renal and breast cancer remains limited [35, 36]. Studying the dendritic cells morphology in intraepithelial and stromal areas led us to assume that two different subpopulations of DC were highlighted, which differ both in maturation and functional status. In our opinion, a particular interest was represented by the quantitative difference of dendritic cells in two studied areas: stromal and intraepithelial. The increased dendritic cells total density was due to the increase of intraepithelial DCs number. Increased number of intraepithelial dendritic cells had a linear feature, which highlight a significant association between the DC density and histological grade of the tumor. Following the obtained results, we suppouse that S100+ stromal dendritic cells can be considered elements of the microenvironment with important anti-tumor effect, and the decrease of their number in prostate adenocarcinoma is an unfavourable prognostic factor. At the same time, the increase in the intraepithelial dendritic cells density can be associated with increased immune tolerance to antitumor mechanisms. Similar data were reported in 2013 by Doros et al. that associated the migration of stromal dendritic cells in the tumor areas of laryngeal carcinomas with a favourable prognosis [37]. Instead, Nagorsen et al. (2007) observed in colorectal carcinoma that better survival depends not only on increased stromal dendritic cell number from the tumor area but also on the increased amount of epithelial DC [38].

Existing vessels in peritumoral tissues can also promote tumor vascularization by co-optation – a process in which existing vessels are surrounded by tumor cells and used to vascularize the tumor. Moreover, our study showed that peritumoral macrophages have often been observed in close contact with the proliferating endothelial cells of capillaries and smooth muscle cells of arterioles and venules. Also, multiple data reported about involvement of dendritic cells in the process of angiogenesis. Depending on the specificity of the antigenic subset, localization, activation status and cytokines, dendritic cells can produce pro- and antiangiogenic mediators. Mostly, *in vivo* studies demonstrated that DC, especially immature DC, promote angiogenesis, while *in vivo* data about antiangiogenic activity of these cells are limited.

Analyzing comparatively the densities of these immune cells, we noted that the expression of CD68 (macrophages) was significantly higher than the expression of S100 (dendritic cells). In order to highlight the interrelationships between the immune cells of prostate carcinoma stroma, the densities of dendritic cells were correlated with those of macrophages. Thus, we obtained statistically significant correlations for both intraepithelial and stromal dendritic cells. The presence of increased densities of immune cells in the modified prostate tissues suggests that hyperplastic epithelial cells have sufficient immunogenicity to recruit immune cells. Antigens, such as PSA (prostate-specific antigen), prostate-specific genes C1, C2, C5, PAGE-1, and prostate stem cell antigen, can induce local activation and proliferation of immune cells [39]. In addition, tumor epithelial cells also produce multiple cytokines and adhesion molecules that recruit more immunocytes to cancer sites. The involve-

ment of epithelial cells in prostate malignancy processes is also supported by our study, which reported in 12 cases of prostate adenocarcinoma about tumor cells expression of S100 marker. Moreover, the presence of these S100+ tumor cells was observed in the areas of tumor invasion, as well as in the lumen of vessels.

Conclusions

In conclusion, the present study has shown that chronic inflammatory processes of the prostate, especially those involving the intratumoral infiltrates with macrophages and dendritic cells, are essential for tumor progression. Thus, the increase in the density of S100+ and CD68+ cells, as well as the significant association of their density with the histological degree of the tumor allow us to consider these cells to be predictive biomarkers of tumor grade, progression and aggressiveness.

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TG designed the study, conducted the laboratory work and performed its technological part, interpreted the data, drafted the first manuscript; LG conducted/performed the laboratory work and drafted the manuscript; VD conducted/performed the laboratory work; PG conducted/performed the laboratory work and drafted the manuscript; EP conducted/performed the laboratory work; LS revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Conflict of Interests
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