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Tryptase and Chymase in Tumor - Associated Mast Cells in Prostate Cancer

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Abstract

Objective: Prostate cancer is one of the most common malignant diseases in men with a steady upward trend in the number of cases every year. Presently, special attention of researchers has been drawn to the search for new methods for diagnosing prostate cancer to provide more accurate diagnosis. In this work, we analyzed the expression of tryptase and chymase in tumor-associated Mast Cells (MC) in the prostate cancer.

Methods: The detection of tryptase in the nuclei of tumor cells may indicate the realization of the anticarcinogenic effects of MC. At the same time, an increase in the number of macrophages with a pro-oncogenic CD68+/CD163+ phenotype in the tumor microenvironment indicates the possibility of the formation of a multidirectional action of immunocompetent cells in the prostate cancer.

Results: We showed that the disease is accompanied by an increase in the MC population in the prostate gland and by an increase in the total pool of specific MC proteases in the tumor microenvironment.

Conclusion: Thus, an increase in the expression of specific MC proteases in the tumor microenvironment of prostate cancer indicates the development of anticarcinogenic effects and may form personalized features of the immune landscape in the pathogenesis of the disease.

Keywords: Prostate cancer; Mast cells; Immunohistochemistry; Tryptase; Chymase

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Introduction

Prostate cancer is one of the most common malignant diseases in men [1]. In 2020, prostate cancer was the second most commonly diagnosed cancer in men worldwide with over 1,400,000 new cases and caused over 375,000 deaths worldwide [2]. Among the causes of deaths from various oncopathologies, prostate cancer ranks fifth after malignant lesions of the lungs, stomach, rectum, and colon [3].

The diagnosis of prostate cancer is based on screening studies, such as digital rectal examination, determination of prostate-specific antigen in blood serum, ultrasound examination of the prostate gland, which have already become traditional [4,5]. It is possible to verify the diagnosis of prostate cancer only with a histological analysis, during which diagnostic errors may still occur due to the similarity of the histological features of prostate cancer with those in hypo- and hyper-biotic processes, benign tumor growth, etc. [6,7]. Therefore, in recent years, special attention of researchers has been focused on the search for new methods for diagnosing prostate cancer, which can provide more accurate diagnosis and assessment of its outcomes, while minimizing the negative effects and maximizing the positive impact of existing treatment methods. Immunohistochemical research methods [8,9], play a special role in the study of prostate cancer, since they currently allow the most accurate diagnosis, reducing possible errors to a minimum [1]. One of the promising areas in immunohistochemistry is the identification and quantification of Mast Cells (MC) in tumor processes in various organs [10,11].

Discovered in the late 1800s by Paul Ehrlich, MCs are multifunctional cells producing a wide

range of mediators [12-14]. Traditionally, MCs are considered to be one of the key cells of inflammation and of allergic reaction of type 1; however, increasing evidence suggests that they may play a central role in many diseases, including tumor processes [11,15] and wound healing [16-18]. MCs, like many other stromal and immunocompetent cells, contribute to the formation of the tumor microenvironment [13,14,19,20].

The general set of MCs is an important and rather stable characteristic of a particular organ; however, it is able to acquire specific features in various pathological conditions [21]. Numerous studies in the field of immunohistochemistry have confirmed that the number of MCs and their phenotype in a tumor are interrelated with the degree of its malignancy [22]. The most well-known and frequently used methods for detecting MCs are based on the metachromatic properties of their secretome [8]. After the introduction of immunohistochemistry protocols into morphological practice, tryptase staining became the most successful method for assessing the total amount of MC in the organ [23,24]. The aim of our work was to analyze the number of tryptase- and chymase-positive MCs associated with prostate cancer, as well as the determination of their profile depending on the macrophage polarization.

Materials and Methods

Case selection

The samples were retrieved from the files of the Belgorod Oncological Dispensary, the Belgorod Pathological and Anatomy Bureau, and the Belgorod Bureau of Forensic Medical Examination from 2017 to 2019. The study group consisted of 10 patients with prostate cancer with histological verification of the disease (5 patients with the 2nd stage of the disease (T1 N0 M0) and 5 with the 3rd stage (T2-3 N0 M0)). The control group consisted of 5 patients - men aged 36 to 50 years who died as a result of traffic accidents, in the prostate gland of which there were no pathological changes. The immunophenotype of the stained objects was checked independently by three pathologists (MT, IP and TP) and after reaching consensus the results were fixed.

All tissues were immediately formalin-fixed and paraffin-embedded. The paraffin blocks were cut into 2 μm sections, which were subsequently subjected to standard dewaxing and rehydration procedures, following the standard procedure [25].

Ethics

This study was conducted in accordance with the principles of World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects" and approved by the Belgorod Pathological and Anatomy Bureau. The samples with written informed consent of patients were redundant clinical specimens that had been de-identified and unlinked from patient information.

Immunohistochemistry

Deparaffinized sections were subjected to antigen retrieval in a steamer with R-UNIVERSAL Epitope Recovery Buffer (Aptum Biologics Ltd., Southampton, UK. Blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted [26]. After antigen retrieval, sections were immunoreacted with primary antibodies. The list of primary antibodies used in this study is presented in Table 1. Antibodies were applied according to the manufacturers' recommendations. For simultaneous visualization of primary antibodies of the same IgG isotype, primary antibodies were non-covalently labeled in vitro with a reporter molecule employing monovalent IgG Fc-specific Fab fragments [25,27]. The reporter molecule was fluorophore Cy3. Primary antibodies were applied in concentration from 1 μg/mL to 5 μg/mL and incubated overnight at +4°C.

Bound primary antibodies were visualized using secondary antibodies (purchased from Dianova, Hamburg, Germany, and Molecular Probes, Darmstadt, Germany) conjugated with Cy3 or Alexa Fluor-488. The list of secondary antibodies and other reagents used in this study is presented in Table 2. The final concentration of secondary antibodies was between 5 and 10 μg/mL PBS. Single and double immunofluorescence labelling were performed according to standard protocols [25]. Nuclei were counterstained with 4′,6-Diamidino-2-Phenylindole (DAPI, 5 μg/mL in PBS) for 15 s, and the sections were then mounted using VectaShield (Vector Laboratories, Burlingame, CA, USA).

Bright-field microscopy

Bound mouse primary antibodies were detected with AmpliStain™ Horseradish Peroxidase (HRP) conjugates (SDT GmbH, Baesweiler, Germany). The HRP label was visualized using the DAB substrate kit (Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with hematoxylin, dehydrated, cleared in xylene and covered with permanent mounting medium.

Controls

The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, or the substitution of primary antibodies with the corresponding IgG at the same final concentration resulted in a lack of immunostaining.

Image acquisition

Stained tissue sections were observed on a ZEISS Axio Imager. A2 equipped with digital microscope cameras (Axiocam 506 color and Axiocam 503 monochrome CCD). The resulting photographs were processed using the ZEN 2.3 program (Carl Zeiss, Jena, Germany). In each micropreparation from one patient, we assayed at least 50 fields of view, each of which, when using a ×40 objective, was 0.0875 mm^2. Thus, for each patient, when obtaining 50 fields of view, we used at least 4.38 mm^2 of tissue area. Nevertheless, the area of the analyzed tissue could be significantly larger.

Statistical analysis

The data were checked for normal distribution using the Kolmogorov–Smirnov test. To identify the significance of differences, the student's t-test was used in the case of a normal distribution or the Nonparametric test including Mann-Whitney U test in the absence of a normal distribution. Differences were considered significant at $p<0.05$.

Results

In the control group, MCs were located mainly in the stroma of the organ, and were less common in the epithelium of prostatic glands (Figure 1a-1d). In the interstitium, MCs were localized near blood vessels, often adjacent to smooth myocytes (Figure 1a). Sometimes MCs were detected in the lumen of prostate glands. MCs, both in the stroma and in the epithelium, had low secretory activity (Figure 1). The expression of chymase in the prostate MC in the vast majority of cases was combined with the expression of tryptase (Figure 1e). As a

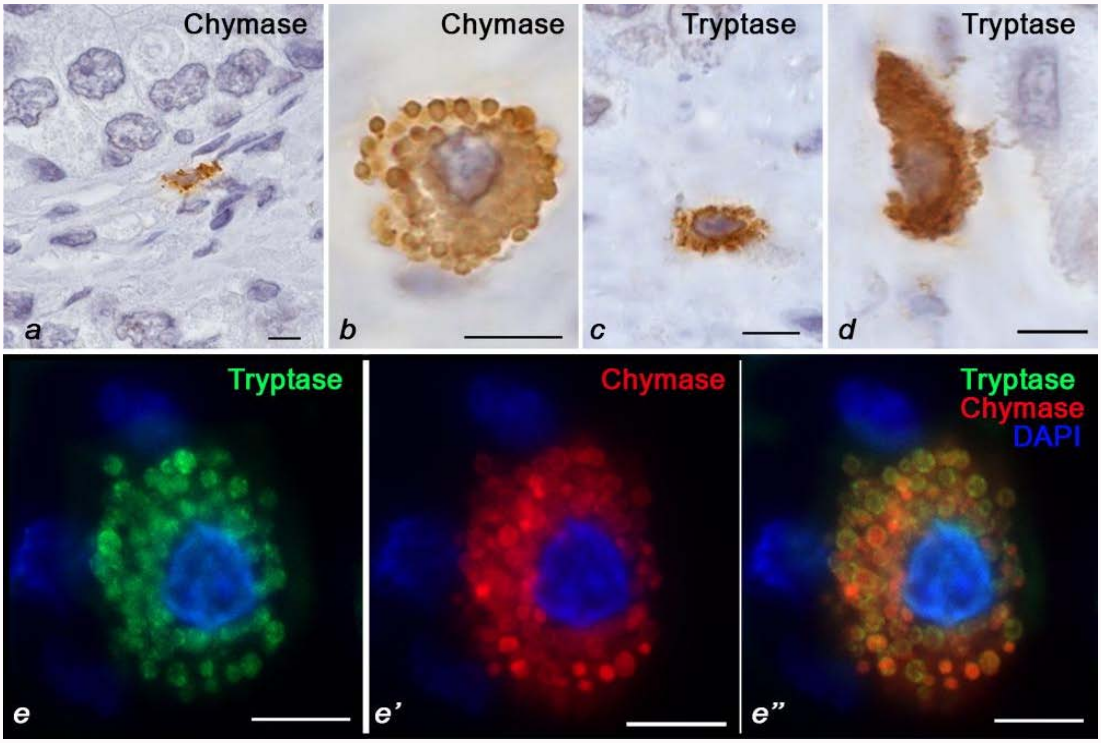

Figure 1: Mast cells in the prostate without pathology. a Mast cell in the stroma of the prostate. b Predominant location of chymase in secretory granules. c With tryptase-positive MC in the interglandular connective tissue. d Directed secretion of tryptase in the composition of the granules to the epithelium of the glands. e Colocalization of tryptase and chymase in large mature secretory granules of MC. Scale bar: 5 μm for the entire layout.

Table 1: Primary antibodies used in this study.

Antibodies Clones	Host	Catalogue Nr.	Dilution	Sourse
Tryptase AA1	mouse monoclonal Ab	#ab2378	2.125	AbCam, United Kingdom
Tryptase EPR9522	rabbit monoclonal Ab	#ab151757	1.43056	AbCam, United Kingdom
Chymase CC1	mouse monoclonal Ab	#ab2377	1.43056	AbCam, United Kingdom
CD68	Rabbit monoclonal Ab	# ab213363 [EPR20545]	0.73611	AbCam, United Kingdom
CD163	Rabbit monoclonal Ab	#ab182422 [EPR19518]	0.38889	AbCam, United Kingdom

rule, proteases were packed into a large number of intracytoplasmic granules (Figure 1b, 1d, 1e). Significantly fewer MCs showed secretion of either only chymase or only tryptase. In general, a significantly low visible secretory activity of MCs should be noted (Table 3).

With the course of the disease, first of all, attention is drawn to the increase in the number of populations of MCs in the prostate (Table 3 and Figure 2a, 2d). MCs were located both in the stroma and in parenchymal cell compartment. The cells often penetrated into the thickness of the stratified epithelium of prostate glands and had contact with epithelial cells, both with basal and luminal cells (Figure 2b, 2c, 2e). Attention is drawn to the increase in the secretory activity of the MCs in prostate cancer, both in the second (Figure 2a-2d) and in the third stage (Figure 2e-2g). At the same time, the change in the morphological characteristics of MCs became obvious. First of all, their size of MCs decreased. The number of MCs with large protease-containing granules was drastically reduced. Their place was occupied by small MCs, the cytoplasm of which contained small secretory granules with a size not exceeding 0.5 μm to 0.6 μm (Figure 2b, 2c, 2e, 2g). Extensive pericellular immunopositivity for specific protease fields around the MC were observed (Figure 2e).

In the composition of the MC population, tryptase-positive cells were predominant, and a smaller part consisted of chymase-positive cells (Table 3). The apparent increase in the number of tryptase-positive cells in the tissues of the prostate cancer by almost two times compared with the control group significantly and directly correlates with the severity of the oncological disease. It should be noted that cells with exclusively chymase expression were practically not observed. Tryptase was found in the nuclei of parenchymal cells, in particular, in the epithelium of the prostate glands in a large number of patients. The presence of tryptase in the nuclei of cancer cells was revealed. In some prostate glands, most of the nuclei were tryptase-positive (Figure 2b, 2c, 2f). At the same time, tryptase could be detected either within limited loci of the karyoplasm or diffusely fill the contents of the nucleus (Figure 2b, 2c, 2e). An interesting fact was the detection in some cases of directed secretion of tryptase in a limited area of glandular cells (Figure 2c, 2e).

When analyzing the population of macrophages in the prostate tissue of the control group, it was found that CD68+ cells were most often single cells. Topographically, they were detected both in the stroma and in the epithelium of the prostate glands. In many

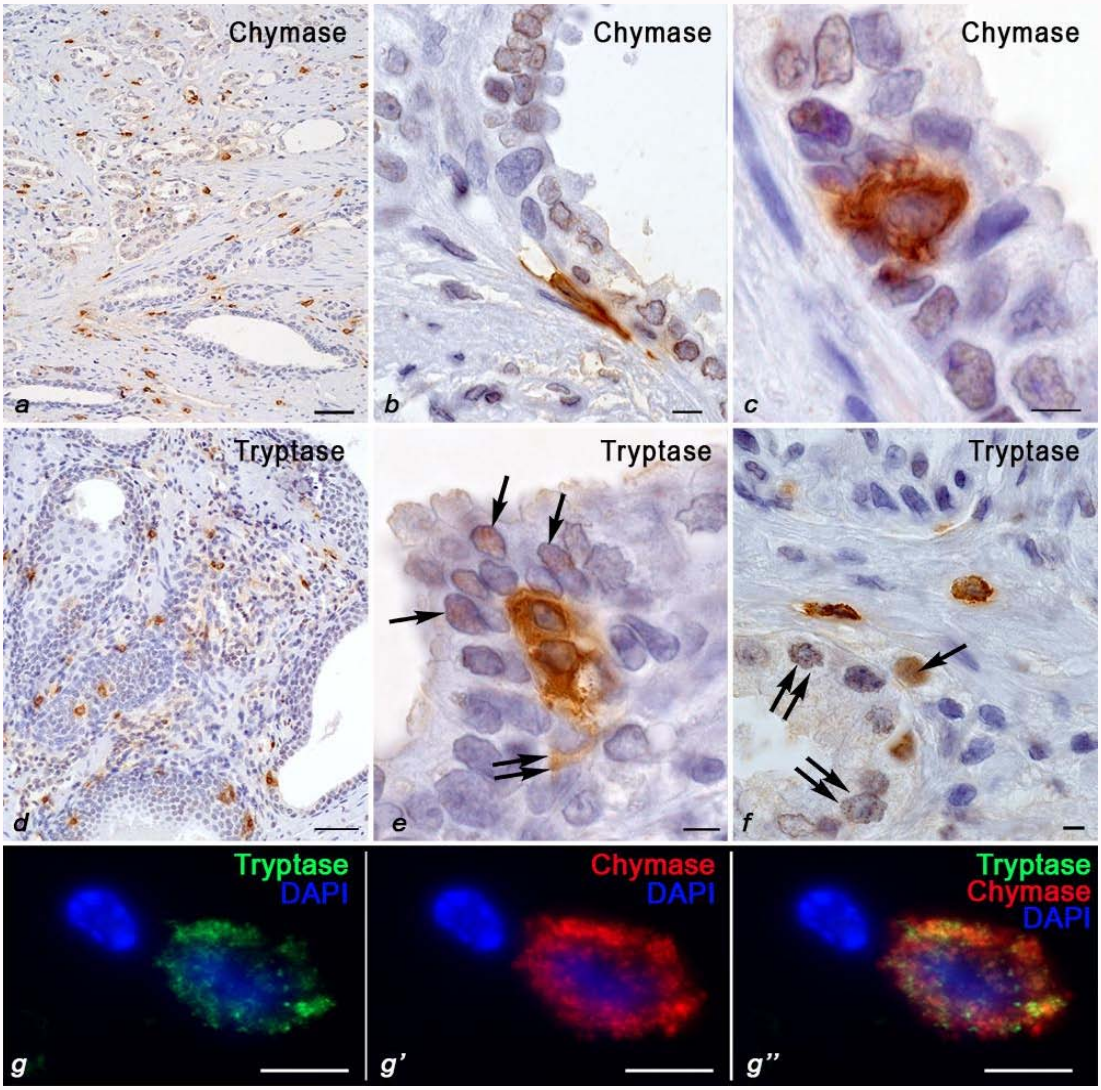

Figure 2: Mast cells in normal prostate tissue of patients with prostate cancer. a-d pT1 patients, e-h pT2-3 patients. a A high amount of MC in the stroma of the prostate cancer, b Adherence to the basement membrane of normal prostate glands. c Intraepithelial MCs of normal prostate glands with increased secretion of chymase. d Increase in the number of tryptase-positive MCs in the stroma and parenchyma of normal prostate tissue. e Tryptase-positive nuclei in a few cells (arrows) of normal glandular epithelium, as well as tryptase secreted by MCs within the glandular epithelium (double arrow). f Stromal localization of tryptase-positive MCs, the presence of tryptase in the nuclei of the stromal cell (indicated by an arrow) and in the nuclei of glandular cells (indicated by a double arrow). g MC with small secretory granules containing both tryptase and chymase. Scale bar: a, d - 50 µm, the rest - 5 µm.

Table 2: Secondary antibodies and other reagents.

Antibodies and other reagents	Source	Dilution	Label
Goat anti-mouse IgG Ab (#ab97035)	AbCam, United Kingdom	1/500	Cy3
Goat anti-rabbit IgG Ab (#ab150077):	AbCam, United Kingdom	1/500	Alexa Fluor 488
AmpliStain™ anti-Mouse 1-Step HRP (#AS-M1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
AmpliStain™ anti-Rabbit 1-Step HRP (#AS-R1-HRP)	SDT GmbH, Baesweiler, Germany	ready-to-use	HRP
4',6-diamidino-2-phenylindole (DAPI, #D9542-5MG)	Sigma, Hamburg, Germany	5 µg/ml	w/o
VECTASHIELD® Mounting Medium (#H-1000)	Vector Laboratories, Burlingame, CA, USA	ready-to-use	w/o
DAB Peroxidase Substrate Kit (#SK-4100)	Vector Laboratories, Burlingame, CA, USA	ready-to-use	DAB
Mayer's hematoxylin (#MHS128)	Sigma-Aldrich	ready-to-use	w/o

cases, CD68+ elements were identified in the lumen of the prostate glands. There was a high polymorphism in the size of CD163+ cells in the prostate tissue. However, for the most part, the cells were large with predominantly eccentric localization of nuclei. Sometimes intracytoplasmic vacuoles were observed, reaching in some cases significant sizes. CD163+ cells were more common in the lumen of

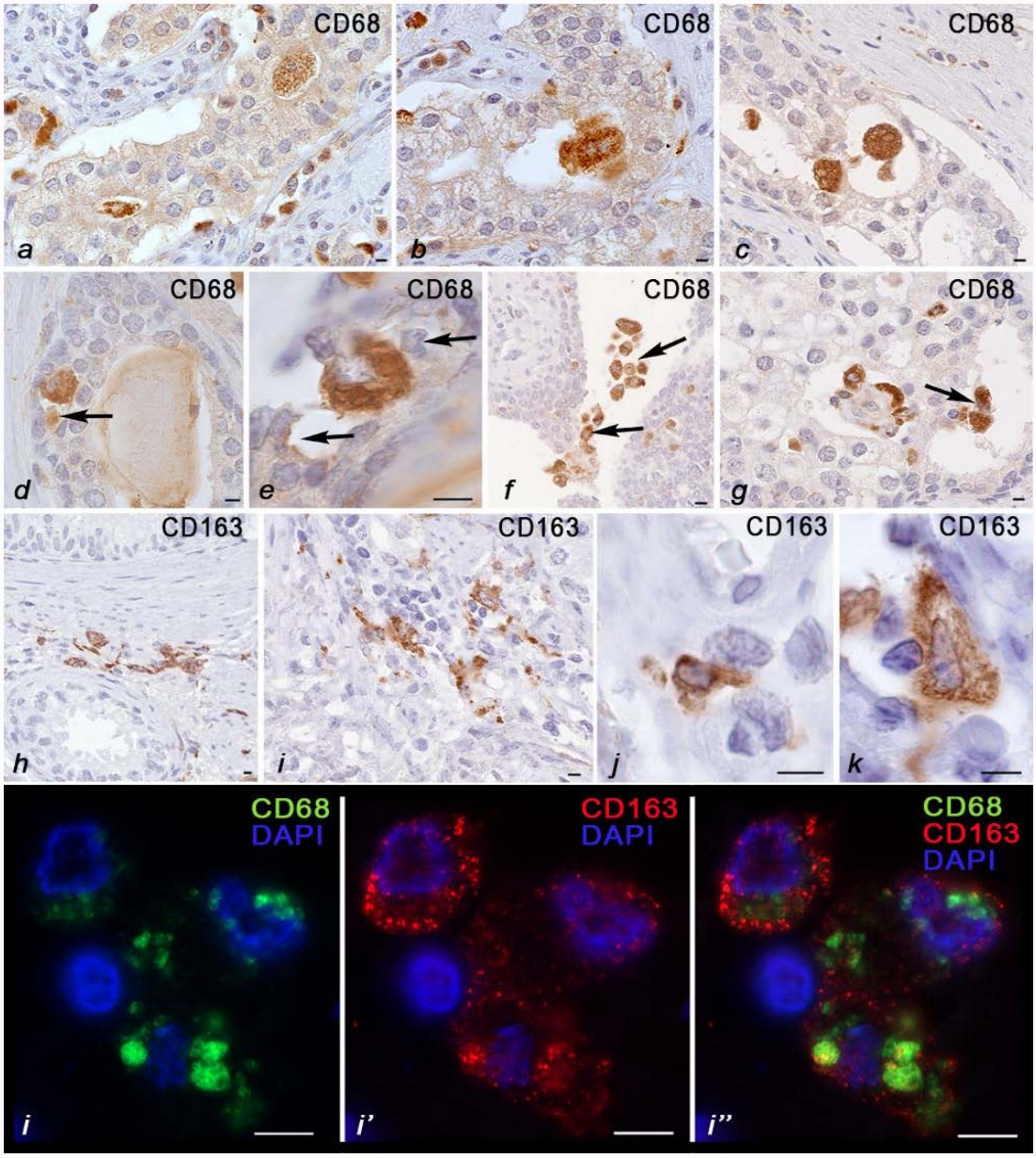

Figure 3: Macrophages in the prostate cancer. a-g Adenocarcinoma of the prostate, stage 2 (T1 N0 M0) a-c, h-k and stage 3 (T2-3 N0 M0) d-g, i. a Location of CD68+ cells in the epithelium of the prostate glands and prostate stroma. b-c Localization of macrophages of large size in the lumen of prostate glands. d-e Intraepithelial localization of macrophages in areas of epithelial alteration (arrows). f-g Formation of multicellular accumulations of macrophages in the vicinity of glandular cells (arrows). h-i Increase in the number of CD163-positive macrophages in the stroma h and in the tumor microenvironment i, contact with cancer cells. j Perivascular location of a CD163+ macrophage. k Intratumoral localization of a macrophage. i Tumor-associated macrophages with a CD68+/CD163+ phenotype.

the prostate glands, or intraepithelially. In the case of large sizes and oval shape, a significant accumulation of CD163-positive material was revealed in the peripheral region of the cytoplasm. The predominant immunophenotype of macrophages was CD68-/CD163+, which accounted for about half (51.3 ± 4.4%) of all marked cells. A smaller number of cells were exclusively expressing CD68 (Table 4).

In prostate cancer at various stages, a significant increase in the total number of CD68+ cells compared with the control group attracted attention (Table 5). At the same time, they also increased in size. The intensity of immunoreactivity to CD68 progressed, which increased as the stage of the disease increased (Figure 3a-3g). At the same time, an increase in the number of CD163+ stromal cells became apparent (Table 5 and Figure 3h, 3i), which could reach quite large sizes. CD163+ macrophages were observed in prostate glands, sometimes penetrating into the lumen of the glands. It should be noted that there is a well-defined peritumoral localization of CD163+ macrophages, which in some cases come into direct contact with

Table 3: The content of mast cells in the human prostate (per 1 mm^2).

Study group	Immunophenotype (proteases)	
	Tryptase-positive MCs	Chymase-positive MCs
Control	34.64 ± 5	7.99 ± 3
Stage 2 (T_1 N_0 M_0)	57.69 ± 4*	15.91 ± 3*
Stage 3 (T_{2-3} N_0 M_0)	68.67 ± 10*	28.52 ± 5*

*: p<0.05 compared to the control group

Table 4: Immunophenotype of tumor-associated macrophages in the prostate.

Study group	Content (in %)		
	CD68+CD163-	CD68+CD163+	CD68-CD163+
Control	14.5 ± 1.5	34.2 ± 2.9	51.3 ± 4.4
Stage 2 (T_1 N_0 M_0)	9.3 ± 1.1*	44.8 ± 3.4*	45.9 ± 3.5
Stage 3 (T_{1-3} N_0 M_0)	9.0 ± 0.8*	40.5 ± 2.5*	41.4 ± 2.8

*: p<0.05 compared to the control group

Table 5: The content of macrophages in the prostate gland (per 1 mm^2).

Marker	Control group	prostate cancer	Prostate cancer
		Stage 2 (T_1 N_0 M_0)	Stage 3 (T_{1-3} N_0 M_0)
	n=5	n=5	n=5
CD68	212.3 ± 55.3	334.1 ± 30.2*	406.43 ± 25.3*
CD163	236.81	337.05*	416.39*

*: p<0.05 compared to the control group

cancer cells. A high frequency of adherence of type 2 macrophages to the vascular bed remained.

When conducting multiple immunolabeling in the prostate tissue, we found that the development of prostate cancer was accompanied by an increase in the level of simultaneous expression of CD163 and CD68 in cells of monocytic origin (Table 3 and Figure 3i). At the same time, a significant decrease in the relative volume of cells expressing exclusively CD68 with the immunophenotype CD68+/CD163- became apparent.

Discussion

The study of the immune landscape of the tumor microenvironment in various variants of the oncological process has for many years occupied a special place both in fundamental research and in clinical practice. The study of MCs and their activity plays a special role. Here we have studied their protease activity of MCs in prostate cancer.

The main conclusions of this observational study are the high intensity of secretion of proteases into the extracellular matrix in prostate cancer. This point of view is confirmed by rather extensive pericellular immunopositive fields of specific proteases around the MC. Simultaneous detection of specific proteases showed that the expression of chymase in MC in most cases was combined with tryptase. A much smaller number of MCs showed only chymase secretion. The differences between tryptase and chymase content in tumor associated MCs in prostate cancer definitely deserve a further study [28,29].

The examined prostate cancer patients did not demonstrate any significant increase in the level of chymase expression. The content of MCs with simultaneous expression of proteases was commensurate with the control group. At the same time, the characteristic immunohistotopographic pattern observed in prostate cancer - tryptase was found in the nuclei of the epithelium of prostate glands. At the same time, tryptase could be detected in limited loci of the karyoplasm or to complete diffuse filling of the nuclei. The presence of tryptase in the nuclei of malignantly transformed prostate glands can be interpreted as an anti-oncogenic effect of MCs, which was shown using other biological models as an example [30,31].

An increase in peritumoral MCs is associated with a poor prognosis; some authors attribute these effects to tryptase and Chymase, including the intensification of angiogenesis [32,33].

In our work, we showed that the intratumoral localization of MCs can be regarded as an indication for tryptase transport to the nuclei of tumor cells as anti-tumorigenic effects. This coincides with the opinion of Hempel on the anti-tumorigenic effects of intratumoral MCs in prostate cancer [34]. Globa et al. believe that MCs play an important role in the pathogenesis of prostate cancer [35].

We also found that the increasing MC population in the prostate cancer was associated with an increase in the total number of tumor-associated CD68+/CD163+ macrophages, and that the intensity of their immunoreactivity to CD68 increased with the severity of the disease.

Recently, Habanjar et al. [10] reported that the detected effects of MC are opposite to the pro-oncogenic properties of tumor-associated macrophages with the CD68+/CD163+ phenotype, including inhibition of lymphocyte function in tumors, suppression of the pro-inflammatory reaction, stimulation of metastasis, stimulation of angiogenesis, suppression of adaptive immunity, etc. However, the biological significance of the interaction of MCs with macrophages in the tumor microenvironment requires further study.

Conclusion

To summarize, we showed that the prostate cancer is associated with an increase in the MC population in the prostate gland and by an increase in the total pool of specific MC proteases in the tumor microenvironment. The study of the expression of specific MC proteases and the assessment of the functional properties of macrophages, open up new ways in the interpretation of these cells in the development and progression of prostate cancer and can serve as an auxiliary method for studying the biology of the tumor microenvironment. Further studies are needed to investigate the role of MCs in prostate cancer.

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