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THE CHANGES OF NO LEVEL AND RNASE ACTIVITY IN TUMOR TISSUE ACCOMPANYING THE PROGRESSION OF PROSTATE CANCER

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Aim: **To assess the inducible NO-synthase activity and the total RNase activity in tissue samples and blood neutrophils of the patients with prostate intraepithelial neoplasia (PIN) and prostate cancer (PCa) of different stages.** ***Materials and Methods*****: NO level was measured in tumor tissue and neutrophils of patients with PIN and PCa of different stages by electron paramagnetic resonance using the spin traps technology. RNase activity in tumor tissue of patients with PIN and PCa was measured by the method of zymography.** ***Results*****: We have found that NO levels in prostate tumor tissue were significantly higher than in the PIN and increased along with the disease progression. Analysis of NO level in neutrophils of the PCa patients demonstrated that the values were not dispersed and did not depend on the stage of disease. NO level in neutrophils of the PCa patients increased manifold as compared with that in healthy donors. At the same time, the RNase activity in the prostate tumor tissue gradually decreased with PCa progression.** ***Conclusion*****: Activities of inducible NO-synthase and RNases change significantly with progression of PCa.**

Key Words**: prostate cancer, intraepithelial neoplasia, NO-synthase, RNase.**

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Prostate cancer (PCa) is among the most common malignancies in men worldwide [1]. In 15–40% of the patients with the localized PCa, the disease progresses due to unknown causes. The involvement of tumor microenvironment in cancer progression is a subject of extensive investigation. Among the important components of tumor microenvironment one could mention RNases and NO.

NO is known to play an ambivalent role in cancer biology: it may stimulate tumor growth as well as trigger antitumor mechanisms [2–5]. NO in comparatively low concentrations accompanying chronic inflammation (~1.5 nmol/g wet tissue, higher than normal) retards apoptosis. Radical NO forms, interacting with DNA, cause mutations and post-translational protein modifications, activation of the signal pathways stimulating cell proliferation, neovascularization and dissemination of tumor cells.

Neutrophils play the key role in the innate immunity and participate in the adaptive immune response. Polymorphonuclear neutrophils (PMN), being activated, may generate biologically active molecules, in particular NO, in the intercellular matrix. In spite of active participation of the neutrophils in different pathologic processes, involvement of inducible NO-synthase (iNOS) generated by PMNs is of special interest. The association of iNOS with PCa pathogenesis is a subject of recent studies [2, 6].

Ribonucleases (RNases) compose a numerous and heterogenic group of the hydrolytic enzymes with various functions [7]. Rapidly proliferating cell populations are characterized by low RNase activity. According to the literature data, evaluation of RNase activity and spectra may be important for PCa diagnosis and prognosis [8]. In particular, Shook *et al*. [9] consider RNase L as a PCa marker. The simultaneous study of iNOS and RNase activity in the biological materials of the PCa patients is challenging.

The aim of this study was to assess the iNOS activity and the total RNase activity in tissue samples and blood neutrophils of the patients with prostate intraepithelial neoplasia (PIN) and PCa of different stages.

MATERIALS AND METHODS

Patients. 50 patients with PCa and 10 patients with PIN treated at the Institute of Urology of the National Academy of Medical Sciences of Ukraine in 2017–2018 as well as 38 patients with PCa (T1–T4) and 5 patients with PIN treated at the National Cancer Institute of the Ministry of Health of Ukraine during 2017–2018 were enrolled in the study. Tumor tissue and blood samples of the patients were studied. As a control, blood samples of 8 healthy donors were provided. The informed consent of the patients for the use of the samples in research purposes was obtained. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committees of the Institute of Urology of the National Academy of Medical Sciences of Ukraine and National

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Abbreviations used: EPR – electron paramagnetic resonance; iNOS – inducible NO-synthase; PCa – prostate cancer; PIN – prostate intraepithelial neoplasia; PMN – polymorphonuclear neutrophils; PSA – prostate-specific antigen; RNase – ribonuclease.

Cancer Institute of the Ministry of Health of Ukraine. All patients did not receive neoadjuvant hormonal therapy. All patients were subjected to common clinical laboratory investigations, prostate biopsy with morphological identification, prostate-specific antigen (PSA) level analysis and sonography, magnetic resonance imaging or computed tomography of the pelvic organs; and additional investigations according to indications. A pathomorphological study of the resected tumor was carried out to determine tumor grade by Gleason score. The stage of the tumor process was determined according to the TNM Classification of Malignant Tumors, 8th Edition (2016).

Sample preparation and electron paramagnetic resonance (EPR). Tumor tissue samples were frozen and kept in liquid nitrogen prior to the study. The fresh blood sample was processed. Neutrophils were isolated in a double density (ρ = 1.077 g/cm^3 and ρ = 1.119 g/cm^3) gradient of Ficoll-sodium diatrizoate at 4 ^{0}C by centrifugation at 400 g for 45 min. NO generation rate in tumor samples and NO level in neutrophils were measured by EPR method using Fe/diethyldithiocarbamate (Fe/DETC) and 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H) (Sigma-Aldrich, USA) as spin traps. EPR measurements were done using RE-1307 (Russia) and Bruker ESP-300 (Bruker, Germany) EPR spectrometers (the spectrometers operate at microwave frequency of 9.5 GHz) at room and liquid nitrogen temperatures (T = 77 K). Technical details of EPR measurements and sample preparations with spin traps are given elsewhere [10–12].

Determination of RNase activity. The tumor material obtained after surgery was immediately frozen in liquid nitrogen and stored at –80 °C until the further studies. Frozen tissue (50 mg) was powdered in liquid nitrogen. The powder was homogenized at 4 °C in a glass-to-glass homogenizer in 5 volumes of Gronow buffer: 50 mM Tris-phosphate buffer (pH 7.5), containing 5 M urea. The tissue homogenate was centrifuged at 5000 rpm for 5 min at 4 °C, supernatant was carefully aspirated and used for enzyme determination. Protein concentration was determined using spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific, USA). Protein extracts (20–100 pg) were diluted with sample buffer (1% SDS, 5% glycerol, 0.125% bromophenol blue, and 25 mM Tris-HC1, pH 6.8) and electrophoresis was performed in Laemmli system in 14% acrylamide gel containing Torula yeast (*Torulopsis utilis*) RNA (Sigma-Aldrich, USA). After electrophoresis, SDS was washed from the gels by treatment with 25% isopropanol, the gels were incubated in 0.05 M acetate buffer pH 5.5 at 37 °C for 60 min and stained in 0.2% toluidine blue (Sigma-Aldrich, USA). Gels were scanned using HP Scanjet 5590 and analyzed using the TotalLab TL120v2006f software (NonLinear Dynamics).

Statistical analysis. Statistical analysis was done using GraphPad Prism 6 and Origin 7.5 programs. Difference between the parameters was considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

NO levels in tumor tissues and neutrophils of PCa patients. We have studied NO levels indicative of iNOS activity in tumor tissue and PBN of 50 patients with PCa (T2N0M0, n = 35, T3N0M0, n = 15) and 10 patients with PIN. According to the level of NO in the tumor tissue, the patients were divided into two groups accounting for the distribution of NO values based on their median (Fig. 1): group 1 — 4.46 ± 0.84 nmol/g tissue; group 2 — 2.18 ± 0.13 nmol/g tissue. In patients with PIN, the mean level of NO in the tumor was 1.75 ± 0.15 nmol/g tissue, in PCa cases the rate of NO generation increased with the stage of the disease (Table 1).

The preoperative PSA values in groups 1 and 2 of patients with PCa are shown in Table 2. Preoperative PSA level was higher in patients with high NO levels in tumor.

Table 1. NO levels in patients with PIN and patients with PCa at different stages of the disease

Tumor tissue	NO levels in tumor (nmol/g tissue)
PIN	1.75 ± 0.16
Localized PCa (T2N0M0)	4.11 ± 0.71*
Locally advanced form (T3N0M0)	5.10 ± 0.08*

Note: *The difference is significant as compared with PIN tissue, $p < 0.01$.

Table 2. PSA levels in blood and NO levels in tumor tissues of PCa patients

Index	PCa patients	
	Group 1 (n = 17)	Group 2 (n = 18)
Age, years	65.4 ± 13.2	64.3 ± 15.4
PSA level before radical prostatectomy, ng/ml	14.3–27.5	8.0–18.2

Analysis of NO level in neutrophils of the PCa patients demonstrated that the values were not dispersed and did not depend on the stage of disease (Fig. 2). At the same time, NO level in neutrophils of the PCa patients increased manifold as compared with that in healthy donors.

Therefore, we have found that NO levels in prostate tumor tissue were significantly higher than in the PIN and increased along with the disease progression. The association of NO level and iNOS activity

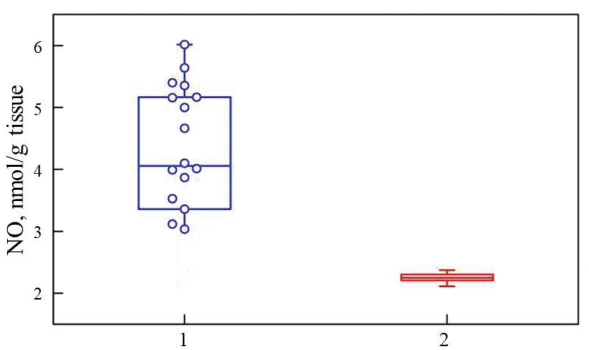

Fig. 1. Distribution of PCa patients by the NO level in the tumor and PSA level in the blood serum: 1 — group 1 (patients with high PSA and NO levels); 2 — group 2 (patients with low PSA and NO level)

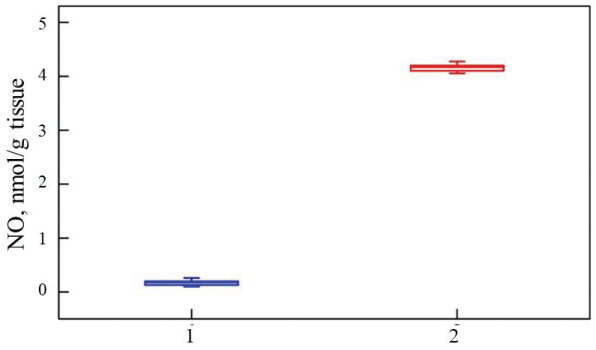

Fig. 2. NO level produced by PMN: 1 — healthy donors; 2 — PCa patients

with pathogenesis of PCa was demonstrated in several studies [2, 6]. Some authors link high iNOS activity with rapid cell proliferation, dedifferentiation and low survival of the patients suggesting the prognostic value of this indicator in PCa [13, 14].

Prolonged action of high NO concentrations leads to stable inhibition of I and IV respiratory chains in mitochondria, their metabolic reprogramming along with cell hypoxia formation [15, 16] and aggressive cell phenotype. As it is known, testosterone and β-estradiol magnify expression of the NO-generating enzymes both in epithelium and in cell microenvironment, in such a way promoting inflammation and redox status modification, being the moving force of the PIN development [17]. Hormonal activation of the NO synthesis create conditions for inflammation through enhanced generation of the radical oxygen species, modifying cell redox status and inducing cell damage. Within a tumor, NO is produced by the tumor cells and, in the intercellular matrix, by the inflammatory cells (neutrophils, macrophages). Among the immune cells immediately interacting with the tumor cells, neutrophils are the most numerous and able to penetrate the tumor hypoxic zone and accumulate there. Due to iNOS, neutrophils and macrophages interact with the tumor cells forming their microenvironment, especially the hypoxic zone.

We demonstrated the significant increase in NO level in PMN of PCa patients independently of their TNM stage. Earlier we have shown the association between neutrophil NO generating activity and treatment outcome in rectal cancer [18].

RNase activity in PCa tumor tissue. RNase activity was studied in tumor tissues of 38 patients with PCa (T1–T4) and 5 patients with PIN. Analyzing the zymograms, we have found two zones of the RNase isoforms, representing activity of the unmodified enzyme and a set of the glycosylated RNases. It is notable that the enzymatic activity in the electrophoregrams is devoid of the interfering effects of the natural RNase inhibitor because the latter has much higher molecular mass and moves significantly slower than RNases in the used electrophoretic system. Table 3 represents the total RNase activity value given in arbitrary units.

Table 3. The total RNase activity in tissue samples of PIN and PCa of different stages

Tumor	RNase activity, a.u.	PSA level (ng/ml) before radical prostatectomy	Gleason score
PIN (n = 5)	641.4 ± 65.7	2.6–5.9	–
PCa (T1N0M0) (n = 4)	460.8 ± 43.2*	7.5–14.5	6
PCa (T2N0M0) (n = 20)	229.3 ± 21.6*, **	5.0–17.4	6–9
PCa (T3N0M0) (n = 4)	186.0 ± 19.4*, **	11.9–27.4	7–9
PCa (T4N0M0) (n = 4)	103.5 ± 12.9*, **, ***	13.0–26.1	7–10

Notes: *difference is significant as compared with PIN ($p < 0.05$); **difference is significant as compared with T1N0M0 ($p < 0.05$); ***difference is significant as compared with T2N0M0 and T3N0M0 ($p < 0.05$).

The data obtained demonstrate the gradual decrease of the RNase activity in the prostate tumor tissue in line with PCa progression. Recently, the investigators pay attention to the stromal tumor microenvironment, for better understanding the mechanisms of cancer initiation and progression.

RNases, in particular RNase L, are implicated in PCa biology [8]. The extracellular RNases in the stromal microenvironment control tumorigenesis by activation of the macrophage migration in the tumor hypoxic zone [19]. Recently, the role of specific RNases in pathogenesis of malignant tumors was established. In particular, overexpressed RNaseT2 inhibits tumor growth by enhancement of the M1-polarized macrophages (producing cytokines, NO and O_2·) infiltration into a tumor [20]. Genetic and epidemiologic studies supported the association of RNase L mutations with PCa, highlighting the role of RNase L in PCa [21].

These data demonstrate the appropriateness of studying RNases in PCa. For this investigation, the method of zymography is the optimal because it permits not only estimate the total enzyme activity but also discover its molecular mass modulations and the presence of the isozymes, most frequently appearing as a result of glycosylation.

In our study, the total RNase activity in tumor tissue decreased along with increasing stage of the disease. When several individual forms of RNases were studied by other authors, the different patterns of specified RNases were evident. In particular, it was shown that RNases κ was significantly decreased in PCa compared to benign prostate hyperplasia, while its overexpression was associated with decreased risk of PCa development [22]. In contrast, RNase4 is up-regulated in PCa, with its expression increasing progressively with aggressiveness of the disease [23]. Therefore, further study of the role of different RNases in PCa could be promising taking into account the possible use of RNases as the therapeutic modalities.

To sum up, we demonstrated the association of iNOS and RNase activities in prostate tumor tissue with PCa progression. While the iNOS activity increased in tumors of increased TNM grade, the total RNase activity significantly decreased. There was observed a clear difference between the activities of these enzymes in PCa and PIN. Further studies should be encouraged to establish the possible benefits of using these enzymes as the diagnostic and prognostic markers in PCa.

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ЗМІНИ РІВНІВ NO ТА АКТИВНОСТІ РНКази В ПУХЛИННІЙ ТКАНИНІ ЗА ПРОГРЕСІЇ РАКУ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ

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Мета: Визначити активність індуцибельної NO-синтази та сумарну активність РНКаз в зразках пухлин та нейтрофілах периферичної крові хворих на внутрішньоепітеліальну неоплазію (ВЕН) та рак передміхурової залози (РПЗ) різної стадії. ***Матеріали та методи:*** Рівень NO визначали в пухлинній тканині та нейтрофілах хворих на ВЕН та РПЗ різної стадії за допомогою електронного парамагнітного резонансу із застосуванням технології спінових пасток. Активність РНКаз у пухлинній тканині визначали методом зимографії. ***Результати***: Рівень NO в пухлинній тканині РПЗ вірогідно вищий, ніж в ВЕН, причому цей рівень зростає з прогресуванням захворювання. Аналіз рівнів NO в нейтрофілах хворих на РПЗ показав, що відповідні значення знаходяться в дуже вузькому діапазоні та не залежать від стадії захворювання. Разом з тим, NO в нейтрофілах хворих на РПЗ зростають багаторазово у порівнянні з відповідними значеннями у нейтрофілах здорових донорів. У той же час активність РНКази в пухлинній тканині РПЗ знижується з прогресуванням захворювання. ***Висновок:*** Активності індуцибельної NO-синтази та РНКази суттєво змінюються з прогресуванням РПЗ.

Ключові слова: рак передміхурової залози, внутрішньоепітеліальна неоплазія, NO-синтаза, РНКаза.