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THE CHANGE OF POLYAMINES AND NITRIC OXIDE QUANTITIES IN HUMAN BLOOD SERUM OF PROSTATE AND BLADDER CANCER

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Currently are shown rapid growth of polyamines and nitric oxide (NO) quantities in blood serum during malignant tumors in different organs. Increased NO generation in cancer cells may contribute to tumor angiogenesis and metastatic ability by up-regulating vascular endothelial growth factor. The goal of workwas to researchin human blood serum the changes of polyamines and NO quantities in different stages of prostate and bladder cancer. Polyamines and NO quantities were determined in blood serum of 11 healthy individuals (34-63 years old) and patients with prostate (28) and bladder (20) cancers (48 patient,I-III stages, 44-73 years old) who were hospitalized in the National Centre of Oncology RA aft. V.A. Fanarjyan. Total polyamines quantity compared with standard was increased by42.5%, 54.5% and 76.8%, respectively in I, II and IIIstages of prostate cancer, and 59.5%, 64.8% and 87.9%, respectively in I, II and IIIstages of bladder cancer.The quantity of nitrite anions was increased by 125% in prostate and bladder cancers patients blood serum.The increase of NO and polyamines concentrations in blood serum in earlier stages and the increase in parallel to cancer development confirm, that this metabolic pathway of L-arginine has a significant role in promoting tumor growth and development. We suggest that downstream of polyamines and NO quantities might have antitumor effect on cancer development.

cancer, polyamine, NO, arginase, tumorigenesis, antitumor potential

Ներկայումս ցույց է տրված պոլիամիններիև NO-ի քանակության կտրուկ աճը արյան շիճուկում տարբեր օրգաններում չարորակ ուռուցքների առկայության ժամանակ: Քաղցկեղային բջիջներում NO-ի և պոլիամինների արտադրման աճը կարող է նպաստել ուռուցքի անգիոգենեզին և մեծացնել մետաստատիկ հնարավորությունները՝ խթանելով անոթային էնդոթելիալ աճի գործոնը: Մեր հետազոտության նպատակն է ուսումնասիրել մարդու արյան շիճուկում պոլիամիններիև NO-ի քանակության փոփոխությունները շագանակագեղձի և միզապարկի քաղցկեղի զարգացման տարբեր աստիճանների ժամանակ: Պոլիամիններիև NO-ի քանակական փոփոխությունները ուսումնասիրվել է 11 առողջ անհատի (34-63 տարեկան), շագանակագեղձի և միզապարկի քաղցկեղ ունեցող հիվանդների արյան շիճուկում (48 հիվանդ, I-III աստիճան, 44-73 տարեկան), որոնք հետազոտության են գրանցվել Ֆանարջյանի անվան ՀՀ Ուռուցքաբանական Ազգային Կենտրոնում: Շագանակագեղձի քաղցկեղի դեպքում պոլիամինների գումարային քանակը առողջ խմբի համեմատ աճում է 42.5%, 54.5% և 76.8%-ով, իսկ միզապարկի քաղցկեղի ժամանակ՝ 59.5%, 64.8% և 87.9%-ով, համապատասխանաբար քաղցկեղի զարգացման I, II և III աստիճաններում: Ստացված արդյունքները ցույց են տվել, որ ստուգիչ խմբի համեմատ արյան շիճուկի նիտրիտ անիոնների քանակը շագանակագեղձի և միզապարկի քաղցկեղի դեպքում աճում է 125%-ով: Հիվանդության վաղ փուլերում պոլիամիններիև NO-ի քանակության աճը արյան շիճուկում և հետագա բարձրացումը զարգացման աստիճանին զուգահեռ՝ հաստատում է L-արգինինի նյութափոխանակային ուղղու այս մետաբոլիտների կարևորագույն դերը ուռուցքի աճի խթանման և զարգացման գործընթացներում: Մենք եզրակացնում ենք, որ պոլիամիններիև NO-ի քանակության նվազումը կարող է ունենալ հակաուռուցքային ազդեցություն քաղցկեղի զարգացման գործընթացում:

քաղցկեղ, պոլիամին, NO, արգինազ, ուռուցքագոյացում, հակաուռուցքային պոտենցիալ

В настоящее время установленно увеличение количества полиаминов и окиси азота (NO) в сыворотке крови во время злокачественных новообразований в разных органов. Увеличение количества NO и полиаминов в раковых клетках может способствовать ангиогенезу опухоли и метастатическим способностям с помощью регулирующего фактора роста эндотелия сосудов. Целью работы было исследование изменения количества полиаминов и NO в сыворотке крови человекана разных стадиях развития рака простаты и мочевого пузыря. Количество полиаминов и NO определяли в сыворотке крови 11 здоровых (34-63 лет) и пациентов с раком предстательной железы (28) и мочевого пузыря (20) (48 пациентов, I-III стадии, 44-73 года), которые были госпитализированы в Национальный Центр Онкологии РА имени Фанарджяна. Общее количество полиаминов по сравнению со стандартом увеличивается на 42.5%, 54.5% и 76.8% соответственно на I, II и III стадиях рака предстательной железы и на 59,5%, 64,8%, 87,9% соответственно на I, II и III стадиях рака мочевого пузыря. Результаты исследований показали, что количество анионов нитрита было увеличено на 125% в сыворотке крови больных раком предстательной железы и мочевого пузыря. Увеличение концентрации NO и полиаминов в сыворотке крови на ранних стадиях и увеличение параллельно с развитием рака подтверждают, что этот метаболический путь L-аргинина играет важную роль в стимулировании роста и развития опухоли. Мы предполагаем, что понижение количества полиаминов и NO может оказать противоопухолевое воздействие на развитие рака.

рак, полиамины, NO, аргиназа, опухолегенез, противоопухолевый

Introduction

The heterogeneous nature of prostate and bladder cancers makes it difficult to understand the molecular mechanisms controlling benign and malignant cell growth [6]. Therefore, it is important to continue to improve current diagnostic and treatment tools and determine new prognostic variables. According to the World Health Organization, the age-standardized death rate due to cancer in Armenia is 39.6 per 100,000; making it the first highest nation globally for cancer deaths [12]. Earlier reports focused on the expression of arginase and NO-synthase (NOS) in murine or human primary cancer tissue as well as malignant cell lines and emphasized its potential role in the promotion of tumour growth via polyamine synthesis or down-regulation of NO-mediated tumour cytotoxicity [5, 9]. Since polyamines are vital for cell proliferation, it is possible that the increased level of ornithine, due to the elevated arginase activity, linked to the development of carogenesis [3]. Over past decades, NO has emerged as a molecule of interest in carcinogenesis and tumor growth progression [2]. NO either facilitates cancer-promoting characters or act as an

anticancer agent. The dilemmain this regard still remains unanswered.Increased NO generation in cancer cells may contribute to tumor angiogenesis by up-regulating vascular endothelial growth factor (VEGF), and VEGF-induced neovascularization may increase the tumors' metastatic ability. Although several reports have addressed the protumoral effects of NO, few have demonstrated the contrasting role of NO in mediating tumor regression. The studies have shown that high levels of NO inhibit epithelial-mesenchymal transition (EMT) and reverses both the mesenchymal phenotype and the invasive properties of human prostate metastatic cells. Although these tumoricidal roles of NO have been proposed, most experiments have been performed in vitro and such findings have not been reported in cancer patients. It has been suggested that NO concentrations found in tumors are insufficient to produce apoptosis and other tumoricidal effect and are likely to facilitate angiogenesis and tumor dissemination.

The goal of this article was to study the involvement of arginase and NO-synthase in prostate and bladder cancers development by the change of polyamines and NO quantities.

Materials and methods

Patients. This study was performed with blood serum of patients with prostate and bladder cancers who were hospitalized in the National Center of Oncology named after V.A.Fanarjyan (Yerevan, Armenia). Arginase activity, polyamines and NO quantities were determined in blood serum of 11 healthy individuals (34-63 years old) and patients with prostate (28, male) and bladder (20, male and female) cancer (48 patients, I-III stages, 44-73 years old). None of the patients had received chemotherapy. The Tumor-Nodes-Metastasis (TNM) system was used to describe the cancer stages (Table 1). The surgical intervention and also other diseasesof the patients in different stages of disease development was not taken into account. The study was approved by the Bioethics Committee of Armenia, and informed consent was obtained from all patients.

Chemicals. Chemicals for determination of arginase activity, NO quantity and TLC were obtained from Sigma Aldrich Co. Ltd. and Carl Roth GmbH + Co. KG (Germany).

Determination of arginase activity. Arginase activity was determined by the colorimetric method of Van Slyke and Archibald with some modifications [1, 8]. In supernatant was determined the final product of the catalysis (urea). 1 ml supernatant, 0.25 ml 3% (w/v) diacetyl monoxime (DAMO) were added and boiled in water bath during 45 min. The intensity was measured with spectrophotometer in 487 nm. Activity of enzyme was evaluated with the received urea, in micromoles in 1 sec (kat).

Separation of serum arginase. For purification of the enzyme the procedure described by Berüter et al. for human liver and erythrocytes, was used with some modifications [8].Separationof arginaseisoenzymes were performed by the method of Kossman with some modifications [1, 8]. The column (2×40 cm) containing Sephadex G-150 was balanced with Na-phosphate buffer (pH 7.2) and 40 fractions each one of 4 ml were collected. 4ml of low-molecular-weight protein fraction after gel-filtration was passed through the column CM-cellulose (CM52, 1x18 cm), balanced against 5 mM Tris-HCl buffer, pH 7.2, elution speed was 24 ml/h, 32 fractions each one of 4 ml were collected. The peak of arginase activity adsorbed on the column with a linear KCl gradient (0.0–0.5 M).

Dansylation and thin layer chromatography (TLC) analysis. The method of Seiler (1970) was used with some modifications as follows [4]. Tissues were extracted in 0.2M cold $HC1O_4$ at a ratio of about 100 mg/ml $HC1O_4$. Dansylpolyamines were extracted in 0.5 ml benzene, and vortexed for 30s. The chromatogram was developed for about 2h with chloroform-triethylamine (25:2, v/v) solvent system. The dansylpolyamine bands were

scraped, eluted in 2 ml ethyl acetate, and quantified in 505 nm.The quantity of polyamines is presented in nMpolyamines in 1 ml serum.

Griess assay for NO quantity. Nitrite was measured by theGriess assay [11]. Briefly, 100 µl Griess reagent were added to 100 µl of each of the above supernatants. The plates were read at550 nm against a standard curve of$NaNO_2$. The values were corrected forthe NO^{2-}+ NO^{3-} content of water, andthe recovery of$NO^{2-}$was calculated.

Data processing. Results were expressed as means ± SD and evaluated by Student's *t*-test (single sample) using Statistica software (StatSoft 10.0).

Results and discussion

The aim of this work was to study the correlation between arginase activity, polyaminesand NO quantities at prostate and bladder cancer and in case of possibility, to suggest use it as a diagnostic test and treatment.We found an increase of arginase activity in 73,8% of patients (True Positive, Table 1). The calculated values ofarginase activity test sensitivity [TP/(TP + FN)], specificity [TN/ (TN+ FP)] and accuracy [(TP+TN)/(TP+FP+FN+TN)] in serum of prostate and bladder cancer patients were 83.4%, 81.7% and 83%, respectively (see Table 1).

Inprostate and bladder cancer group of stage I activity of serum arginase was increased by 61,52%, in the group of stage II by 118,27% and the group of stage III by 194,23% comparing to the healthy group (Fig. 1). This insinuates that arginase may have some role in promoting tumor growth and development and its activity can be considered as an important marker to determine disease progression or regression.

Table 1. Characteristics of patients and arginase activity test in blood serum from patients with prostate and bladder cancer.

Patients	Sex	Age	Cancer	Stage	TNM	Characteristic of test	Number of patients	%
11	Male and Female	49±15	-	-	-	True positive (TP)	40	67.8
16		60±11	Prostate (men, 28) and bladder (women and men, 20)	I	$T_1N_0M_0$	False positive (FP)	2	3.4
19		58±14		II	$T_{2a}N_0M_0$, $T_{2b}N_0M_0$	True negative (TN)	9	15.2
13		64±9		III	$T_{3a}N_0M_0$, $T_{3b}N_0M_0$, $T_{4a}N_0M_0$	False negative (FN)	8	13.5

In blood serum patients with prostate and bladder cancers Gel chromatography (GC) revealed 1 peaks for arginase activity (*Ve* = 104-116 ml) and 2 peaks for protein quantity (*Ve* = 44-52 and 104-116 ml) (Fig. 2).

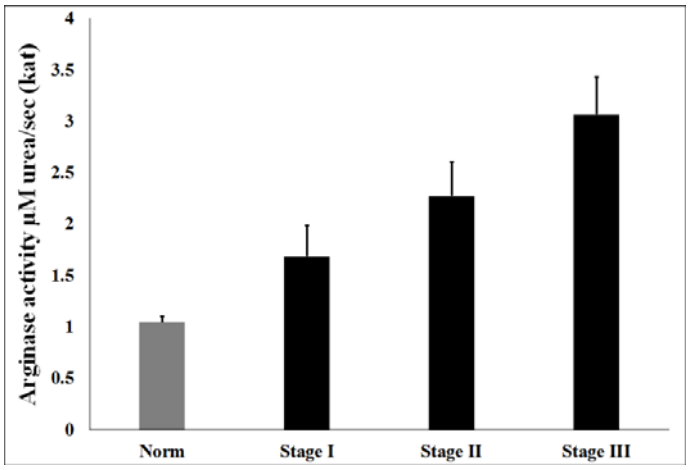

Fig. 1. The change of arginase activity in blood serum during different stage of prostate and bladder cancer (n=patients in table 1, $p<0.001$).

The influence of substrate concentrations, varying from 0.1 to 2 mM, on enzymic activity (fractions after Gel chromatography, 104-116ml, for each cancer group n=7, $p<0.05$) was studied in 0.2 M Glycine buffer (pH 9.5), 0.2 ml 5μM $MnCl_2$. From this data a Lineweaver-Burk plot with a *Km* value of 2 mM was calculated for human serum arginase with prostate and bladder cancers, which is near by characteristicfor the liver and erythrocytes type isoform of arginase (arginase I).

To confirm this fact, Ion-Exchange chromatography (IEC) of the second peak fraction (low-molecular fraction, *Ve* = 116 (A) and 104 (B) ml) was performed. In Ion-Exchange chromatography arginase activity curve is presented with 1 cationic isoenzyme peak (*Ve* = 92 ml), (see Fig. 2, Cand D). For prostate and bladder cancers, it was revealed that the only isoenzyme expressed in serum was cationic form (arginase I) (see Fig. 2).

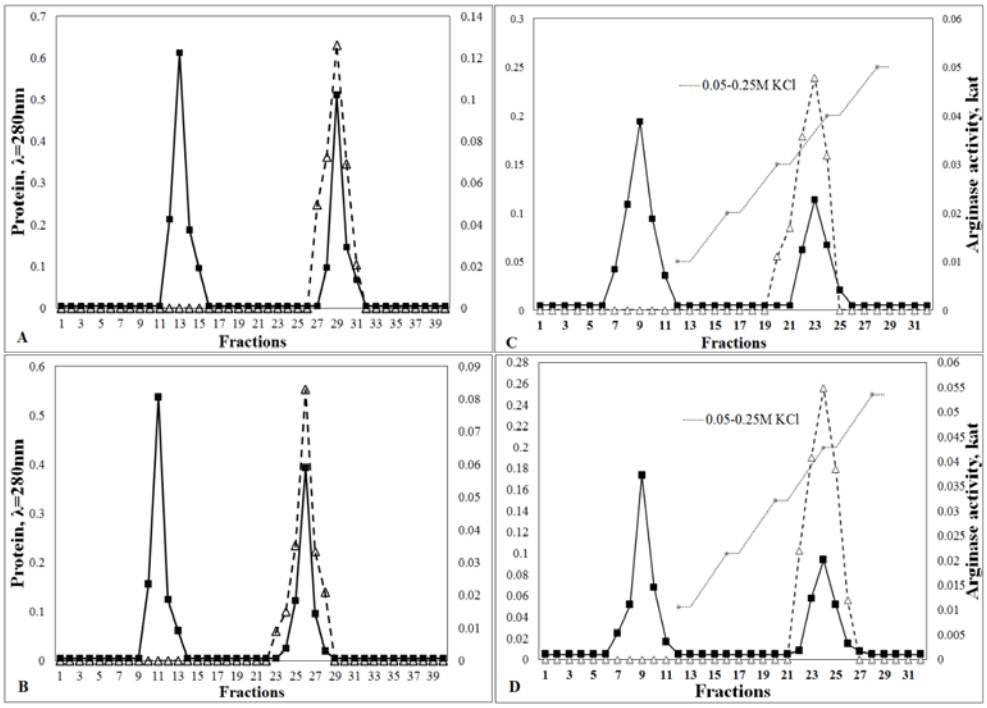

Fig. 2. Arginase activity (Δ) and protein quantity (■) curves after Gel (A, B) and Ion-Exchange (C, D) chromatography of serum proteins prostate (A, C) and bladder (B, D) cancers patients (n=7, $p<0.05$) (stage II, For Gel chromatography the protein obtained after several steps of purifications (see materials and methods) was applied to a Sephadex G-150 column (2 x 40 cm) and eluted with Na-phosphate buffer (pH 7.2). The enzyme obtained after Gel chromatography (*Ve* = 116ml) to a CM-cellulose column (1 x 18 cm) equilibrated with 5 mM Tris-HCI, pH 7.5, eluted with a KCl concentration gradient.

Total polyamines quantity compared with standard is increased by 42.5%, 54.5% and 76.8%, respectively in I, II and III stages of prostate cancer, and 59.5%, 64.8% and 87.9%, respectively in I, II and III stages of bladder cancer (Fig. 3 and Table 2). The increase of polyamines quantity coincides with the increase of arginase activity, what shows the correlation between them during the disease.

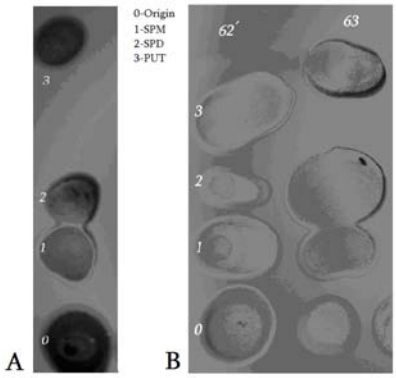

Fig. 3. Quantitative and qualitative analysis of blood serum polyamines of a healthy (A), prostate and bladder (B) cancer patients (A, R_fSPM – 0.29, R_fSPD – 0.45, R_fPUT – 0.82); B: 62'- bladder (stag II), 63-prostate (stage II),62'- R_fSPM-0.32, R_fSPD-0.48, R_fPUT-0.68; 63-R_fSPM-0.33, R_fSPD-0.45, R_fPUT-0.76 (n=7, $p<0,05$, PUT – putrescine, SPD – spermidine, SPM - spermine).

Table 2.The change of polyamines quantity in blood serum during different stage of prostate and bladder cancers (n=10, p<0.001).

Stage of cancer	Polyamine	nM Polyamine/ml serum	
		Bladder	Prostate
Norm	PUT	10.2±0.23	
	SPD	8.6±0.14	
	SPM	15.3±1.4	
I	PUT	17.4±1.1	15.7±1.5
	SPD	18.4±1.6	15.6±0.9
	SPM	18.6±1.8	17.3±0.8
II	PUT	17.9±1.3	16.4±1.3
	SPD	18.2±1.1	17.7±1.1
	SPM	20.1±1.9	18.6±1.3
III	PUT	18.4±1.1	17.3±1.1
	SPD	21.9±1.9	20.2±1.9
	SPM	23.8±1.2	22.8±1.2

Our results have shown, that the quantity of nitrite anions was increased by 125% in stage II prostate and bladder cancers patients blood serum (Fig. 4).It indicates the acceleration of metabolism as a result of disease, because a large amount of a building material is needed, and polyamines and NO are essential factor for tumor growth.

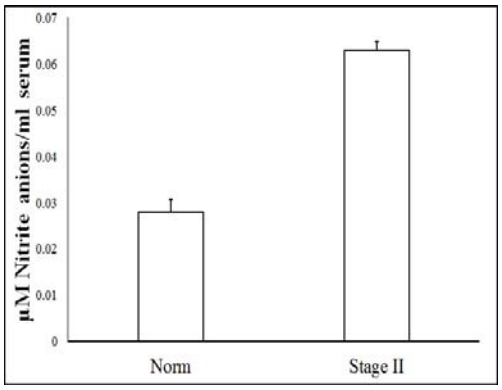

Fig. 4. The change of nitrite anions quantity in blood serum of prostate and bladder cancer patients of stage II (n=7 for norm, n=11 for cancer of stage II, p<0.05).

Analyzed results show that arginase activity, polyamines and NO quantity are significantly increased during prostate and bladder malignant cancers and that this increase is in correlation with the cancer stages. So, we can draw a conclusion that arginase and NO-synthase (NOS) are the important partakers of cancerogenesis and can be considered as an important diagnostic and treatment tools for cancer development. The practical importance of our work is through the change of arginaseand NOS activity, we can influence on polyamines and NO quantity, thus influencing on cancer cell's metabolism.

The feature of the arginase and NOS family that makes them attractive as therapeutic targets is that there are a host of small molecules that inhibit these enzymes [7, 10]. Since

modern medicine has no effective cure for the malignant cancers and tumors, scientists are interested in finding a potent agent with non-cytotoxic properties. We suggest that arginase and NOS inhibition may have some antitumor effects on the cancer development as it inhibits polyamines and NO levels, precursor of cancercell proliferation, tumor angiogenesis and metastasis. Our results can be as a base to use this model for the study of other inhibitors of arginase and NOS for the determination of productive and non-cytotoxic antitumor concentration. The inhibitors of arginase and NOS will allow changing the course of cancer development. Taking into account the above mentioned, in our next project we will research the anti-tumor potential of the arginase and NOS activity inhibition by nor-NOHA (N^{ω}-hydroxy-nor-Arginine), L-NAME (L-N^{G}-Nitroarginine methyl ester) and mixed, administered for 5 weeks against 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis in rats.

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