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The effects of NETosis on fibrinolysis in colon cancer patients

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ABSTRACT

Aim. To investigate formation of neutrophil extracellular traps (NETs) and their impact on fibrinolysis in patients with colon cancer.

Materials and methods. The study was performed in two groups. The experimental group consisted of patients with stage 2–3 non-metastatic colon cancer (n = 17, average age – 67 years). The control group included healthy volunteers matched by sex and age (n = 30, average age – 68 years). An experimental model was created from the whole blood. It included platelet-poor plasma and an isolated culture of neutrophils, previously induced to NETosis by adding 100 nmol PMA. The samples were incubated for 4 hours, then the test tubes were centrifuged to pellet cells and their remnants, and the plasma was transferred for subsequent examination. The plasma incubated with intact neutrophils was used as a control. The levels of interleukin-8 (IL-8) and P-selectin glycoprotein ligand-1 (PSGL-1) were used to determine the degree of cell activation. NETosis was confirmed by enzyme-linked immunosorbent assay (ELISA) and fluorescent microscopy. Fibrinolysis was assessed using the thrombodynamics test. The results were compared with the levels of fibrinolytic system components measured by flow cytometry.

Results. In the control group, NETosis induction contributed to pronounced neutrophil activation that was accompanied by an increase in the IL-8, PSGL-1, and plasminogen levels, a decrease in PAI-1, and enhancement of fibrinolysis, compared with the intact samples. Higher levels of IL-8, PSGL-1, plasminogen, and PAI-1 and intensified fibrinolysis were detected in the intact samples. However, PMA-induced NETosis did not result in an increase in the degree of activation and significant changes in the given parameters.

Conclusion. NETosis promotes both formation and lysis of fibrin clots. However, in cancer patients, suicidal NETosis does not contribute to fibrinolysis due to intracellular protease depletion, which may be one of the mechanisms causing hypercoagulation and insufficient fibrinolysis in cancer.

Key words: cancer, neutrophil extracellular traps, fibrinolysis, immunothrombosis, NETosis, hypercoagulation.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by Chita State Medical Academy (Protocol No. 86 of 01.11.2017).

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Влияние нетоза на лизис фибринового сгустка при раке толстого кишечника

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РЕЗЮМЕ

Цель – изучение особенностей формирования нейтрофильных внеклеточных ловушек, а также их влияния на фибринолиз у лиц, имеющих злокачественные новообразования толстого кишечника.

Материалы и методы. Группу пациентов составили лица с впервые выявленным раком толстого кишечника 2–3-й стадии без метастаза (17 человек, средний возраст 67 лет); контрольную группу – доноры, сопоставимые по полу и возрасту, не имеющие злокачественных опухолей (30 человек, средний возраст 68 лет). Из цельной крови создали экспериментальную модель, включавшую бедную тромбоцитами плазму крови и изолированную культуру нейтрофилов, предварительно индуцированных к нетозу внесением 100 нмоль PMA, инкубировали 4 ч, клетки осаждали центрифугированием, плазму отбирали для дальнейшего исследования. В качестве контроля использовали плазму, инкубированную с интактными нейтрофилами. О степени активации клеток судили по уровню интерлейкина (IL) 8 и PSGL-1. Нетоз подтверждали измерением уровня нуклеосом и флуоресцентной микроскопией. Оценку фибринолиза проводили в тесте тромбодинамики. Результаты сопоставляли с концентрацией компонентов фибринолитической системы, измеренных методом проточной цитометрии.

Результаты. В контроле индукция нетоза вызывает выраженную активацию нейтрофилов, сопровождающуюся повышением уровня IL-8, PSGL-1, плазминогена, снижением PAI-1 и усилением фибринолиза, в сравнении с интактными образцами. У пациентов зафиксирован больший, чем в группе контроля, уровень IL-8, PSGL-1, плазминогена, PAI-1 и показателей фибринолиза в интактных образцах. При этом индукция нетоза не привела к увеличению степени активации и значимому изменению данных показателей.

Заключение. Гибель нейтрофилов путем нетоза в местах тромбообразования может способствовать как формированию, так и растворению фибринового сгустка. Однако у лиц со злокачественными новообразованиями «смертельный» нетоз не приводит к локальному увеличению фибринолитического потенциала ввиду истощения внутриклеточных резервов протеаз нейтрофилов, что может являться одним из механизмов развития гиперкоагуляции и недостаточности фибринолиза при онкопатологии.

Ключевые слова: рак, нейтрофильные внеклеточные ловушки, фибринолиз, иммунотромбоз, нетоз, гиперкоагуляция.

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INTRODUCTION

The hypercoagulable state and thrombotic events are known to accompany malignancy. Thrombophilia in cancer is based on synthesis and release of procoagulants by tumor cells, as well as a complex of reactions, including activation of and (or) damage to the endothelium, activation of platelets and immune cells, and synthesis of cytokines. Neutrophils, being effectors of innate immunity, are essentially involved in inflammatory processes and hemostatic responses. Indeed, neutrophils and NETosis products – neutrophil

extracellular traps (NETs) – are detected in thrombi of various localizations [1]. However, in numerous studies on the prothrombotic properties of NETs [2–7], the possible role of NETs in fibrinolysis, especially in cancer, remains understudied [8, 9].

MATERIALS AND METHODS

The study was carried out in two groups. The experimental group consisted of patients with newly diagnosed, stage 2–3 non-metastatic colon cancer (n = 17, average age – 67 years). The control group included healthy volunteers matched by sex and age (n = 30, average age – 68 years). Preliminary selection of donors was carried out on the basis of a questionnaire. Then, the absence of cancer in the control group was estimated by the results of a clinical examination. Measurement of body temperature and complete blood count were performed in each donor. Donor exclusion criteria were the following: 1) body temperature > 37 °C; hemoglobin (HGB) < 100 g/l, red blood cells (RBC) < 3.5×10^{12}/l, white blood cells (WBC) > 10×10^{9}/l, erythrocyte sedimentation rate (ESR) > 12 mm per hour; 2) intake of anticoagulants / platelet antiaggregants, chemotherapy; 3) acute inflammation or exacerbation of chronic inflammation, inherited blood disorders, diabetes mellitus, traumas or surgeries in the last 6 months.

All patients and healthy donors signed an informed consent to participate in the study. 25 ml of peripheral venous blood was collected in tubes with 3.2% sodium citrate solution as an anticoagulant once. To obtain platelet-poor plasma (PPP), whole venous blood was centrifuged for 15 min at 1,500 rpm, and then the plasma was taken for repeated centrifugation at 3,000 rpm for 10 min. 90% of prepared PPP was transferred into separate tubes.

To isolate neutrophilic granulocytes (NG), Ficoll – Urographin density gradient centrifugation was performed (1.077 / 1.093). The granulocyte layer was transferred into separate tubes, washed with phosphate-buffered saline (PBS), and then centrifuged to pellet cells. The remained red blood cells were lysed (VersaLyse, Beckman Coulter, USA), isolated neutrophils were washed again with PBS, and the supernatant was discarded. Then, 4 ml of RPMI1640 was added to the cell pellet and mixed, and a suspension of isolated neutrophils was obtained. Cell viability was assessed by staining with a 0.4% methylene blue solution; the culture with the viability of at least 98% was used. The number of cells was adjusted to $4.5–6 \times 10^{6}$/ml.

Then 1 ml of the suspension was added into two separate test tubes and centrifuged to pellet cells. The culture medium was removed. After that, 5µl of PBS and then 1 ml of PPP were added to the cell pellet in the first test tube, mixed, and, thus, a suspension of intact neutrophils was prepared. 5µl (100 nm) of phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich, USA) was added to the cell pellet in the second test tube to induce NETosis, then 1 ml of PPP was added, mixed, and, thus, a suspension of neutrophils stimulated for NETosis was obtained. The obtained samples were labeled in the following way: PPP – platelet-poor plasma; INT – plasma containing intact neutrophils; PMA – plasma containing PMA-stimulated neutrophils.

All samples were incubated for 4 hours at 37 °C. Then the INT and PMA samples were centrifuged to pellet cells and their remnants, and the plasma was transferred into separate test tubes for further research. Each plasma sample was assessed using the thrombodynamics test (HemaCor, Russia): lysis onset time (LOT; min), lysis progression (LP; %/min), and lysis time estimation (LTE; min) were recorded. Fibrinogen (Fib), plasminogen / plasmin system (PLS), tissue plasminogen activator (tPA), plasminogen activator ingibitor-1 (PAI-1), P-selectin glycoprotein ligand-1 (PSGL-1), and IL-8 in each plasma sample were determined using multiplex assay kits (Human Thrombosis Panel, BioLegend, USA; Human Fibrinolysis Panel, BioLegend, USA) by flow cytometry. NETosis induction was estimated by the level of extracellular DNA (ecDNA), measured by ELISA (Cell Death Detection Kit ELISA^{plus}, Roche, Germany). Additionally, NETs were visualized using fluorescence microscopy.

For this purpose, 1 ml of prepared INT and PMA samples were placed into the Poly-L-Lysine-coated (Sigma Aldrich, USA) cell culture plates and incubated at 37 °C for 4 hours. After that, the plates were washed with PBS three times, the adherent cells were stained with SYTOX Green (Beckman Coulter, USA), and NETs were visualized using the ZOE Fluorescent Cell Imager (BioRad, USA), green channel, objective ×20.

Statistical processing of the results was performed using Microsoft Excel and Statistica 10 (StatSoft Inc., USA) software. The data obtained are presented as the median and the interquartile range Me [Q_1; Q_3]. The Wilcoxon signed-rank test was used to compare the results within the groups, and the Mann – Whitney U-test was used for comparison between the experimental and control groups. The differences were considered significant at $p^* < 0.05$.

RESULTS

NETosis in the control and experimental groups was confirmed by an increase in the level of ecDNA in the PMA samples compared with the INT samples ($p_1 = 0.008$ and $p_2 = 0.05$, Table). Additionally, microscopy of NG cultures made it possible to visualize NETs. The figure demonstrates intact neutrophils and NETs in the control group (*a* and *b*, respectively) and in cancer patients (*c* and *d*, respectively). Neutrophil-specific IL-8 and free PSGL-1 were considered as activation markers. The levels of IL-8 and PSGL-1 in the control group were the highest in the PMA samples; however, no significant increase in the indicated molecules in the corresponding plasma samples was detected in the experimental group (Table). The effect of NETs on fibrinolysis was evaluated in the control group: increased LP and reduced LTE were registered (Table). In the experimental group, no significant changes in fibrinolysis parameters were recorded.

Changes in the level of NETosis-associated fibrinolytic system components were detected in the control group: a significant increase in plasminogen and a decrease in PAI-1 were found in the PMA samples compared with the intact ones. In the experimental group, changes in the levels of fibrinolytic system components were not significant (Table).

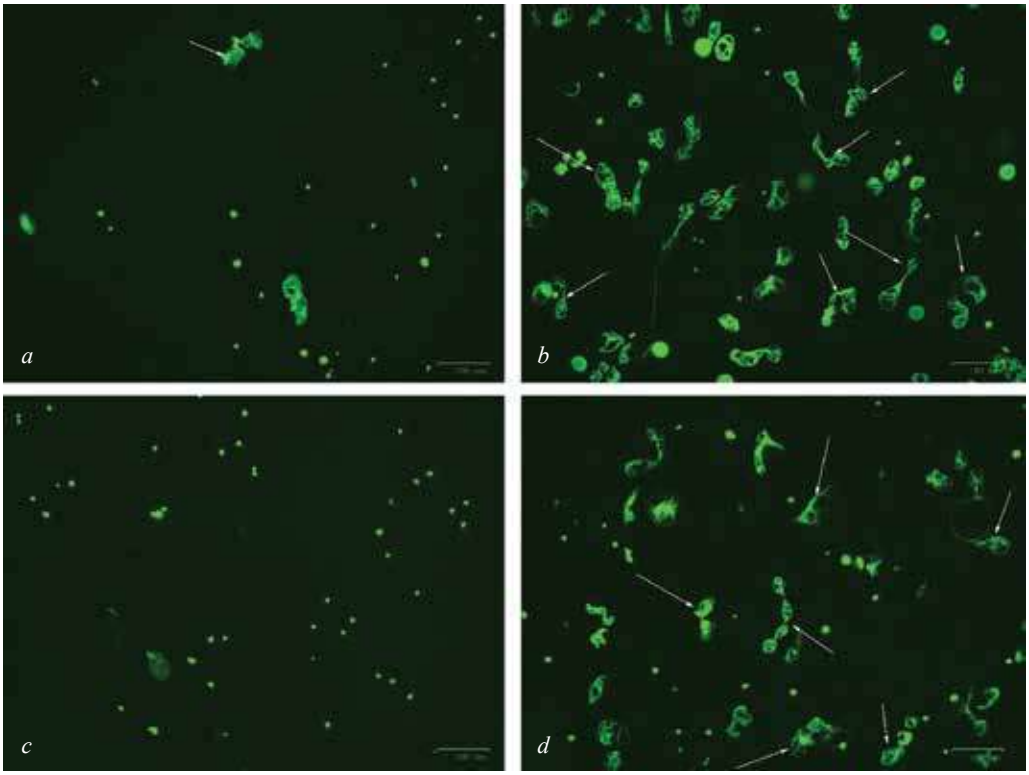

Figure. Intact neutrophils: in the control group (*a*), in the experimental group (*c*); neutrophil extracellular traps: in the control group (*b*); in the experimental group (*d*). SYTOX Green staining, objective ×20. White arrows mark neutrophil extracellular traps

Table

Parameters of cell activation and the effect of intact and PMA-stimulated neutrophils on fibrinolysis and the level of fibrinolytic system components, *Me* [Q_1; Q_3]

Parameter	Control group			*p*	Experimental group	
	Value		p_1		Value	p_2
ecDNA, OD	INT	0.38 [0.22; 0.48]	0.008*	0.1	0.26 [0.18; 0.33]	0.01*
	PMA	0.5 [0.4; 0.6]		0.03*	0.34 [0.26; 0.41]	
PSGL-1, pg / ml	INT	865 [620.5; 1018.7]	0.01*	0.3	781.25 [431.8; 926.5]	0.2
	PMA	1236.7 [1071.2; 1340.5]		0.05*	791.5 [538.8; 1254.3]	
IL-8, pg / ml	INT	2422 [880.8; 4852.5]	0.007*	0.7	3150.7 [2007; 5470.8]	0.6
	PMA	7836.2 [5422.5; 10786.]		0.07	1677 [1006.8; 7777]	
LOT, min	INT	30.2 [28.3; 40.7]	0.7	0.02*	24.8 [16.7; 28.6]	0.5
	PMA	29 [28.1; 36.7]		0.01*	24.1 [19; 25.5]	

Table (continued)

Parameter	Control group			p	Experimental group	
	Value		p_1		Value	p_2
LP, %/min	INT	2.7 [2.2; 2.9]	0.01*	0.0003*	8.8 [5.1; 12.4]	0.4
	PMA	3 [2.9; 3.8]		0.001*	9.2 [4.7; 17.1]	
LTE, min	INT	40.6 [36.8; 50.6]	0.02*	0.002*	16.9 [12.6; 22.4]	0.8
	PMA	35.2 [27.8; 37.1]		0.001*	16 [12.4; 24.8]	
PLS, mcg/ml	INT	547.1 [475; 732.5]	0.008*	0.007*	805.1 [783.7; 825.6]	0.9
	PMA	767.7 [662; 864.1]		0.7	820 [787.9; 845]	
tPA, pg/ml	INT	63.5 [61.6; 64.7]	0.4	0.0001*	440.5 [227.5; 607.75]	0.5
	PMA	61.3 [59.7; 65.7]		0.0001*	389.7 [247; 672]	
AI-1, pg/ml	INT	2101.5 [508; 2681.3]	0.008*	0.001*	6376.2 [5258.5; 9428.8]	0.6
	PMA	1328.2 [1044.4; 2157.8]		0.0002*	7956.7 [5332.5; 10645.6]	
Fib, mcg/ml	INT	2882.7 [1877.3; 3142.6]	0.9	0.04*	3203.4 [2749.6; 6229.9]	0.6
	PMA	2355.8 [1745; 3206.6]		0.08	3384.7 [2723.7; 8997]	

Note: INT – plasma sample after incubation of intact neutrophils; PMA – plasma sample after incubation of PMA-stimulated neutrophils; ecDNA – extracellular DNA, OD – optical density; p – level of statistical significance between the control and experimental groups (Mann – Whitney U-test); p_1 – level of statistical significance between the INT and PMA samples in the control group (Wilcoxon signed-rank test); p_2 – level of statistical significance between the INT and PMA samples in the experimental group (Wilcoxon signed-rank test); * – statistically significant difference (at $p < 0.05$).

DISCUSSION

The results obtained demonstrate different effect of NETosis induction in the investigated groups. Lower ecDNA level in the plasma samples in the experimental group indicates that less neutrophils underwent NETosis in the presented experimental model (Table). Presumably, it could be caused by alteration of intracellular signaling due to predominance of other signaling pathways. It is known that cancer cells are capable of regulating the activity of leukocytes, contributing to tumor progression [10–14]. Different cytokine microenvironment might be a factor determining NETosis propensity and (or) the prevailing signaling pathway (suicidal or vital NETosis) [15].

PMA induced lytic (suicidal) NETosis accompanied by the release in the plasma of not only chromatin, but also all intracellular proteases, which activate both the plasminogen system and non-specific proteolysis of fibrin. Our previous study [16] demonstrates failure of NETs-associated fibrin plate lysis in the presence of the protease inhibitor aprotinin. Besides a higher number of NETosis-affected neutrophils, a more pronounced effect of NETs formation on fibrinolysis in the control group could be explained by the ability of the crucial NETosis enzyme PAD4 to modify the fibrinogen structure. It results in impaired fibrin formation and changes in its structure [17, 18]. In this study, altered fibrin structure could be one of the factors underlying fibrinolysis boost in the PMA samples in the control group (Table).

No increase in fibrinolysis in cancer patients could be associated with cell activation failure (low ecDNA and PSGL-1), higher concentration and activity of PAI-1, and prevalence of procoagulant factors in malignancy. Additionally, in cancer, initially high activity of intact neutrophils suggests lower intracellular protease level due to their fast and persistent release into the extracellular space, which, therefore, causes their insufficient increase in suicidal NETosis (that also concerns IL-8). Considering literature and our own data, it could be assumed that NETs formed following suicidal or vital NETosis have different effects on clotting and fibrinolysis due to the differences in the amount of released chromatin and the final composition of extracellular traps, which depends on NETosis inductor signal [15]. Hence, it is more relevant to *in vivo* studies. However, that should become the objective of further investigations.

CONCLUSION

NETs formation can provide not only a thrombogenic effect, implemented due to interactions of nucleosomes with platelets, endothelium, and coagulation factors, but also a fibrinolytic effect, based on the plasminogen activation system and non-specific lysis of insoluble fibrin by neutrophil granule proteases, which, along with thrombi phagocytosis by other leukocyte types, provides final recanalization of blood vessels. However, cancer disrupts the coagulation / fibrinolytic balance. It contributes to thrombophilia by the development of a local and systemic inflammatory response, alteration of the NETs formation pathway

and, therefore, prevalence of prothrombotic effects of ecDNA over the fibrinolytic activity of NG. When combined with an increased concentration of fibrinolysis inhibitors, it eventually contributes to the development of a chronic hypercoagulable state typical of cancer.

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Authors contribution

Parshina A.A. – conception and design, carrying out of the experiment, analysis and interpretation of data, drafting of the manuscript. Tsybikov N.N. – conception, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Tereshkov P.P. – carrying out of the experiment, critical revision of the manuscript for important intellectual content. Karavaeva T.M. – analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Maksimenya M.V. – critical revision of the manuscript for important intellectual content.

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