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DNA-Containing Extracellular Structures of Tumor Cells Inhibit the Formation of Neutrophil Extracellular Traps in Vitro

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ABSTRACT

Aim. To study the parameters of formed DNA-containing extracellular structures during co-cultivation of neutrophils from healthy donors, HCT116 adenocarcinoma cells and K562 myeloblastoma.

Materials and methods. Erythrocyte sedimentation in EDTA-treated blood was carried out using Dextran 500. The neutrophil-enriched layer of blood plasma was collected. The admixture of mononuclear cells was less than 1%. Platelets were removed using differential centrifugation. Isolated neutrophils in RPMI-1640 medium were used in short-term culture experiments. HCT116 adenocarcinoma and K562 myeloblastoma cells were obtained from the American Type Culture Collection. In the experiments, donor neutrophils and tumor cells were co-cultivated for 3 hours. SYBR Green fluorescence microscopy was used to visualize the DNA-containing extracellular structures formation by cells cultured in RPMI-1640 medium.

Results. Neutrophils recognize tumor cells and respond to contact interactions, forming neutrophil extracellular traps in the form of neutrophil networks. Contacts with HCT116 adenocarcinoma cells cause rapid formation of neutrophil web-like structures – within 1 hour. The opening of neutrophil web-like structures induced by contacts with K562 myeloblastoma cells requires a longer incubation (2 hours). HCT116 cells form large bundles of DNA-containing fibers, which completely inhibit the formation of neutrophil networks. K562 cells suppress neutrophil defense responses by reducing the number and size of neutrophil networks. The effect of inhibition of neutrophil networks by K562 cells is probably due to the action of a soluble factor that suppresses neutrophil functions described earlier.

Conclusion. The study shows that both tumor cell lines are capable of suppressing innate immune cell responses through different mechanisms. Adenocarcinoma cells inhibit neutrophil network formation upon direct contact due to the large size DNA-containing fibers they produce. Myeloblastoma cells produce the same effect, probably acting by secreting humoral factors.

Keywords: HCT116, K562, NETs, DNA-containing fibers, oncology, pathophysiology

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ДНК-содержащие экстраклеточные структуры опухолевых клеток подавляют формирование нейтрофильных экстраклеточных ловушек *in vitro*

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РЕЗЮМЕ

Цель. Исследование параметров формируемых ДНК-содержащих экстраклеточных структур при совместном культивировании нейтрофилов здоровых доноров, клеток аденокарциномы HCT116 и миелобластомы K562.

Материалы и методы. Осаждение эритроцитов в крови, обработанной ЭДТА, проводили, используя декстран 500. Слой плазмы крови, обогащенный нейтрофилами, отбирали. Примеси мононуклеарных клеток составляли менее 1%. С помощью дифференциального центрифугирования освобождались от тромбоцитов. Выделенные нейтрофилы в среде RPMI-1640 использовали в экспериментах по кратковременному культивированию. Клетки аденокарциномы HCT116 и миелобластомы K562 были получены из American Type Culture Collection. В экспериментах проводили совместное культивирование нейтрофилов доноров и опухолевых клеток в течение 3 ч. Для визуализации формируемых ДНК-содержащих внеклеточных структур клетками, культивированными на среде RPMI-1640, использовали флуоресцентную микроскопию с красителем SYBR Green.

Результаты. Нейтрофилы распознают опухолевые клетки и реагируют на контактные взаимодействия, формируя нейтрофильные экстраклеточные ловушки в форме нейтрофильных сетей. Контакты с клетками аденокарциномы HCT116 вызывают быстрое формирование нейтрофильных сетей – в течение 1 ч. Раскрытие нейтрофильных сетей, индуцированное контактами с клетками миелобластомы K562, требует более продолжительной инкубации – в течение 2 ч. Клетки HCT116 формируют пучки ДНК-содержащих волокон значительного размера, которые полностью ингибируют формирование нейтрофильных сетей. Клетки K562 подавляют нейтрофильные защитные реакции, уменьшая количество и размеры нейтрофильных сетей. Эффект ингибирования нейтрофильных сетей со стороны клеток K562 обусловлен, вероятно, действием растворимого фактора, подавляющего функции нейтрофилов, описанного ранее.

Заключение. Исследование показывает, что обе клеточные линии опухолевых клеток способны подавлять реакции клеток врожденного иммунитета с помощью различных механизмов. Клетки аденокарциномы ингибируют формирование нейтрофильных сетей при непосредственных контактах за счет продуцируемых ДНК-содержащих волокон значительного размера. Клетки миелобластомы вызывают тот же эффект действуя, вероятно, путем секреции гуморальных факторов.

Ключевые слова: HCT116, K562, НЭЛ, ДНК-содержащие волокна, онкология, патофизиология

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INTRODUCTION

One of the basic positions of the modern oncological and immunological concept describing the interaction of tumor cells and neutrophils is that neutrophils forming neutrophil extracellular traps (NETs) are capable of causing tumor recurrence and metastasis [1, 2]. It is suggested that NETs can capture circulating cancer cells and promote their spread. In addition, it has been reported that NETs awaken dormant cancer cells, triggering tumor recurrence and metastasis. Therefore, it is quite natural that researchers propose to inhibit the formation of neutrophil extracellular traps to prevent tumor growth [3].

They also see the reason for the activation of tumor cell growth in the adverse effect of tumor-associated fibroblasts, which are able to support carcinogenesis. The authors acknowledge that the lack of knowledge about the tumor microenvironment (TME) is an obstacle to the introduction of innovative methods of treating tumor diseases.

Many researchers note that both neutrophil extracellular traps and tumor-associated fibroblasts as cellular factors of the tumor microenvironment have not been sufficiently studied [4, 5]. The complex composition of the components of the tumor microenvironment (humoral and cellular components) creates an environment necessary for the growth, proliferation, phenotypic flexibility and variability of tumor cells, which is at the same time rigid and immunosuppressive for the body, with a deficiency of nutrients [6]. NETs may be involved in the process of carcinogenesis and cancer progression. However, it is still difficult to decide whether netosis plays a pro- or antitumor role [7]. At the same time, some researchers have recently noted the dual nature of the effects of NETs regarding antitumor therapy, studying their potential to either neutralize or even improve treatment results [8]. It is also believed that NETs play a key role in the formation of a positive response to chemotherapy and have significant potential to increase the effectiveness of treatment [9].

It is possible to clarify the role of neutrophils and neutrophil extracellular traps formed by them in carcinogenesis only with direct studies of the interaction of tumor cells and cells of the innate immune system. It should be noted that such works are rare, and this circumstance determines the relevance of this study.

Aim. To study the parameters of the formed DNA-containing extracellular structures during the co-cultivation of neutrophils from healthy donors, adenocarcinoma cells HST116 and myeloblastoma K562.

MATERIALS AND METHODS

Obtaining Neutrophil Cell Fractions from Healthy Donors, as well as Adenocarcinoma Cells and Myeloblastoma K562 Cells

The isolation of neutrophils, in order to study the cellular reactions developing in the patient's body, requires the exclusion of exposure to chemical or mechanical stimuli on these cells *in vitro*, therefore, standard methods of isolation in the ficoll density gradient are of little use.

Neutrophils were isolated from the venous blood of volunteers treated with EDTA using Dextran 500. To do this, 1 ml of a 10% Dextran 500 solution (Fluka) prepared in a sodium phosphate buffer solution (50 mM, pH 7.4) was added to 10 ml of peripheral blood and gently mixed. After precipitation of erythrocytes for 30 minutes at +37°C, a layer of blood plasma (200 µl) was taken, closely adjacent (at a distance of 1 mm) to the layer of erythrocytes.

The blood plasma from this layer contains only neutrophils, platelets and a small number of red blood cells. The impurities of mononuclear cells are less than 1%. To get rid of platelets, 10 ml of 50 mM sodium phosphate buffer solution, pH 7.4, was added to 200 µl of blood plasma containing neutrophils and precipitated by centrifugation at 1,200 rpm (400 g), 15 min. The supernatant fluid was removed and the precipitate was resuspended in 1 ml of RPMI-1640 medium. Isolated neutrophils in RPMI-1640 medium were used in short-term cultivation experiments. The viability of the isolated neutrophils was at least 95% (test with 0.1% trypan blue solution).

The human colon adenocarcinoma HCT116 cell line was obtained from the American Type Culture Collection (Manassas, Virginia). Myeloblastoma cells K562 were obtained from the Laboratory of Mechanisms of tumor Cell Death of Blokhin National Medical Research Center of Oncology.

Cell Culture

All the studied cells in RPMI-1640 medium were incubated in an atmosphere of 5% CO_2 at 37°C in all experiments for 3 hours. The final concentration of cells in the culture medium was 2×10^5 cells/ml.

Lipopolysaccharides (LPS) (*Klebsiella pneumoniae*, Sigma, Japan), 25 mcg/ml, were added to some of the samples to activate neutrophils from

healthy donors and acquire the ability to form neutrophil extracellular traps. This technique makes it possible to obtain NETs in a characteristic morphological form – neutrophil networks. Of the four main morphological structures of neutrophil extracellular traps (web-like structures, single filaments, fibers and veils) [10], only the web-like structures have functional activity [11].

Immunofluorescence Staining of Cells Forming Extracellular DNA-Containing Structures

Fluorescence microscopy was used to visualize and determine the parameters (number and size) of extracellular DNA-containing structures of the studied cells (neutrophils of healthy donors, adenocarcinoma cells HST116 and myeloblastoma K562) [12]. The results were expressed as a percentage, as the ratio of the number of DNA-containing extracellular structures to the total number of cells in the field of view. An eyepiece micrometer was used to determine the size of DNA-containing extracellular structures. Extracellular DNA-containing structures were detected using the fluorescent dye SYBR Green (Evrogen; Russia), which specifically interacts with double-stranded DNA. Microscopy, counting and photo registration of cells and extracellular structures were performed at a ×1000 magnification.

The results were processed using Statistica 12.0 software (StatSoft Inc., USA). The data are presented as a mean and the standard error of the mean ($M \pm m$). Quantitative characteristics were compared based on the results of the Student's t-test and analysis of variance. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Observations of the behavior of neutrophil cells from healthy donors, adenocarcinoma HST116 and myeloblastoma K562 cells during their short-term co-cultivation in various combinations for 3 hours demonstrate the active formation of DNA-containing extracellular structures by these cells. The determination of the parameters (number and size) of these DNA-containing extracellular structures makes it possible to identify characteristic dynamic changes in the extracellular structures formed during the entire observation period.

Cells were cultures in the following sample variants: 1) intact neutrophils of healthy donors; 2) neutrophils of donors with the addition of LPS; 3) neutrophils of donors and adenocarcinoma cells HCT116; 4) neutrophils of donors with the addition of LPS and adenocarcinoma cells HCT116; 5) neutrophils of donors and myeloblastoma cells K562; 5) neutrophils of donors with the addition of LPS and myeloblastoma cells K562.

The results of co-cultivation in different combinations for 1 hour (Table 1) indicate that neutrophils of healthy donors quickly respond to contact interactions with adenocarcinoma cells and form neutrophilic networks. However, the reaction of neutrophils to contacts with cells of myeloblastoma K562 in the form of the formation of neutrophilic networks during 1 hour of cultivation is practically absent. A characteristic feature of the studied tumor cells (HCT116 and K562) is the formation of DNA-containing extracellular structures of a considerable size. HCT116 cells form single filaments with a length of 54.92±6.82 microns, and K562 cells form 99.00±8.41 microns. The revealed differences between HCT116 and K562 cells in their contacts with neutrophils from healthy donors consist in the fact that the reaction of neutrophils and K562 cells is clearly slowed down. During 1 hour of co-cultivation of K562 cells with neutrophils, the formation of neutrophil networks does not occur, this reaction manifests itself later.

Cultivation of HCT116 cells with neutrophils in the presence of LPS does not significantly change the parameters of cellular reactions (Table 1). Neither the number of DNA-containing extracellular structures (8.87 ± 1.20% and 7.41 ± 0.39%, respectively) nor their sizes (54.92 ± 6.82 μm and 53.92 ± 6.06 μm, respectively) change.

Cultivation of K562 cells with neutrophils in the presence of LPS shows a sharp increase in the number of DNA-containing extracellular structures (Table 1). The presence of LPS in samples with K562 cells and neutrophils causes an increase in the number of neutrophil networks (from 2.97 ± 0.32% to 10.70 ± 1.81%), i.e. almost 3 times. At the same time, the sizes of DNA-containing single strands from K562 cells are significantly (twofold) reduced (from 99.00 ± 8.41 microns to 57.00 ± 9.34 microns). The decrease in the size of single strands from K562 cells is probably due to spontaneous enzymatic degradation of DNA under the action of DNAases localized on chromatin itself, which can be activated during chromatin despiralization.

After 2 hours of cultivation (Table 2) the number of cells producing DNA-containing structures during the co-cultivation of neutrophils and HCT116 cells is 6.47 ± 0.46%.

Table 1

Parameters of Extracellular Structures during Co-Cultivation in Various Combinations of Neutrophils from Healthy Donors, Adenocarcinoma Cells HCT116 and Myeloblastoma K562 for 1 hour, *M ± m*

Cells studied	Number of extracellular structures, %	Dimensions of extracellular structures, μm	Description of emerging extracellular structures
Neutrophils (N)	0.00	0.00	Do not form extracellular structures, retain a segmented structure
N + HCT116	8.87 ± 1.20	54.92 ± 6.82	Neutrophils form networks, and HCT116 adenocarcinoma cells form single filaments
N + LPS	4.69 ± 0.29	32.25 ± 2.60	Neutrophils form networks
N + LPS + HCT116	7.41 ± 0.39	53.92 ± 6.06	Combination of neutrophil networks with single fibers of adenocarcinoma cells
N + K562	2.97 ± 0.32	99.00 ± 8.41	There are no neutrophil networks. Only single filaments of K562 myeloblastoma cells are observed
N + LPS + K562	10.70±1.81*	57.00 ± 9.34*	Neutrophil networks in combination with single filaments of K562 myeloblastoma cells

* $p < 0.05$ compared to samples without LPS – here and in Tables 2, 3.

Table 2

Parameters of Extracellular Structures during Co-Cultivation in Various Combinations of Neutrophils from Healthy Donors, Adenocarcinoma Cells HCT116 and Myeloblastoma K562 for 2 Hours, *M ± M*

Cells studied	Number of extracellular structures, %	Dimensions of extracellular structures, μm	Description of emerging extracellular structures
Neutrophils (N)	0.00	0.00	Do not form extracellular structures, retain a segmented structure
N + HCT116	6.47 ± 0.46	44.25 ± 2.47	HCT116 adenocarcinoma cells form fiber bundles
N + LPS	23.65 ± 2.35	39.67 ± 2.81	Neutrophils form networks
N + LPS + HCT116	10.75 ± 0.84*	46.67 ± 5.54	Combination of neutrophil networks with bundles of adenocarcinoma cell fibers
N + K562	8.25 ± 0.59	21.58 ± 2.80	Small neutrophil networks
N + LPS + K562	9.64 ± 0.97	18.08 ± 1.08	Small neutrophil networks

Moreover, there is a clear change in the morphology of DNA-containing extracellular structures that form HCT116 cells. Bundles of single fibers are formed, and the number of NETs forming networks decreases sharply (it does not exceed 10% of the total number of DNA-containing extracellular structures). Under the LPS influence, the total number of DNA-containing extracellular structures increases to 10.75±0.84%, due to an increase in structures produced by neutrophils (neutrophil networks).

Co-incubation of neutrophils with HCT116 cells in the presence of LPS leads to the detection of neutrophil networks in the samples together with bundles of DNA-containing fibers of adenocarcinoma cells. The presence of LPS in the samples causes the activation of neutrophils and the formation of NETs. The sizes of fiber bundles produced by HCT116 cells practically do not change with the addition of LPS (44.25 ± 2.47 microns and 46.67 ± 5.54 microns, respectively, without LPS and in the presence of LPS).

Neutrophils cultured with K562 cells for 2 hours form exclusively neutrophil networks (NETs) numbering 8.25 ± 0.59%; in the presence of LPS, their number is 9.64 ± 0.97%, so it practically does not change (Table 2). K562 cells do not form any DNA-containing extracellular structures during this observation period. A characteristic feature of K562 cells is their ability to suppress the activating effect of LPS on neutrophils, which these cells must receive through innate immunity receptors (TLRs). Probably, the K562cells line inhibit the activity of neutrophils through humoral factors. The sizes of neutrophilic networks in the presence of K562 cells are small and amount to 21.58 ± 2.80 μm. In the presence of LPS, even a slight decrease in the size of neutrophilic networks to 18.08 ± 1.08 μm was noted. This circumstance confirms the inhibitory effect of K562 cells on activated neutrophils.

The results of co-cultivation of tumor cells with neutrophils from healthy donors for 3 hours are shown in Table 3.

Table 3

Parameters of Extracellular Structures during Co-Cultivation in Various Combinations of Neutrophils from Healthy Donors, Adenocarcinoma Cells HCT116 and Myeloblastoma K562 for 3 Hours, *M ± M*

Cells studied	Number of extracellular structures, %	Dimensions of extracellular structures, μm	Description of emerging extracellular structures
Neutrophils (N)	0.00	0.00	Do not form extracellular structures, retain a segmented structuree
N + HCT116	20.13 ± 1.71	135.92 ± 12.43	HCT116 adenocarcinoma cells form bundles of fibers. There are no neutrophil networks
N + LPS	21.92 ± 1.41	30.33 ± 3.57	Neutrophils form networks
N + LPS + HCT116	29.46 ± 5.09	> 350	Bundles of adenocarcinoma cell fibers. There are no neutrophil networks
N + K562	8.26 ± 0.32	28.00 ± 2.56	Small neutrophil networks
N + LPS + K562	14.82 ± 1.27*	25.08 ± 2.62	Small neutrophil networks

During this period, the number of cells producing DNA-containing structures during co-cultivation of neutrophils and HCT116 cells is 20.13 ± 1.71%, and these are exclusively DNA-containing extracellular structures that produce adenocarcinoma cells (Fig.). They are represented by bundles of DNA-containing fibers, and neutrophil networks (NETs) are completely absent. The number of DNA-containing extracellular structures in the presence of LPS increases to 29.46 ± 5.09%. Neutrophils of healthy donors cultured for 3 hours together with HCT116 cells practically do not respond to the activating effects of LPS. The addition of LPS to the samples does not cause an increase in the formation of neutrophilic networks. The fiber bundles originating from HCT116 adenocarcinoma cells during this period are long, exceeding 100 μm (135.92 ± 12.43). Under the influence of LPS, the length of fibers from HCT116 cells increases even more (it becomes more than 350 μm), which lies outside the measuring range.

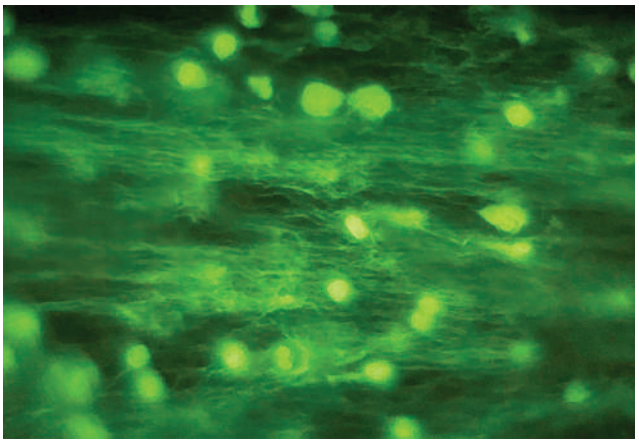

Fig. Adenocarcinoma HCT116 cells spontaneously form bundles of single DNA-containing strands. Neutrophils of a healthy donor present in the preparation do not form neutrophil networks. Incubation time is 3 hours. Staining with CYBR Green. The magnification is x1000.

In studies on experimental animals, data were obtained on the ability of NETs to change the metabolic program of cancer cells and, due to the release of neutrophilic networks, promote the growth of cancer cells [13]. The relationship between the formation of NETs, the frequency of metastasis, and survival rates was also revealed [14]. It should be noted that these results [13, 14] were obtained by indirect methods. These studies did not determine the number of NETs, but only obtained signs of the presence of NETs in the body of experimental animals and patients in the form of soluble factors (neutrophil elastase, myeloperoxidase, citrullinated histone H3). These cited results are in some contradiction with the results of our *in vitro* study. However, it should be noted that the true morphological structure of NETs in cancer patients has not been determined and this issue has not been comprehensively investigated.

Neutrophils cultured with K562 cells form only 8.26 ± 0.32% NETs, and under the influence of LPS their number increases to 14.82 ± 1.27, while filamentous structures from K562 cells are no longer recorded after 3 hours of incubation (Table 3). Possibly single DNA-containing strands, myeloblastomas originating from cells, which we observed during incubation for 1 hour, underwent spontaneous enzymatic degradation and therefore are not detected in later samples. The sizes of neutrophilic networks are small and do not depend on the presence of LPS in the samples. So, without LPS, the sizes of neutrophilic networks are 28.00 ± 2.56 μm, and in the presence of LPS – 25.08 ± 2.62 μm.

The results of the study of co-cultivation of K562 cells with neutrophils from healthy donors for 3 hours indicate a possible inhibitory effect of myeloblastoma cells on the formation of neutrophil networks by neutrophils, which has a protective

nature. Our assumption is confirmed by other data from researchers, where it was determined that K562, a chronic myeloid leukemia cell line, secretes a soluble factor (K562-IF) with a low molecular weight (6–8 kDa), which suppresses the functions of neutrophils during inflammation [15].

CONCLUSION

The results of the study indicate the ability of neutrophils to recognize tumor cells (HCT116 and K562) and respond to interaction with them, forming neutrophil extracellular traps in the morphological form of neutrophil networks. The rate of development of the neutrophil reaction that opens the neutrophil networks varies. Interaction with HCT116 adenocarcinoma cells causes very rapid formation of neutrophil networks – within 1 hour. The opening of neutrophil networks induced by contacts with K562 myeloblastoma cells requires a longer incubation – for 2 hours. The cultivation of tumor cells (HCT116 and K562) for 3 hours demonstrates the ability of these cells to form DNA-containing extracellular structures.

HCT116 adenocarcinoma cells form bundles of DNA-containing fibers of a considerable size. These DNA-containing fibers completely inhibit the development of neutrophilic reactions in the form of the formation of neutrophilic networks. These observations allow us to conclude that the cells of the HCT116 tumor line turn off the protective reactions of the innate immune system in the form of the opening of neutrophil networks.

K562 myeloblastoma cells suppress neutrophil defense responses, reducing the number and size of emerging neutrophil networks. The effect of inhibition of neutrophil networks by K562 cells is probably due to the action of a soluble factor that suppresses neutrophil functions, described earlier.

Our study shows that both types of tumor cell lines are capable of suppressing the reactions of innate immune cells using various mechanisms. Adenocarcinoma cells inhibit the formation of neutrophilic networks in direct contact due to the production of DNA-containing fibers of a considerable size. Myeloblastoma cells cause the same effect, probably by secreting humoral factors.

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