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ROS PRODUCTION BY CIRCULATING PHAGOCYTES AND GUERIN CARCINOMA RESISTANCE TO CISPLATIN

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***Background*: Tumor drug resistance remains a primary cause of unsuccessful cancer therapy. The search for biological markers of the sensitivity/resistance of malignant neoplasms to drug therapy is an urgent and important task, the solution of which will increase the effectiveness of anticancer chemotherapy. *Aim:* To study the relationship between the functional activity (parameters of the phagocytosis and reactive oxygen species (ROS) production) of neutrophils and monocytes in the peripheral blood of rats with transplanted Guerin carcinoma and the degree of its sensitivity to cisplatin (Cpt). *Materials and Methods*: The original and Cpt-resistant variants of Guerin carcinoma were transplanted to female Wistar rats 2.5 months old. The parameters of the phagocytic activity of circulating neutrophils and monocytes were determined by the degree of ingestion of inactivated and FITC-labeled staphylococci using flow cytometry. The number of ROS-generating cells and the intensity of ROS production by phagocytes were determined by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate. *Results*: The growth of both variants of Guerin carcinoma caused a statistically significant decrease in the intensity of neutrophil phagocytosis by more than 47% with a tendency to the reduction of the intensity of phagocytosis by monocytes. The phagocytic activity of circulating neutrophils and monocytes did not differ significantly between the groups of animals with the original and Cpt-resistant variant of Guerin carcinoma. In contrast, the intensity of ROS generation by both monocytes and neutrophils in the peripheral blood of animals with Cpt-resistant tumor increased by more than 86% as compared to original carcinoma-bearing rats. *Conclusion*: This study provides evidence that the intensity of ROS production by circulating monocytes and neutrophils may reflect the degree of tumor sensitivity to Cpt. Increased intensity of ROS production could serve as a pretreatment predictor of the formation of tumor drug resistance.**
***Key Words*: cisplatin resistance, pretreatment markers, circulating phagocytes, phagocytic activity, ROS production.**

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Modern tactics of cancer treatment based on an increased knowledge of cancer pathways, has improved technologies for diagnostics, surgical and radiation therapy, and precision therapeutics (chemotherapy) results in better patients outcomes. This is especially true for chemotherapy, which over the past decades represents the backbone of cancer treatment at different stages of the disease [1]. Nevertheless, tumor drug resistance remains a primary cause of unsuccessful cancer therapy. In clinical practice, the main sign of the formation of resistance of malignant tumors to chemotherapy is its decreased effectiveness revealed upon a long course of cancer drug administration. Monitoring of the acquired anticancer drug resistance of malignant neoplasms is a relevant and important topic, as it allows to timely change or stop chemotherapy when it becomes ineffective [2, 3].

Despite the availability of various methods for assessing the sensitivity of the tumor to the action of cancer drugs, they all have their drawbacks and limitations. For a long time, the studies of the drug sensitivity of tumor cells *in vitro* were considered as a promising method for predicting the clinical effectiveness of appropriate antitumor therapy [4].

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Abbreviations used: Cpt – cisplatin; NLR – neutrophil-to-lymphocyte ratio; ROS – reactive oxygen species.

Meanwhile, the spatial heterogeneity of malignant tumors causes significant errors in the *in vitro/in vivo* assessment of the degree of their sensitivity/resistance to the action of anticancer agents using a small part of the tumor material. Therefore, the American Society for Clinical Oncology does not recommend *in vitro* tests for the prediction of tumor chemosensitivity [5].

To assess the direct response of the tumor development to therapy, the criterion for the treatment of solid tumors includes determining the minimum size of the studied lesion [6]. To do this, modern methods such as computed tomography, magnetic resonance imaging, or positron emission tomography are used. Each of these methods has its own disadvantages and limitations. For example, small tumor lesions (< 10–15 mm) cannot be measured by these techniques. In addition, the change in tumor size is not always a reliable sign of the effect of treatment.

In clinical practice, one could assess the effectiveness of the therapy using tumor markers, the number of which is increasing every year [7–9]. As a rule, a decrease in the level of tumor markers is considered as an indicator of the efficacy of cancer therapy and the high sensitivity of the tumor [10]. Meanwhile, such a correlation may not take place, since the level of tumor markers can significantly increase with a high efficiency of treatment due to a substantial disintegration of the tumor mass and the release of a great amount

of protein products from tumor cells, some of which are tumor markers.

The search for biological markers of the sensitivity/resistance of malignant neoplasms to drug therapy is thought to be associated with peripheral blood, which is not only one of the main systemic carriers of information about physiological and pathophysiological processes occurring in the body at all levels of its organization, but also the most accessible system for analysis of this information [11]. In addition to numerous blood soluble and insoluble factors which are often considered as diagnostic and therapeutic biomarkers, blood cells (leukocytes, erythrocytes, and platelets, etc.) are of significant interest for monitoring the effectiveness of cancer therapy [12].

In particular, many studies have shown the existence of an associative relationship between some inflammation markers (such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio) and clinical outcome of cancer patient treatment [13]. Based on the analysis of overall survival and progression-free survival of cancer patients, as a rule, one could conclude on a relationship between the NLR level and the resistance of malignant neoplasms to the action of anticancer drugs, although such a statement needs a more specific evidence base.

Earlier we have proved that in rats with the transplanted Walker carcinosarcoma, the intensity of reactive oxygen species (ROS) production by monocytes and neutrophils of peripheral blood and their functional activity can reflect the degree of tumor sensitivity to doxorubicin and an increase of these indices predicts the formation of tumor drug resistance [14]. The predictive value of these indices is especially important for dynamic monitoring of the development of tumor drug resistance during long-term chemotherapy. Considering the standard 2–3 weeks interval between the courses of cancer therapy and the short lifetime of circulating phagocytes, the measurement of indicators of their functional activity before each subsequent course can be used for a pretreatment assessment.

However, it remains to be established for which malignant tumors and for which cancer drugs really exists the correlation between the functional activity of circulating phagocytes and tumor drug resistance.

Cisplatin (Cpt) is the most widely used chemotherapeutic agent against lung cancer, ovarian cancer, head and neck squamous cell carcinoma, breast cancer, brain tumors, etc. [15]. The major obstacle in its clinical use is an inherent and acquired resistance [16]. Therefore, predicting the effectiveness of the antitumor effect of Cpt is one of the key needs of clinical oncology [17].

In this regard, the aim of this work was to study the relationship between the functional activity (parameters of the phagocytosis and ROS production) of neutrophils and monocytes in the peripheral blood of rats with transplanted Guerin carcinoma and the degree of sensitivity of this tumor to the action of Cpt.

MATERIALS AND METHODS

Experimental tumor models. In the study, the original and Cpt-resistant variants of Guerin carcinoma were transplanted to female Wistar rats 2.5 months old weighing 165–185 g, bred in an animal facility at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine. Animals were kept in the vivarium with the standard light and food regimen (water and nutrition *ad libitum*). The research was carried out under the provisions of the European Convention on the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Starburg, 1986) and "Procedure for Conducting Scientific Experiments on Animals by the Scientific Institutions".

The animals were distributed into 5 groups (6 animals per group): the 1st group — intact animals; the 2nd and 3rd groups — animals with transplanted original variant of Guerin carcinoma; and the 4th and 5th groups — animals with transplanted Cpt-resistant variant. Animals of the 3rd and 5th groups received a course of Cpt therapy, which included 5 intraperitoneal Cpt injections at a total dose of 6 mg/kg body weight. On the 2nd day after the termination of the therapy (16 days of tumor growth), tumor volumes were measured by the formula $V = a \times b \times c$, where a, b, c are the maximum diameters of the tumor node in three mutually perpendicular planes, and analysis of blood parameters and functional activity of peripheral blood phagocytes was performed. Peripheral blood indices of rats were determined using an automatic hematology analyzer Particle Counter PCE-210 (ERMA Inc., Japan).

Phagocytic activity of circulating neutrophils and monocytes. To determine the level of phagocytic activity of neutrophils and monocytes, suspension of inactivated and FITC-labeled staphylococci (200×10^6/ml) was added to blood samples placed in flow cytometry tubes. After incubation for 30 min at 37 °C, samples were triply washed with phosphate buffer and fixed in 0.4% formalin solution for flow cytometry analysis. Each sample (at least 3×10^3 cells) was analyzed in triplicate. The relative counts of neutrophils and phagocytic monocytes and the intensity of bacterial cell engulfing were determined [18].

ROS generation by circulating neutrophils and monocytes. To determine the ROS production by neutrophils and monocytes, 90 µl of blood were placed in a flow cytometer tube, 10 µl of 2',7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich, USA) was added and the specimens were incubated for 30 min at 37 °C. After incubation, the samples were triply washed with phosphate buffer and fixed in 0.4% formalin solution for further flow cytometry analysis. At least 3×10^3 cells in three replicates were analyzed. The relative number of ROS-producing neutrophils and monocytes and the intensity of ROS production were determined [18].

Statistical analysis of the data was performed using descriptive statistics, Student's *t*-test,

Mann — Whitney U-test using Microsoft Excel, Microcal Origin and Statistica Programs. Data are presented as M ± m, where M is the mean value, m is the standard error of the mean.

RESULTS AND DISCUSSION

Characteristics of the original and Cpt-resistant variants of Guerin carcinoma. The original variant of Guerin carcinoma was characterized by a higher growth rate compared to the resistant counterpart. On the 16th day after tumor transplantation, the tumor volume of the original variant was by 56% ($p < 0.001$) higher than that of the resistant tumor (17.4 ± 2.2 *vs.* 7.6 ±0.4 mm^3). Administration of Cpt to rats with the original variant of Guerin carcinoma inhibited tumor growth by 50% ($p < 0.001$) as determined on the 2nd day after the end of Cpt therapy. On the contrary, the volume of resistant Guerin carcinoma on the 2nd day after the end of Cpt therapy was significantly larger (by 29%, $p < 0.01$) as compared to rats with resistant tumor without Cpt treatment.

Blood indices of rats with original and Cpt-resistant variants of Guerin carcinoma. The growth of both original and Cpt-resistant variants of Guerin carcinoma is accompanied by the significant leukocytosis in tumor-bearing rats. As can be seen from Table 1, the total leukocyte count in the blood of animals with the original carcinoma strain increased by 97.4% ($p < 0.01$) and almost by 140% ($p < 0.01$) in animals with resistant variant of the tumor compared with intact control. This change in white blood cells count is characteristic of a malignant process and occurred mainly due to an increase in the level of blood granulocytes, both their quantitative and percentage content. In particular, in rats with the original carcinoma strain, granulocyte counts increased by almost 339% ($p < 0.001$), which led to an increase in their percentage in the blood of animals by almost 114% ($p < 0.05$) compared with intact animals. The development of resistant tumors caused a statistically significant increase in the counts and percentage of granulocytes in the blood of rats by 416.3% ($p < 0.001$) and 109.7% ($p < 0.001$), respectively, compared with intact animals, without differing significantly from the corresponding indices for rats with the original tumor strain (Table 1).

Despite the fact that the lymphocyte count in the blood of rats did not change during the growth of both variants of Guerin carcinoma, their percentage decreased significantly: in the case of the original tumor — by 43% ($p < 0.05$), and resistant — by almost 41% ($p < 0.05$). At the same time, the number and percentage of monocytes in both groups of animals remained at the level of intact control.

Table 1. Blood leukogram of rats with original and Cpt-resistant Guerin carcinoma

Blood indices	Intact animals	Rats with transplanted tumors	
		Original variant	Cpt-resistant variant
WBC	11.5 ± 1.4	22.7 ± 3.2*	27.5 ± 3.4*
Ly(× 1000/μl)	7.8 ± 1.0	8.7 ± 1.0	11.0 ± 1.1*
Gr (× 1000/μl)	3.0 ± 0.4	12.8 ± 2.0*	15.2 ± 2.4*
Mo (× 1000/μl)	0.8 ± 0.3	1.2 ± 0.2	1.4 ± 0.2
Ly (%)	67.8 ± 2.6	38.6 ± 1.8*	40.0 ± 1.2*
Gr (%)	26.2 ± 2.0	56.0 ± 1.7*	54.8 ± 1.8*
Mo (%)	6.1 ± 1.5	5.5 ± 0.5	5.2 ± 0.9

Note: *the differences are significant ($p < 0.05$) compared with corresponding indices of intact animals. Mo – monocytes; Ly – lymphocytes; Gr – granulocytes.

We have revealed that the growth of Guerin carcinoma is associated with an increase in the NLR in the peripheral blood of animals: by 274.4% ($p < 0.001$) in the group with the original tumor variant, and by 251.3% ($p < 0.001$) in animals with the resistant variant compared with that in intact control. There were no significant differences in this index between the groups of animals with two variants of the tumor (1.46 ± 0.10 and 1.37 ± 0.09).

Phagocytic activity of monocytes and neutrophils in the peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma. Analysis of the results showed that the growth of Cpt-sensitive and -resistant variants of Guerin carcinoma is accompanied by a significant increase in the absolute number of phagocytic neutrophils without change in their percentage. As can be seen from Table 2, the number of phagocytic neutrophils in the blood of rats with the original tumor strain significantly increased by 320% ($p < 0.001$) compared with intact rats. The development of Cpt-resistant carcinoma was associated with an increase in the counts of phagocytic neutrophils by 384% ($p < 0.0005$) compared with intact control. This increase in the counts of phagocytic neutrophils in the peripheral blood of rats with both original and resistant variants of Guerin's carcinoma is entirely due to granulocytosis against the background of tumor development. Along with this, there was registered a significant ($p < 0.05$) decrease in the intensity of phagocytosis by neutrophils — by 47.3% in rats with the original variant and by 49% in rats with the resistant variant.

Regarding peripheral blood monocytes in rats with tumors, the growth of both variants of Guerin carcinoma also caused an increase in both their absolute number and the percentage of their phagocytic fraction (Table 3). In the case of a resistant tumor variant, such increase was only a trend, while in the case of the original variant the absolute count of peripheral blood monocytes increased by 70% ($p < 0.05$). The percentage of phagocytic monocytes increased significantly by more than 130% ($p < 0.01$) compared with intact control. Meanwhile, the intensity of phagocytosis showed a slight downward trend.

It should be noted that in contrast to the results of similar studies using Walker carcinosarcoma and doxorubicin [12], significant differences in the phagocytic activity of circulating neutrophils and monocytes between the groups with original and Cpt-resistant variants of Guerin carcinoma were not found. This may be partly due to the relatively low sensitivity (and relatively high resistance) of the original variant of Guerin carcinoma to Cpt, the administration of which in a total dose equal to MTD causes only 50% inhibition of tumor growth.

On the other hand, the pathophysiological differences between Walker carcinosarcoma and Guerin carcinoma may also significantly affect the predictive power of the studied parameters. It is also important to note the fundamental differences between doxorubicin and Cpt both in the mechanisms of their antitumor action and the formation of drug resistance. These differences may influence the predictor indices of extratumoral markers of malignant neoplasm resistance. For example, a comparison of the results of the *in vitro* short-term test and clinical chemotherapy of human tumors showed that doxorubicin (compared to several other drugs) was the most accurate compound to predict clinical responsiveness to chemotherapy [2].

ROS generation by monocytes and neutrophils in the peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma. The study has shown that the development of both the original and resistant variants of Guerin carcinoma was accompanied by a statistically significant increase in the number of ROS-producing monocytes and neutrophils as compared with intact controls (Tables 4, 5). At the same time, the recorded increase in ROS generating phagocytes in the blood of tumor-bearing animals was completely proportional to the increased absolute number of these cells. This was especially pronounced in the case of neutrophils, the absolute number of which, as well as their ROS-producing fraction, increased by more than 350% compared with the corresponding indices of intact animals. It should be noted that these indices of circulating phagocytes did not depend on the sensitivity of the tumor to the action of Cpt.

The increase in the number of ROS-producing monocytes and neutrophils in the blood of animals with tumors was accompanied by an increased level of ROS production. However, the increase in the intensity of ROS formation by both monocytes (see Table 4) and neutrophils (see Table 5) in the blood of animals with original Guerin carcinoma compared with intact rats showed just a statistical tendency due to their significant variability. In the group of animals with Cpt-resistant variant of carcinoma, a significant increase in the intensity of ROS production by both monocytes by 279% ($p < 0.01$) and neutrophils by more than 400% ($p < 0.001$) was recorded compared to intact animals.

Particular attention should be paid to the significant difference in the intensity of ROS generation by phagocytes in the blood of rats with Cpt-resistant variant of Guerin carcinoma in comparison with the corresponding index in animals with the original variant. In particular, the level of ROS in rat blood monocytes was 86.7% ($p < 0.05$), and in neutrophils — 115% ($p < 0.05$) higher than the corresponding index of phagocytes in the blood of animals with the original variant of Guerin carcinoma. It should also be noted that the most informative indices of peripheral blood phagocytes to predict the degree of Walker carcinosarcoma resistance to doxorubicin was the intensity of ROS production [12].

In conclusion, our study has shown that the growth of two variants of Guerin carcinoma with different sensitivity to the action of Cpt is accompanied by the development of leukocytosis, more pronounced in the case of the resistant variant. The change in the leukocyte formula in animals with tumors is entirely due to significant granulocytosis. The development of both variants of Guerin carcinoma causes a statistically significant decrease in the intensity of neutrophil phagocytosis, and a tendency to the reduction of the intensity

Table 2. Indices of phagocytosis by neutrophils in peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma

Group of rats	Neutrophil counts (× 1000/μ)	Number of phagocytic neutrophils (× 1000/μ)	Percentage of phagocytic neutrophils	Intensity of phagocytosis (r.u.)
Intact	3.0 ± 0.3	2.5 ± 0.3	85.9 ± 2.6	315.9 ± 17.8
Original carcinoma	12.8 ± 1.3*	10.4 ± 1.3*	82.6 ± 4.3	166.6 ± 12.8*
Cpt-resistant carcinoma	13.8 ± 1.5*	12.1 ± 2.6*	77.4 ± 6.4	161.0 ± 16.7*

Notes: *the differences are significant ($p < 0.05$) compared with corresponding value of intact rats.

Table 3. Indices of phagocytosis by monocytes in peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma

Group of rats	Monocyte counts (× 1000/μl)	Number of phagocytic monocytes (× 1000/μl)	Percentage of phagocytic monocytes	Intensity of phagocytosis (a.u.)
Intact	0.85 ± 0.20	0.27 ± 0.09	36.4 ± 1.9	86.9 ± 7.9
Original carcinoma	1.45 ± 0.06*	0.63 ± 0.12	50.4 ± 4.1*	62.6 ± 17.2
Cpt-resistant carcinoma	1.2 ± 0.1	0.66 ± 0.20	47.0 ± 5.1	60.3 ± 14.0

Notes: *the differences are significant ($p < 0.05$) compared with corresponding value of intact rats.

Table 4. Indices of ROS generating monocytes in peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma

Group of rats	Monocyte counts (× 1000/μl)	Number of ROS generating monocytes (× 1000/μl)	Number of ROS generating monocytes (%)	Intensity of ROS production by monocytes (a.u.)
Intact	0.85 ± 0.2	0.74 ± 0.2	88.3 ± 9.2	72.1 ± 27.3
Original carcinoma	1.45 ± 0.06*	1.3 ± 0.1*	96.6 ± 2.7	147.3 ± 24.7
Cpt-resistant carcinoma	1.2 ± 0.1	1.2 ± 0.1*	97.6 ± 2.0	272.8 ± 40.6*,#

Notes: the differences are significant ($p < 0.05$) compared with corresponding value of: *intact rats; #rats bearing original variant of carcinoma.

Table 5. Indices of ROS generating neutrophils in peripheral blood of rats with original and Cpt-resistant variants of Guerin carcinoma

Group of rats	Neutrophils counts (× 1000/μl)	Number of ROS generating neutrophils (× 1000/μl)	Number of ROS generating neutrophils (%)	Intensity of ROS production by neutrophils (a.u.)
Intact	3.0 ± 0.3	2.7 ± 0.3	89.9 ± 4.1	117.6 ± 48.5
Original carcinoma	12.8 ± 1.3*	12.4 ± 1.1*	97.0 ± 1.5	275.6 ± 66.1
Cpt-resistant carcinoma	13.8 ± 1.5*	12.7 ± 1.6*	91.0 ± 4.5	592.2 ± 71.4*,#

Notes: the differences are significant ($p < 0.05$) compared with corresponding value of: *intact rats; #rats bearing original variant of carcinoma.

of phagocytosis by monocytes. The phagocytic activity of neutrophils and peripheral blood monocytes did not differ significantly between the groups of animals with the original and Cpt-resistant variant of Guerin carcinoma.

In contrast to phagocytic activity, the study has revealed an obvious relationship between the degree of sensitivity/resistance of Guerin carcinoma to Cpt and the intensity of ROS generation by both monocytes and neutrophils in the peripheral blood of tumor-bearing animals, the level of which significantly increased in animals with Cpt-resistant tumor variant.

This study provides evidence that the intensity of ROS production by circulating monocytes and neutrophils, may reflect the degree of tumor sensitivity to Cpt, and the increase in these indices could serve as a pretreatment predictor of the formation of tumor drug resistance.

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ПРОДУКЦІЯ АФК ЦИРКУЛЮЮЧИМИ ФАГОЦИТАМИ І РЕЗИСТЕНТНІСТЬ КАРЦИНОМИ ГЕРЕНА ДО ЦИСПЛАТИНУ

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Стан питання: На сьогодні резистентність до протипухлинних препаратів залишається основною причиною безуспішної терапії раку. Тому пошук біологічних маркерів чутливості/резистентності злоякісних новоутворень до медикаментозної терапії є актуальним і важливим завданням, вирішення якого підвищить ефективність протипухлинної хіміотерапії. ***Метою*** роботи було вивчення взаємозв'язку між функціональною активністю (показниками фагоцитозу та продукцією АФК) нейтрофілів та моноцитів у периферичній крові щурів з перещепленою карциномою Герена та ступенем чутливості цієї пухлини до дії цисплатину. ***Матеріали та методи***: Вихідний та резистентний до цисплатину варіанти карциноми Герена перещеплювали щурам-самкам лінії Wistar віком 2.5 міс. Оцінку фагоцитуючої активності циркулюючих нейтрофілів та моноцитів проводили на проточному цитометрі з використанням інактивованого *Staphylococcus aureus*, міченого флуоресцеїн ізотіоціонатом. Відносну кількість АФК-генеруючих клітин та інтенсивність продукції АФК фагоцитами крові визначали з використанням 2',7'-дихлордигідрофлуоресцеїн діацетату методом проточної цитометрії. ***Результати***: Ріст обох варіантів карциноми Герена супроводжується статистично достовірним зниженням інтенсивності фагоцитозу нейтрофілами більш ніж на 47%. Відзначається також тенденція до зниження інтенсивності фагоцитозу моноцитами. Фагоцитуюча активність циркулюючих нейтрофілів та моноцитів суттєво не відрізнялася між групами тварин з чутливим та резистентним до цисплатину варіантами карциноми Герена. На відміну від фагоцитуючої активності, дослідження виявило очевидну залежність між ступенем чутливості/резистентності карциноми Герена до дії цисплатину та інтенсивністю продукції АФК як моноцитами, так і нейтрофілами в периферичній крові тварин-пухлиноносіїв, рівень яких підвищився більш ніж на 86% у тварин з резистентним