Morphofunctional state of neutrophils and cytokine status in patients with ovarian cancer

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ABSTRACT

Introduction: The purpose of this study was to evaluate the morphofunctional state of polymorphonuclear neutrophils (PMN) and cytokine levels in the lysate of blood neutrophils of ovarian cancer (OC) patients. Methods: The absolute and the relative number of peripheral blood PMN in 120 primary OC patients (III-IV clinical stage FIGO) and 80 healthy women were determined. The expression of CD11a, 11b, 15 and CD95 in PMN was assessed by fluorescent microscopy. Cytokine priming on PMN cell membrane, the content of cationic proteins (CP), the activity in the spontaneous nitroblue tetrazolium (NBT)-test and phagocytic activity in PMN was determined. The amounts of cytokines tumor necrosis factors (TNF-α), interleukin (IL-1β), 1Ra, 6, 18, and granulocyte colony-stimulating factor (G-CSF) in serum were measured by the enzyme immunoassay method. Rigidity and topology of native PMN were assessed by scanning probe microscopy. To detect the differences between the data obtained in the evaluation of OC patients and healthy people, a non-parametric U-Mann–Whitney test was used. Reliable difference was considered at P ≤ 0.05. Results: These studies found an increase of the total number of PMN in peripheral blood, while reducing their cytotoxicity parameters. We observed reduced MPO activity and level of CP. Reducing in the levels of CD15 and CD95 expression on the background of increasing PMN activity in the spontaneous NBT-test may indicate a decrease in ability to complete phagocytosis. The dynamics of CD11a and CD11b expression in PMN was multidirectional, and the changes were not statistically significant. Polymorphonuclear neutrophils membrane stiffness decreased in Stage IV OC. We also observed the levels of IL-1β reduced up to 248.5 ± 9.00 pg/ml in Stage III OC, up to 230.9 ± 15.04 pg/ml in Stage IV OC versus 36.8 ± 10.74 pg/ml in control, reduced levels of 1Ra, and IL-18. We found that the level of IL-6 increased at Stage III OC up to 34.38 ± 9.74 pg/ml versus 7.66 ± 2.28 pg/ml in control and the levels of TNF-α and G-CSF decreased. Conclusion: Thus, these results suggest a state of secondary immunodeficiency in patients with OC at III-IV clinical stages of the disease.

KEY WORDS: Cytokines, immunodeficiency, ovarian cancer, polymorphonuclear neutrophils

INTRODUCTION

The role of polymorphonuclear neutrophils (PMN) in the antitumor immune reactions is currently considered important, but not unique. The direct cytostatic and cytotoxic effects of PMN tumor cells in vitro [1], and their migration into the tumor at the early stages of its formation were shown [2]. At the same time, it was found that H2O2 produced by granulocytes suppresses adaptive immune response reactions, promotes angiogenesis and metastasis at the advanced stages of tumor development via different factors, including cytokines [3,4]. Furthermore, a change in functional activity of PMN [5] and their secreted cytokine profile [6] was demonstrated in patients with advanced forms of cancer. The assumption that cytokines launch the processes that activate the main intracellular regulatory system in PMN finds experimental support [7,8]. The cytokine priming on PMN cell membrane results in increased expression of adhesion molecules while connecting with interstitial matrix and vascular endothelium. The migration of tumor cells and of PMN occurs by means of the same integrins [9]. A consequence of increasing of the number of cells - precursors in the bone marrow is the increase in absolute number of PMN during the tumor progression [10]. The mechanism of changes in cell size and rigidity of the cell membrane in this process, however, remains unclear. Thus, the literature data and the results of our studies [11] indicate that a malignant tumor modifies the morphofunctional state of peripheral PMN. Presently, it is established that PMN constitute a significant part of immunocompetent cells infiltrating the tumor. It was suggested to distinguish these tumor - PMN into a special population, which, as the authors suggest, is very different in their morphofunctional characteristics from...
the peripheral blood PMN [12] and can have both pro- and anti-tumor phenotypes. There is a view that the parameters of the morphofunctional state of tumor-PMN may serve as independent prognostic factors in assessing survival in squamous head and neck tumors [13]. At the same time, ovarian cancer (OC), which is the subject of our research, is accompanied by a significant increase in the quantitative indicators of pathogenic organisms in the area of tumor growth, and therefore it is not possible to differentiate the factors - the influence of microbial or tumor cells - determining morphofunctional state of tumor-PMN. Information about the dynamics of the cytokines levels in carcinogenesis also seems important in developing immunotherapy strategies, depending on the biological pattern of the tumor and the stage of tumor progression. The aim of this study was to investigate the morphofunctional state of peripheral blood PMN and evaluate the cytokine status in OC patients.

MATERIALS AND METHODS

Observed group consisted of 120 Stage III - IV (by FIGO) primary OC patients. The control group consisted of 80 healthy women. Selection of patients was based on strictly defined criteria: Age 28-60 years (inclusive); absence of acute inflammatory infectious and non-infectious diseases; the absence of surgical procedures performed during the previous year (including dental). PMN were isolated from 5 ml of heparinized blood on the double density Ficoll-Urografin gradient (ρ = 1.117 and 1.077 g/ml). The neutrophils suspension was washed thrice with 0.85% NaCl. The purity of neutrophil fraction is 92-94%. Viability of PMN in the test with a 0.5% trypan blue was 95%. Spontaneous production of cytokines interleukin (IL-1β), IL-1Ra, IL-6, IL-18, tumor necrosis factors (TNF-α), granulocyte colony-stimulating factor (G-CSF) (JSC “Vector-Best-Volga” Russia) was determined by solid-phase enzyme immunoassay in the serum. The serum and PMN lysate were stored for a month at −25°C.

Neutrophils were labeled with monoclonal antibodies to CD11a, 11b, 15 and CD95 conjugated with fluorescein isothiocyanate. Results were checked visually on a fluorescent microscope (×700) with immersion.

To assess the phagocytic activity of PMN, the common method using yeast was employed. 150 μl of Hanks solution and 150 μl of yeast working solution were added to 300 ml of blood in a test tube and incubated for 5, 30 and 60 min in an oven at a temperature 37°C. Phagocytic index (PI) was calculated according to Hamburgers, phagocytic number (PN) according to wright and the index of completeness of phagocytosis (ICP): ICP = PN 5'/PN 60'. The activity of myeloperoxidase (MPO) was assessed by the Graham-Knoll method with benzidine. Fresh slides were fixed in 4% formalin-alcohol solution, then applied to the incubation medium (250 mg benzidine dissolved in 6 ml 96° alcohol and 4 ml of water with the addition of 0.02 ml of 3% hydrogen peroxide). Cytoplasm and nucleus were then stained with azure cosin. The level of cationic proteins (CP) was assessed by the M. G. Shubich method with bromophenol blue. Fresh slides were fixed with a 5% aqueous solution of sulfosalicylic acid. Further, slides were stained with 0.1% bromophenol blue solution in borate buffer, nuclei stained with 1% aqueous solution of safranine.

The proportion of active neutrophils was in a spontaneous version of the test of reduction of nitroblue tetrazolium to insoluble dipheormazane was determined cytochemically. Dried slides were placed in a container with May-Grunwald stain for 3-5 min. Then slides were placed in a cell with diluted (1:10) Romanovsky stain solution. Further, they were washed with running water and the nitroblue tetrazolium solution with a concentration of 1 mg/ml was applied on the wet slides in a dark place. Results were expressed as mean cytochemical coefficient (MCC). Each slide was counted 50 or 100 PMN, among which the percentage of cells containing the corresponding enzyme deposits was determined, and MCC was calculated by the formula:

$$MCC = \frac{0•a + 1•b + 2•c + 3•d}{100}$$

where a - quantity of PMN without granules.

b, c, d - quantity of PMN with varying degree of cytoplasmic staining.

The numbers indicate the intensity of cytoplasmic staining:

0: No staining;
1: Presence of single granules in the cytoplasm;
2: Granules fill up ⅓ of the cytoplasm;
3: Granules fill up more than ⅓ cytoplasm.

To assess the morphology and stiffness of the red blood cells membrane we used the scanning probe microscopy (SolverPro, NT-MDT, Zelenograd, Russia). The proprietary silicon probes with the rigidity of 0.2 N/n were used, and the radius of curvature of the probe tip was approximately 50 nm. Red blood cells were scanned in tapping mode. Rigidity of the membranes was evaluated by the Young’s modulus, which was calculated according to the Hertz theory [14]. As the central characteristics, the median was used, and a nonparametric Mann-Whitney test was used when comparing.

RESULTS

The studies found that while the total number of white blood cells in OC patients stays the same, the amount of PMN increases (4.1 ± 0.1 × 10⁹/l in Stage III and 4.9 ± 0.1 × 10⁹/l in Stage IV OC versus 3.7 ± 0.1 × 10⁹/l in the control group (P1<0.05; P2<0.05)). PI and phagocytic number ranged within the framework of standards, but absolute phagocytic index, which reflects the absolute number of phagocytic cells in 1 liter of blood (API) increased (3.1 ± 0.2 × 10³/l in Stage III and 3.6 ± 0.1 × 10³/l in Stage IV OC versus 2.8±0.2 × 10³/l in the control group (P<0.05; P<0.05)). The microbicidal oxygen-dependent mechanism is associated with activation of the NADPH oxidase complex, secretion of the oxygen radicals, superoxide anion (O₂⁻) with the subsequent formation of hydrogen peroxide (H₂O₂). The level of reactive oxygen species is determined using NBT-test. The number of PMN in NBT-test did not change significantly. We also found a decrease of aerobic
and anaerobic cytotoxicity. The PMN killer effect is due to MPO secretion, which in combination with H2O2 and extracellular halides forms reactive oxidants damaging target cells and taking part in the cytotoxic reactions against tumor cells. The MPO activity in OC patients PMN was reduced (1.44 ± 0.07 MCC in Stage III and 1.30 ± 0.04 MCC in Stage IV against 2.03 ± 0.01 MCC in the control group (P1 < 0.05; P2 < 0.05)). The CP, enclosed in a protoplasmic granules, have a microbicidal activity under anaerobic conditions, in the absence of respiratory burst. Release of lyosomal proteins in a modified form occurs into a phagolysosome or intercellular space. Cationic antimicrobial proteins are specific PMN markers. They are an essential component of oxygen-independent microbicidal system. CP level decreased (1.52 ± 0.04 MCC in Stage III and 1.23 ± 0.12 MCC in Stage IV against 1.73 ± 0.06 MCC in the control group (P1 > 0.05; P2 < 0.05) [Figure 1].

In terminal stages of OC we observed reduction of PMN expressing the differentiation marker CD15 (33.2 ± 5.02% - in Stage III and 40.0 ± 7.17% - in clinical Stage IV against 58.0 ± 4.60% in the control group (P1 < 0.05; P2 < 0.05) [Figure 2]. The dynamics of CD11a + and CD11b + cells was multidirectional, and changes were not statistically significant.

![Figure 1: The absolute amount, phagocytic activity, oxygen-dependent and anaerobic cytotoxicity of blood neutrophils in the terminal stages of ovarian cancer](image1)

![Figure 2: The number of CD15 +, CD95 + and rigidity of membranes of blood neutrophils in ovarian cancer patients](image2)

We found that the expression of CD95 on the PMN in OC decreased from 58.0 ± 5.98% - in the control group to 39.3 ± 9.91% - in Stage III and 34.2 ± 7.62% - in Stage IV (P1 < 0.05; P2 < 0.05).

Stiffness of the membrane of PMN increased and amounted to 87.78 ± 2.750 MPa in Stage III OC and 71.99 ± 7.712 MPa in stage IV OC against 56.68 ± 2.445 MPa in the control group [Figure 2].

We found that the level of IL-1β significantly increased in Stage III OC up to 248.5 ± 9.00 pg/ml, in Stage IV OC up to 230.9 ± 15.04 pg/ml versus 36.8 ± 10.74 pg/ml in the control group (P1 < 0.05, P2 < 0.05).

We found an increase in the level of IL-1Ra in the progression of OC: 403.3 ± 104.112 pg/ml in the III and 457.45 ± 74.64 pg/ml in the IV Stage OC versus 219.078 ± 29.44 pg/ml in the control group (P1 < 0.05, P2 < 0.05). We have established significant increase in the level of IL-6 in Stage III OC: 34.38 ± 9.74 pg/ml versus 7.66 ± 2.28 pg/ml in the control group (P1 < 0.05).

In our studies, the level of IL-18 in the serum of patients with OC at III (488.07 ± 203.78 pg/ml) and IV (285.43 ± 45.28 pg/ml) stages of the disease was significantly higher relative to those indicators in healthy women (87.59 ± 33.79 pg/ml) (P1 < 0.05; P2 < 0.05).

According to our data, the level of TNF-α in the advanced stages of OC is reduced relative to controls (2.71 ± 0.36 pg/ml in the III and 2.89 ± 0.68 pg/ml in the IV stage OC versus 16.95 ± 2.82 pg/ml in the control group [P1 < 0.05, P2 < 0.05]).

We have shown the decrease of G-CSF in the progression of OC: Stage III - up to 15.85 pg/ml (10.06-36.35) and IV - to 31.54 pg/ml (1.63-96.29) against 157.69 (52.39-368.10) in the control group [Table 1].

### DISCUSSION

PMN have long been considered terminally differentiated effector cells, playing a major role during the acute phase of

### Table 1: Cytokine levels in the serum of OC patients

<table>
<thead>
<tr>
<th>Cytokines, pg/ml</th>
<th>Control</th>
<th>OC (III)</th>
<th>OC (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>36.8</td>
<td>248.5</td>
<td>230.9</td>
</tr>
<tr>
<td>(20.17-48.02)</td>
<td>(118.37-355.73)</td>
<td>(107.75-318.21)</td>
<td></td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>219.078</td>
<td>403.3</td>
<td>457.45</td>
</tr>
<tr>
<td>(119.98-325.435)</td>
<td>(100.469-779.84)</td>
<td>(20.88-800.551)</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>47.228</td>
<td>34.38</td>
<td>14.814</td>
</tr>
<tr>
<td>(21.69-95.62)</td>
<td>(4.5-115.8)</td>
<td>(1.10-55.8)</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>87.59</td>
<td>488.07</td>
<td>285.43</td>
</tr>
<tr>
<td>(0-215.752)</td>
<td>(133.7-1480.51)</td>
<td>(94.98-651.45)</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>16.95</td>
<td>2.714</td>
<td>2.891</td>
</tr>
<tr>
<td>(5.92-25.59)</td>
<td>(0-4.249)</td>
<td>(1.21-4.572)</td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>157.698</td>
<td>15.855</td>
<td>31.5396</td>
</tr>
<tr>
<td>(52.39-368.101)</td>
<td>(10.063-36.532)</td>
<td>(1.635-96.292)</td>
<td></td>
</tr>
</tbody>
</table>

OC: Ovarian cancer, G-CSF: Granulocyte colony-stimulating factor, TNF-α: Tumor necrosis factors alpha, IL: Interleukin
inflammation and in the resistance against microbes. Recent evidence questioned this limited point of view, indicating that neutrophils can interact with distinct cell populations and produce a wide number of cytokines and effector molecules [15]. The main function of PMN is phagocytosis. Respiratory burst, which occurs after activation of PMN and, leads to the formation of reactive oxygen species (ROS) and activation of the hexose monophosphate shunt enzymes, is an important element of phagocytosis. Reaction of reduction of NBT to diaphormazane used to assess the ability of PMN to generate ROS. Definition of MPO activity and CP allows us to estimate the oxygen-dependent and anaerobic cytotoxicity. There is a decrease in bactericidal activity in the end-stage OC.

As the granulocytes mature, the differentiation markers appear or disappear on their surface. As a result of the emergence of cellular receptors, PMN permeates through the vascular endothelium. Under the influence of co-stimulatory factors present in the tissue, the PMN realizes its functions - adhesion, chemotaxis, respiratory burst, absorption, degranulation, extracellular secretion, antimicrobial and cytotoxic effect. CD11a is the most important surface adhesion molecule that provides the adhesion and transmembrane migration of PMN from the circulation into the tissues, as well as PMN apoptosis. CD11b is present not only in a cytoplasmic form, but in the PMN granules and after a chemotactic stimulation, can be translocated to the cell membrane. The CD95 expression level determines the readiness for Fas-induced apoptosis of the cell when contacting with the appropriate ligand [16]. End-stage OC reduces the amount of PMN able to apoptosis, and CD15 + PMN.

The method of scanning probe microscopy allows to measure local and elastic properties of the cell surface. For neutrophilic granulocytes, exploring the mechanical properties of the membrane is of paramount importance, since the processes of diapedesis and migration in the tissues and the process of phagocytosis are accompanied by complex elastic-mechanical response. In reconnaissance reactions of PMN great importance, belongs to cytoskeletal rearrangements and receptor activation. Reduced membrane plasticity probably has a pathogenetic significance, defining a modification of the functional activity of PMN membrane receptors. In end-stage OC PMN membrane becomes less elastic, the stiffness of the membrane increases markedly.

Imbalance in the cytokine system is considered an important mechanism for the development of many pathological processes. In tumorigenesis, two systems interact with cytokines: Neoplasm - cytokines and immune system - cytokines. Thus tumor cells may express the corresponding receptor as well as they produce cytokines. IL-1β determines tumor growth, metastasis and angiogenesis by affecting the production of matrix metalloproteinases, growth factors and adhesion molecules [17]. IL-1Ra allows neoplasm to keep inflammation at the level necessary to create optimal living conditions [17]. End-stage OC increases levels of IL-1β and IL-1Ra. IL-6 is a potent proinflammatory cytokine as IL-1 and TNF, but the former is produced later. It is known that IL-6 stimulates the growth of a number of experimental tumors. The expression of IL-6 is shown to increase in a number of malignant tumors, accompanied by adverse clinical course of the disease. IL-18 is a proinflammatory cytokine, and may play a significant role in antitumor protection of the organism. At present, it is shown that IL-18, as well as its receptor, IL-18-binding protein are polymorphic structures, which are formed on the basis of allelic polymorphism of these proteins. Thus, 9 allelic variants were detected for the IL-18 gene and 11 allelic variants for the IL-18-binding protein. The association of IL-18 allelic variant with increasing frequency of the corresponding pathologic process was shown [18]. End-stage OC significantly increases levels of pro-inflammatory cytokines IL-6 and 18. TNF-α is called so because of its main biological effect - lysis of tumor cells. Many tumor cells have the ability to produce TNF-α, including OC cells [19]. TNF-α is known to be the only cytokine with a direct cytotoxic effect against tumor cells [20]. With the progression of OC, level of TNF-α significantly reduces in the blood serum. G-CSF increases the functional activity of mature neutrophils, enhances the production of oxygen radicals and antibody-dependent cellular cytotoxicity, stimulates the synthesis of IL-10, IL-1β, TNF-α. In advanced OC activity of G-CSF is reduced.

Thus, the literature data [21] and the results of our studies suggest that a malignant tumor modifies the morphofunctional state of peripheral PMN.

CONCLUSION

The results can be used to develop schemes and immunotherapy of patients with OC immunorehabilitation. Thus, the growing number of PMN in the peripheral blood observed in patients with advanced stage OC is accompanied by a simultaneous reduction of their cytotoxicity. Besides, the absolute amount of phagocytic cells increases and the readiness of these cells to Fas-induced apoptosis decreases. Increasing in the rigidity of PMN membranes was accompanied by reduced expression of differentiation marker CD15 on their surface. Cytokine spectrum of blood serum of patients with advanced stage is characterized by a significant increase in the levels of proinflammatory cytokines, while reducing levels of TNF-α and G-CSF, which stimulates the functional activity of the PMN. The results can be used in developing immunotherapy schemes and immunorehabilitation of patients with OC.

ACKNOWLEDGMENTS

The work was performed within the public task of the Ministry of Education and Science of the Russian Federation.

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Abakumova, et al.: Neutrophils and cytokines in ovarian cancer

Physiol 2013;228:1404-12.

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Source of Support: Nil, Conflict of Interest: None declared.