Functional activity of natural killer cells in biological fluids in patients with colorectal and ovarian cancers

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Abstract

Aim of the study: To compare the functional activity of natural killer cells depending on the presence of a malignant process and its dissemination.

Material and methods: The study included 20 patients with Stage IIIb, C (FIGO, 2009) ovarian cancer, 10 patients with benign ovarian tumours (BOT), and 20 patients with colorectal cancer (T2-4N0-2M0). The control group consisted of 9 healthy donors. To evaluate the number and functional activity of NK cells, multicolour flow cytometry was performed.

Results: In cancer patients, the relative number of activated NK cells secreting granzyme B (GB) (CD56+CD107a+GB+PF–) was significantly decreased, and the proportion of degranulated NK cells (CD56+CD107a+GB–PF–) was significantly increased, compared to those observed in healthy donors. The total number of NK cells in peripheral blood was low in ovarian cancer patients (p < 0.05). The proportion of activated peripheral blood NK cells containing cytolytic granules GB and perforin (PF) in colorectal cancer patients increased with tumour growth. However, lymph node metastasis did not affect the content and activation of NK cells. Comparative analysis of NK-cell populations in patients with benign and malignant ovarian tumours revealed that the level of CD56+ cells was significantly higher in ascites than in peripheral blood. However, CD56+CD107a+ activated cells and CD56+CD107a+GB–PF– cells were found more frequently in ascites of BOT patients than in ovarian cancer patients. The degranulated population of NK cells (CD56+CD107a+GB–PF–) was mainly observed in the peripheral blood of ovarian cancer patients.

Key words: colorectal cancer, ascites, peripheral blood, ovarian cancer, natural killer cells, functional activity.

Introduction

The immune system, especially the functional activity of natural killer cells, may play an important role in the process of tumor invasion and metastasis. Natural killer cells (NK cells) are a heterogeneous subset of lymphocytes of the innate immunity system. NK cells account for about 15% of total circulating lymphocytes. They were also found in the liver, spleen, and peritoneal fluid [1, 2]. Moreover, a number of clinical and experimental studies have shown that the current state of the immune system, including the level of functional activity of NK is the important condition for high efficiency of antitumor treatment [3–5]. NK cells use various mechanisms to perform their functions, but the most important one is contact cytolysis. This effect is realized with participation of cytotoxic granules containing granzymes, perforin, granulysin, cathepsins, and lysosomal-associated membrane proteins (LAMP). Surface expression of LAMP-1 (CD107a) is a marker of degranulating NK cells [6, 7]. The value of surface and intracellular expression of LAMP-1 in NK-cell biology is currently being studied. It has been shown that downregulation of LAMP-1 significantly slows down the movement of lytic granules to the synaptic gap, but also leads to a reduction in their concentration of perforin, but not granzyme B. The reduced levels of lytic granules in combination with their impaired movement in LAMP-1-deficient cell lines leads to the inhibition of NK cells cytotoxicity [7].
It is known that the realization of the cytotoxic cellular immune response against tumor target cells occurs locally, thus determining the prognostic significance of local immunity. There is evidence of significant differences in the content of NK cells and other cell populations of the immune system between ascitic fluid and peripheral blood of patients with ovarian cancer [8]. Although there are published data on the total amount of NK cells in biological fluids and cell subsets of NK cells in colorectal and ovarian cancers, data on the functional activity of NK cells are virtually absent. The aim of the study was to compare the NK-cell functional activity depending on the presence of cancer and cancer dissemination.

Material and methods

The study was approved by the Local Ethics Committee of the Cancer Research Institute of the Tomsk National Research Medical Center. Blood and ascites samples were obtained from 20 patients with IIIB, C stages of ovarian cancer (FIGO, 2013). All patients were fully informed of the purpose and nature of the treatment and provided informed written consent. Between 2015 and 2016, the patients were treated at the Department of Gynecology of the Cancer Research Institute of the Tomsk National Research Medical Center. They were Caucasian and resided in the Western Siberia region. Histological sections were prepared from paraffin blocks according to the standard hematoxylin and eosin staining techniques. In our study, all ovarian cancers were characterized as high-grade serous adenocarcinomas. Verhaak et al. in their multicenter study reported that up to 80% of all malignant epithelial ovarian tumors were high-grade serous adenocarcinomas [9]. All patients were tested for the presence of BRCA1/BRCA2 mutations allele-specific PCR in real time. Patients included in this study were BRCA1/BRCA2-negative. All patients with disseminated ovarian cancer underwent optimal debulking surgery followed by adjuvant chemotherapy. The Gynecologic Oncology Group (GOG) currently defines optimal cytoreduction as leaving residual disease less than 1 cm in maximum tumor diameter. The adjuvant chemotherapy regimen was administered as follows: carboplatin plus paclitaxel, 175 mg/m², carboplatin AUC 6 on day 1, every 21 days, for 6 cycles. The comparison group consisted of 10 patients with benign tumors (BOT) of the ovary (serous and endometrioid BT) with ascitic fluid. The median age of patients with ovarian cancer was 53 years (range 39–69). The median age of patients with BT was 58 years (range 51–68).

The study also included 20 patients (9 males and 11 females) with stage T2–4N0–2M0 colorectal cancer. The median age of these patients was 58 years. Between 2015 and 2016, the patients were treated at the Department of Abdominal Oncology of the Cancer Research Institute of the Tomsk National Research Medical Center. All colorectal cancer patients underwent radical surgery with lymph node dissection. Patients with lymph node metastases (N1–2) received the standard adjuvant chemotherapy. All tumors were histologically proven to be adenocarcinomas. The patients did not have a family history of cancer or other synchronous malignancy (such as ovarian cancer, prostate cancer, breast cancer). The control group consisted of 9 healthy donors (3 males and 6 females) with a median age of 52 years.

Heparinized test tubes were used to collect peripheral blood from the cubital vein. Samples of ascites fluid were obtained during surgery in an amount of about 20 ml before any treatment in tubes with heparin solution in a final concentration of 25 U/ml. Cell fraction of ascites was isolated by centrifugation for 20 minutes at 900 g, then erythrocytes were removed from cell suspensions and cells were washed twice with Cell Wash (BD). We added an appropriate volume of fluorochrome-conjugated monoclonal antibody to 100 µl cell suspension obtained from ascites taking into consideration the concentration of cells in suspension. We added also an appropriate volume of fluorochrome-conjugated monoclonal antibody to 100 µl of whole blood in a 12 × 75 mm tube, then we vortex samples gently and incubate 20 minutes in the dark at room temperature (20° to 25°C). To remove erythrocytes, 1 ml of BD FACS Lysing Solution was added to samples. After incubation for 12 minutes in the dark at room temperature, all samples were centrifuged at 500 × g for 5 minutes twice. The supernatants were removed. Then we used BD Cytofix/Cytoperm Fixation and Permeabilization Solution (250 µl in each sample for 20 minutes in dark at 4°C) before to stain the intracellular molecules as perforin and granzyme B. The samples were washed twice with using BD PermWash Buffer then the anti-Perforin and anti-Granzyme B antibodies were added to samples. Staining duration was 20 minutes at 4°C. Then samples were washed twice. The BD Flow solution was added to each sample. Then samples were analyzed on a BD FACS CANTO II flow cytometer [10, 11]. Figure 1 provides a scheme of a gating strategy. Briefly, we created a “lymphocyte gate” on a forward scatter/side scatter plot for samples from peripheral blood (part A). Then we applied the “lymphocyte gate” to all peripheral blood samples. For samples obtained from ascites we initially selected CD45+ cells as leucocytes (Part E). Then we selected the gate of CD45+CD56+ cell as NK (part B, F). CD56+CD107a+ cells were detected as activated NK (part C, E). Parts D and H show the production of cytotoxic molecules perforin and granzyme B by NK from peripheral blood and ascites, respectively.

Compensation matrix was generated automatically in BD FACSDiva. Software after acquisition of data is presented in the Supplemental file. Instrument configuration and antibody panel are presented in Table 1. Statistical analysis was performed using Statistica 10.0 software package. In the tables the results are presented as medians with interquartile ranges, Me (25–75%). Significance of differences was assessed by the Mann-Whitney U test and Kruskal-Wallis test.
Fig. 1. The flow cytometry gating for NK-cell populations from peripheral blood and ascites in patients with ovarian cancer. Figure provides a representative dot plots gating for ascitic fluid and peripheral blood. Part A presents a lymphocyte gate on a forward scatter/side scatter plot for samples from peripheral blood. Part E selects CD45+ cells as leucocytes for following gates of cells obtained from ascites. Parts B, F show the gates of CD45+CD56+ cell as NK. Part C, G select CD56+CD107a+ cells detected as activated NK. Parts D and H show the production of cytotoxic molecules perforin and granzyme B by NK from peripheral blood and ascites, respectively.
Results

Comparative characteristics of the content of NK cells and their functional status of the control group, patients with ovarian and colorectal cancers are shown in Table 2. The content of NK cells in the peripheral blood of healthy donors and colorectal cancer patients did not differ. The content of NK cells in peripheral blood of ovarian cancer patients was significantly reduced. In healthy donors and colorectal cancer patients less than 1% of the population of peripheral blood NK cells were activated, namely they had a surface expression of CD56+CD107a+.

Table 1. Instrument configuration and antibody panel

| Laser (nm) | PMT position | Filter (nm) | Fluorochrome | Utilized panel | Dose | Localization of target |
|------------|--------------|-------------|--------------|----------------|------|------------------------|
| 488        | A octagon    | 780/60      | PE-Cy7       | CD56           | 5 μl | Surface                |
| 488        | B octagon    | 670         | PerCP-Cy5.5  | CD45           | 20 μl| Surface                |
| 488        | D octagon    | 585/42      | PE           | Granzyme B     | 5 μl | Intracellular          |
| 488        | E octagon    | 530/30      | FITC         | Perforin       | 20 μl| Intracellular          |
| 633        | C trigon     | 660/20      | APC          | CD107a         | 5 μl | Surface                |

Table 2. Amounts of NK cells and their subpopulations in peripheral blood of healthy donors, ovarian cancer and colorectal cancer patients, $Me$ (25–75%)

| Cell population       | Healthy donors   | Ovarian cancer | Colorectal cancer | $p$ |
|-----------------------|------------------|----------------|-------------------|-----|
|                       | Healthy donors   | Ovarian cancer | Colorectal cancer |     |
| CD45+CD56+            | 12.3 (8.1–14.5)  | 5.7 (2.9–6.7)  | 10.10 (7.9–13.4)  |     |
| CD56+CD107a+          | 0.13 (0.1–0.5)   | 2.75 (0.5–7.7) | 0.65 (0.5–1.1)    |     |
| CD107a+GB+PF–         | 16.6 (6.3–31.1)  | 3.28 (0.4–16.4)| 3.49 (0.20–18.2)  |     |
| CD107a+GB+PF+         | 27.6 (2.9–49.3)  | 32.3 (1.10–43.4)| 13.4 (0.80–50)    | > 0.05 |
| CD107a+GB–PF–         | 47.9 (10.8–78.2) | 59.2 (53.2–91.7)| 78.5 (61.4–92.3)  |     |
| CD107a+GB–PF+         | 7.80 (0.5–8.7)   | 5.14 (0.5–25.0)| 4.58 (0.4–20.0)   | > 0.05 |

Note: $p_{1,2} < 0.05$ – significance of differences between the control group and ovarian cancer patients, $p_{1,3} < 0.05$ – significance of differences between the control group and colorectal cancer patients, $p_{2,3} < 0.05$ – significance of differences between parameters in patients with ovarian and colorectal cancers, GB – granzyme B, PF – perforin

Table 3. Amounts of NK cells and their subpopulations in patients with colorectal cancer depends on the size and nodal status, $Me$ (25–75%)

| Cell population       | T2     | T3+T4   | $p$    | N0     | N1+N2   | $p$ |
|-----------------------|--------|---------|--------|--------|---------|-----|
| CD45+CD56+            | 9.10   | 11.1    | > 0.05 | 10.0   | 10.1    | > 0.05 |
| CD56+CD107a+          | 0.70   | 0.58    | > 0.05 | 0.65   | 0.80    | > 0.05 |
| CD107a+GB+PF–         | 8.90   | 0       | > 0.05 | 5.81   | 2.80    | > 0.05 |
| CD107a+GB+PF+         | 7.14   | 20.7    | < 0.05 | 8.25   | 2.80    | > 0.05 |
| CD107a+GB–PF–         | 87.5   | 75.0    | > 0.05 | 77.9   | 83.7    | > 0.05 |
| CD107a+GB–PF+         | 6.16   | 4.30    | > 0.05 | 4.95   | 2.80    | > 0.05 |

Note: GB – granzyme B, PF – perforin, T2, T3, T4 – tumor size, N0 – group of patients with no lymph node involvement, N1+N2 – group of patients with lymphogenous metastases

Table 4. Amounts of NK cells and their subpopulations in patients with colorectal cancer depends on the size and nodal status, $Me$ (25–75%)

| Cell population       | T2     | T3+T4   | $p$    | N0     | N1+N2   | $p$ |
|-----------------------|--------|---------|--------|--------|---------|-----|
| CD45+CD56+            | 9.10   | 11.1    | > 0.05 | 10.0   | 10.1    | > 0.05 |
| CD56+CD107a+          | 0.70   | 0.58    | > 0.05 | 0.65   | 0.80    | > 0.05 |
| CD107a+GB+PF–         | 8.90   | 0       | > 0.05 | 5.81   | 2.80    | > 0.05 |
| CD107a+GB+PF+         | 7.14   | 20.7    | < 0.05 | 8.25   | 2.80    | > 0.05 |
| CD107a+GB–PF–         | 87.5   | 75.0    | > 0.05 | 77.9   | 83.7    | > 0.05 |
| CD107a+GB–PF+         | 6.16   | 4.30    | > 0.05 | 4.95   | 2.80    | > 0.05 |

Note: GB – granzyme B, PF – perforin, T2, T3, T4 – tumor size, N0 – group of patients with no lymph node involvement, N1+N2 – group of patients with lymphogenous metastases

The content of NK cells in peripheral blood of ovarian cancer patients was significantly reduced. In healthy donors and colorectal cancer patients less than 1% of the population of peripheral blood NK cells were activated, namely they had a surface expression of CD56+CD107a+. This population in ovarian cancer patients was signifi-
cantly higher. Subpopulations of activated NK cells of healthy donors were significantly different from patients with malignant tumors. CD107a+GB+PF– NK cells were more frequently detected in healthy individuals. The percentage of CD107a+GB–PF– NK cells in healthy donors was lower than in cancer patients. Comparative characteristics of the NK-cell subpopulations in patients with colorectal cancer depending on the size and nodal status are presented in Table 3. The analysis of the content of NK cells and their subpopulations in peripheral blood of patients with colorectal cancer showed that invasive growth resulted in NK cells functional activity with a decreased percentage of CD107a+GB+PF– and increased percentage of CD107a+GB+PF+ NK cells. However, involvement of lymph nodes in colorectal cancer patients did not affect the content and the activation of NK cells.

Comparative characteristics of the content and NK-cell subpopulations in ascites and the peripheral blood in patients with ovarian cancer and BOT are shown in Table 4 and in Figure 1. As in BOT and in advanced ovarian cancer patients, both the number NK cells and the number of activated killers in ascites were significantly higher compared with their number in the peripheral blood. However, the percentage of activated NK cells in ascites in BOT patients was significantly higher than that in ascites in ovarian cancer patients. Activated NK cells population containing a complete set of lytic enzymes in the granules were observed in peripheral blood and ascites of BOT patients. While CD107a+GB–PF– population (85.6%) totally dominated in patients with ovarian cancer in the peripheral blood, CD107+GB+PF– and CD107+GB–PF– populations were equally observed in ovarian cancer patients in ascites.

**Discussion**

Our results as to the number of NK cells in peripheral blood of patients with colorectal cancer and ovarian cancer are consistent with literature data. The number NK cells in peripheral blood is significantly decreased in patients with disseminated forms of ovarian cancer [12]. However, there are contradictory data on both reduction and increase in the number of peripheral blood NK cells in colorectal cancer patients [13–15]. In our study no significant differences in the number of peripheral blood NK cells between colorectal cancer patients and healthy donors was found.

It should be noted that although the absolute number of peripheral blood NK cells in ovarian cancer patients was reduced, the percentage of activated cells having surface expression of CD107a, was the highest among all groups. According to data of Carlsten et al., about 1–2% of NK cells of healthy donors have surface expression of CD107a [16]. In our study, the appearance on the surface CD107a is viewed as a sign of degranulation of the NK cell in response to intrinsic environment factors of patients with malignant tumors.

Functional activity of peripheral NK cells in cancer patients was significantly changed compared to healthy donors. Namely, the percentage of CD107a+GB+PF– NK cells was reduced and the percentage of CD107a+GB–PF+ NK cells was increased. Population of CD107a+GB–PF– NK cells could be considered as a pool of functionally defective cells that do not contain active cytotoxic enzymes in the granules, and as a subset of degranulated cells, since surface-expressed CD107a protects NK cells from apoptosis after degranulation [6].

It has previously been shown that the peripheral blood of colon cancer patients, NK cells showed an altered phe-
and defects in the ability to activate degranulation and IFNγ-production [17]. We were the first to show the relationship between the functional activity of peripheral blood NK cells in colorectal cancer patients and invasive tumor growth and lymphogenous metastasis. The increase in the size of primary tumors in colorectal cancer patients led to the increase in the proportion of activated peripheral blood NK cells, cytolytic granules containing enzymes GB and PF. Despite the fact that along with the lymphogenous metastasis formation, hematogenous metastases to the liver is the major form of tumor progression of colorectal carcinomas, the presence or absence of lymphogenic dissemination had no significant effect on the amount and subpopulations of NK cells.

Although the number of NK cells in the ascites in patients with ovarian cancer is high, their functional usefulness is quite contradictory. In our study, the number of perforin and granzyme B positive-positive cells in the peripheral blood and ascites of ovarian cancer patients was significantly reduced. According to data obtained by Lukesova et al., the population of CD56 bright NK cells, having a weak cytolytic activity in the ascites of patients with ovarian cancer prevailed [12]. It is currently believed that the population of CD56 bright and CD56 dim reflect different stages of maturation and differentiation of lymphocytes. More mature population is expressed by CD56 dim. It is believed that in ascites, NK cells arise from lymph nodes as a result of their tumor compression of the conglomerates and the blockade of the lymphatic pathways. CD56 bright accounts for up to 75% of the NK cells in normal lymph nodes, they have low cytotoxicity, but high cytokine-producing activity, are capable of interacting with dendritic cells and play an important role in the regulation of adaptive immune response [18].

In 2007, Bamias et al. showed a significant difference in the amount and subpopulations (based on the combination of surface antigens and receptors) of lymphocytes in the peripheral blood and ascites of patients with ovarian cancer with dominant subpopulations of Treg CD4+CD25+ and NKT-cells with the phenotype of CD3+CD56+ in ascites [8]. The association of these populations with VEGF and TNF alpha levels in ascites, as well as an association with resistance to platinum-based chemotherapy were revealed [19]. These data are confirmed by studies of different subpopulations in peripheral blood and ascites cytotoxic cells in ovarian cancer patients [12]. According to our data, the level of NK cells in peripheral blood was significantly reduced as compared to ascites in BOT patients. A similar trend was found in patients with advanced ovarian cancer. However, the percentage of activated NK cells in the ascites was significantly higher in BOT patients compared to ovarian cancer patients. The difference in functional activity of NK cells in ascites and peripheral blood of ovarian cancer patients requires further investigation of the lymphocyte receptor status and cytokine-producing function of NK cells. Due to the constant accumulation of data on the state of the local immune system in patients with ovarian cancer, the development of intraperitoneal chemotherapy and intraperitoneal immunotherapy of ovarian cancer [20], there is no doubt that the studies on the subpopulations and functional activity of NK cells are relevant to study possible ways of regulating the expression of NK-cell receptors and its intracellular major components.

Conclusions

The presence of malignant tumor and its dissemination influence the content and the functional activity of NK cells. The increase in the size of primary tumors in colorectal cancer patients led to the increase in the proportion of activated peripheral blood NK cells, cytolytic granules containing enzymes granzyme B and perforin. However, involvement of lymph nodes in the tumor process in colorectal cancer did not affect the content and activation of NK cells. Accumulation of free fluid in the abdominal cavity in patients with benign as well as in patients with malignant ovarian tumors was accompanied by a significant decrease in the level of NK cells in peripheral blood and its increase in ascites. The differences in the functional activity of NK cells in ascites and the peripheral blood in ovarian cancer patients require further investigation in the lymphocyte receptor status and cytokine-producing function of NK cells.

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The authors declare no conflict of interests.

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