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IL-2 AND TNF-α ACTIVITY IN OVARIAN CANCER DEPENDS ON THE STAGE OF DISEASE

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In present study the changes of immunoregulatory cytokine IL-2 and pro-inflammatory cytokine TNF-α activity in pathophysiology of ovarian cancer has been investigated. IL-2 and TNF-α activity have been determined by the ELISA method in plasma and tissue samples of the primary oncologic patients with different stages of disease (I, II, III). Results obtained have shown that IL-2 and TNF-α levels for both plasma and tumor tissue samples demonstrate stage dependent changes. Data obtained expand the knowledge about the complicity of the stage dependent interplay between host immunity, inflammation and ovarian cancer.

Interleukin-2 – tumor necrosis factor alpha – ovarian cancer – inflammation

Ներկայացված աշխատանքում ուսումնասիրվել են իմունակարգավորիչ ցիտոկին ինտերլեյկին-2-ի և նախաբորբոքային ցիտոկին ուռուցքների նեկրոզի գործոն-ալֆայի ակտիվության փոփոխությունները ձվարանների քաղցկեղի պաթոֆիզիոլոգիայում: Ցիտոկինների ակտիվությունները չափվել են ELISA մեթոդով՝ հիվանդության տարբեր փուլերում գտնվող (I, II, III) առաջնային քաղցկեղային հիվանդների պլազմայի և հյուսվածքային նմուշներում: Ստացված արդյունքները ցույց են տալիս, որ նշված ցիտոկինների ակտիվության փոփոխությունները և պլազմայի, և հյուսվածքային նմուշներում կախված են հիվանդության փուլից: Այս տվյալներն ընդլայնում են մեր գիտելիքներն իմունային համակարգի, բորբոքման և ձվարանների քաղցկեղի միջև փուլից կախյալ փոխազդեցությունների ողջ բարդության վերաբերյալ:

Ինտերլեյկին-2 – ուռուցքի նեկրոզի գործոն ալֆա – ձվարանների քաղցկեղ – բորբոքում

В статье были изучены изменения активности иммуномодуляторного цитокина ИЛ-2 и противовоспалительного цитокина ФНО-альфа в патофизиологии рака яичников. Активность ИЛ-2 и ФНО-альфа была определена методом ИФА в плазме и в тканях первичных онкобольных с различными стадиями болезни (I, II, III). Полученные результаты показывают, что и в плазме, и в ткани изменения активности ИЛ-2 и ФНО-альфа зависят от стадии заболевания. Полученные данные расширяют знания относительно всей сложности стадиязависимых взаимодействий между иммуной системой, воспалительным процессом и раком яичников.

Интерлейкин-2 – фактор некроза опухолей альфа – рак яичников – воспаление

Ovarian cancer (OC) is the 8th most common type of cancer and is still the deadliest form of all gynecologic malignancies in women worldwide [20]. In Armenia, OC is the 4th leading gynecological cancer with the prevailing age above 45 years [20]. Overall, ovarian cancer patients respond to cytoreductive surgery and platinum and taxane

based combination chemotherapy. However, advanced cases have a high recurrence rate and the 5-year survival rate has remained largely unchanged since the 1980s, being close to 30 % [16]. This, however, is skewed, given the mass of recent evidence suggesting that histological subtypes of ovarian cancer represent unique diseases, but are still treated with a 'one size fits all' approach.

Epidemiological and experimental data have implicated hormonal fluctuations in sex steroids, and androgen exposure in particular, in the pathogenesis of ovarian cancer [12]. Moreover, ovarian cancer cells share a number of estrogen regulated pathways with other hormone-dependent cancers such as breast and endometrial cancer [15]. These pathways were studied in more details already [7, 15]. It is considered that the inflammatory environment caused by repetitive ovulation over the life time of women increases the risk of genetic error and mutation during the repair process, leaving the ovarian surface epithelium susceptible to neoplastic transformation in subsequent ovulation and repair cycles [17]. However, ovarian cancer is a heterogeneous disease and its subtypes are quite different with respect to mutations, origins, marker and prognosis, and respond differently to standard chemotherapy.

The immune system is thought to be an important mediator of ovarian carcinogenesis [4] due to persistent infiltration of immune cells during the rupture and remodeling phases of ovulation. This causes injury to surrounding epithelium, damage DNA through release of reactive oxygen species, or produce cytokines that promote proliferation. Interactions between tumor and host immune system are objectives of special interest among current topics of research. Recent studies also show that anti-tumor immunity is often negated by immune suppressive cells present in the tumor microenvironment. These suppressive immune cells also directly enhance the pathogenesis through the release of various cytokines and chemokines, which together form an integrated pathological network. IL-2 is a potent pro-inflammatory cytokine that is secreted by Th-1 cells and effectively participates in the activation of T cells to produce the cytokines tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [4]. Recent studies showed that IL-2 plays a critical role in the differentiation and survival of regulatory T cells, thereby ensuring their significance in the control of the immune response [4]. Thus, IL-2 plays multiple roles in immune functions by contributing to the generation and propagation of antigen-specific immune responses [3]. The ability of IL-2 to expand T cells with maintenance of functional activity has been translated into the first reproducible effective human cancer immunotherapies. Anti-humoral therapy associated with IL-2 administration led to the remission of metastatic renal cell carcinoma in up to 30% of patients and increased the survival of patients with melanoma and acute myeloid leukemia [3]. In these cases, the administration of high doses of IL-2 was associated with improved survival, although, the related adverse effects were considerably severe in most patients [3]. While adoptive transfer of these activated T cells could mediate modest antitumor activity, the inability to grow these cells ex vivo severely limited progress, and the lack of human cells with specific antitumor reactivity was a major obstacle to the application of this approach in humans.

In contrast to these immunomodulatory agents that directly increase or decrease specific immune cell subsets, another concept of antitumor immunity that has recently been introduced is the disruption of integrated cytokine networks [10]. The tumor milieu in which ovarian carcinoma develops has been described as one enriched with a broad spectrum of pro-inflammatory cytokines and chemokines. In particular, several of these cytokines (such as TNF-α, IL-1β, and IL-6) produced by tumor itself or/and activated immune cells, besides stimulating cancer cell growth, have been shown to influence clinical disease status and prognosis, by reducing responsiveness to chemotherapy and

symptoms such as anorexia, altered energy metabolism, anemia, weight loss, depression and fatigue [13].

Recent data show that cytokine antagonists may have a role in the treatment of ovarian cancer. Their action by inhibiting both production and activity of inflammatory cytokines seems to obtain the control of angiogenetic and apoptotic events, the reversal of chemoresistance, the improvement of systemic symptoms and prognosis [21]. Although as suggested by its name, TNF was described initially as a killer of cancer cells, however recent data indicate that TNF can be considered mostly as a pro-tumor cytokine. Numerous studies have shown that anti-TNF drugs reduce tumor growth in different types of malignances. This deleterious effect of TNF was further supported with the results in TNF-knockout mice model that display reduced tumor growth [18]. Currently, clinical trials aimed at inactivating TNF-α in solid tumors are under evaluation. A recent phase I study of the specific TNF-α antagonist etanercept (TNFRII:Fc fusion protein) in ovarian cancer provided biochemical (CA125) and radiological evidence for prolonged disease stabilization in 6/30 patients with advanced disease [21].

Thus, to understand how inflammation and cytokine expression in a tumor microenvironment can impact both host defense against the tumor and tumor cell survival, it is important to continue careful studies of cytokine networking to identify their transcriptional and posttranscriptional regulation. Complex understanding of posttranscriptional regulation of this cytokines, in particular, may lead to more potent drugs that target their expression. Recently we have shown the involvement of Ca^{2+}/CaM-dependent protein phosphatase calcineurin (CN) in pathophysiology of ovarian cancer, as well as demonstrated that changes in CN activity in plasma and tumor tissue of patients with primary ovarian cancer depend on stage of disease and degree of tissue differentiation [20]. Considering all abovementioned and the fact that CN participates in the synthesis of several cytokines, including IL-2 and TNF-α, via dephosphorylation and activation of NFAT (nuclear factors of activated T cells) transcription factors [9, 14], we considered advisable to study the changes in IL-2 and TNF-α activity in both tissue and plasma samples from primary ovarian cancer patients depending on the stage of disease.

Materials and methods. *Sample collection and preparation.* The blood and tissue samples from postoperative material of untreated patients with the I (n=6), II (n=7) and III (n=5) stages of primary ovarian cancer were provided by the National Centre of Oncology after V.A. Fanarjyan (NCO MOH RA). The plasma of healthy donors (n=6) and histologically checked healthy parts of remote tissue (n=8) were used as a control. Histological study of the postoperative material was conducted by the Laboratory of Clinical Pathomorphology at the NCO MOH RA. The most cases of ovarian cancer were diagnosed as a moderately and poorly differentiated adenocarcinoma. Age of patients ranged from 45-80, and the average age was 61 years.

Blood (1.5-2 ml) was collected into sodium citrate (3.2 %)-coated vacutainer tubes and centrifugated at 1500 rpm for 10 min. Plasma was separated and stored at -32°C. Tissue samples were homogenized with 5 volumes of 50 mM Tris-HCI, pH 7.5 buffer, containing 0.05 % Triton-X-100, 0.1 mM EDTA, 1 mM ditiothreitol (DTT), protease inhibitors, and centrifugated at 20000×g for 60 min at 4ºC. The supernatant was separated and stored at -32°C as well.

The protein content in samples was determined by Bradford assay [2].

TNF-α and IL-2 assays. TNFα and IL-2 levels in plasma and tissue samples were measured using human TNFα ELISA MAX kit (BioLegend Inc., USA) and human IL-2 ELISA MAX kit (BioLegend Inc., USA) according to the manufacturer's recommendation. The optical density was measured in each well at the wavelength of 450 nm using LABLine-022 microplate reader (LABLINE Diagnostics, Austria).

Statistical analysis. Data were analyzed statistically by one-way ANOVA using Origin 61 software. Statistical significance – $p<0.05$. All data were expressed as mean±SEM.

Results and Discussion. As one can see, the significant increase in plasma level of IL-2 of ovarian cancer patients takes place (4.4 fold) in the I stage of disease compared to healthy control group (fig. 1). The statistically significant decreases in plasma levels of IL-2 were observed in II stage (1.7 fold) and III stage (2.6 fold) of disease compared to the I stage.

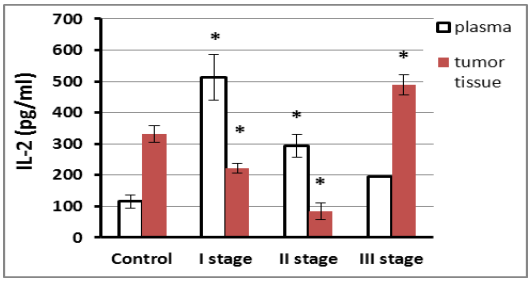

Fig. 1. IL-2 levels in plasma (white) and tumor tissue (red) primary ovarian cancer patients.
*-p<0,05 for the I stage compared to control, and for II, III stages compared to the I stage.

In contrary, as shown in fig.1, tumor tissue level of IL-2 was found to be decreased in the I stage of disease by 1.5 times compared to control group and by 2.6 times in the II stage compared to the I stage. For the III stage of disease the significant increase (2.2 fold) takes place in tissue level of IL-2 compared to the I stage.

As one can see from fig. 2, TNF-α level have shown to be decreased for the first stage of disease in both plasma and tumor tissue samples by 1.18 and 4.5 times respectively compared to the control groups (fig. 2). However, in plasma the decrease in TNF-α level was not statistically significant. Plasma and tissue levels of TNF-α have shown statistically significant increase in the II and III stages of disease compared to the I stage. In plasma of ovarian cancer patients TNF-α level has been shown to be increased by 1.5 and 1.6 times for the II and III stages, respectively, compared to the I stage. Similarly, tumor tissue TNF-α level has been shown to be increased by 4.4 and 5 times for the II and III stages, respectively, compared to I stage.

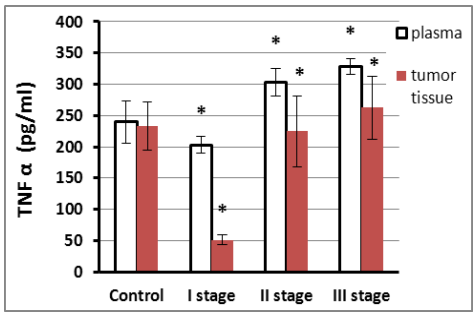

Fig. 2. TNFα levels in plasma (white) and tumor tissue of (red) of primary ovarian cancer patients.
*-p<0,05 for the I stage compared to control, and for II, III stages compared to the I stage.

Immune system is widely integrated in all steps of OC carcinogenesis, including host response to tumor initiation and progression. The main components of host-tumor immune response including pro/anti-inflammatory networks are in part regulated by CD4+ helper T-lymphocytes, Th1 and Th2 cells. Th1 cells produce pro-inflammatory cytokines

and drive the cell-mediated immune response, while Th2 cells regulate humoral immunity by expressing anti-inflammatory expressing anti-inflammatory cytokines. The Th1-Th2 balance is closely correlated with cancer, but the correlation in untreated OC patients remains unconfirmed [8]. IL-2 is a potent pro-inflammatory cytokine secreted by activated macrophages, Th1, DC, CD8+ cells. As mentioned above, transcription and expression of IL-2, as well as TNF-α, is regulated by Ca^{2+}/CaM/calcineurin/NFAT signaling pathway which has a key role in activation of the immune system [9, 14, 24]. Our data obtained have been indicated that the activity of IL-2 is changed differently in plasma and tumor tissue of OC patients among the subsequent stages of OC development. Via activation of IL-2 expression CN plays a central role in the activation of immunosuppressive T_{reg} cells to produce anti-inflammatory cytokines [22]. However, clinical manipulation of IL-2 levels remains complex as IL-2 can act both as an immune stimulating or suppressive cytokine, depending on the dose. On one hand, low-doses of IL-2 favor T_{reg} survival and suppressive function and lead to a better control of autoimmune and inflammatory diseases [1]. On the other hand, high-dose IL-2 administration in ovarian cancer may result in the conversion of tumor-associated T_{reg} into Th17 cells, relieving T_{reg}-mediated suppression, and contributing to enhance antitumor immunity via changing a non-inflamed tumor into an inflamed tumor, shifting produced cytokines type onto TNF-α and IFN-γ direction, perhaps thereby increasing sensitivity of the tumor to further immune attack [12]. From this point of view the significant increase of plasma IL-2 activity and decrease of tumor tissue IL-2 activity in the I stage of disease may be a protective response of the organism to fight against malignant neoplasm. However, similar chronic activation of the immune system contributes to chronic systemic release of inflammatory cytokines and formation of the chronic inflammatory environment. Thus, the immune system initially playing a protective role for the organism during tumorigenesis, diminishes gradually and eventually this could contribute to tumor progression. Indeed, there are different mechanisms by which tumors evade the control of the immune system. Many types of tumor cells secrete immunosuppressive cytokines (IL-6, IL-10, IL-13) and chemokines that negatively regulate the immune system. In current study increasing tumor tissue activity of IL-2 in the III stage of disease is most probably evidencing the existence of tumor milieu/stage dependent shift from anti-inflammatory T_{regs} towards inflammation favoring Th17. This IL-2 driven exacerbation of inflammation could also lead to overproduction of other pro-inflammatory cytokines, including TNF-α, as the major one. In ovarian cancer, several cytokines associated with cellular immunity were correlated to cancer development and patient prognosis, including TNF-α, IFN-γ, IL-6, and MHC molecules [16]. TNF-α plays a complex role in cancer and tumor immunity because of its pleiotropic effect and the fact that it has two receptors. It is now recognized that the expression of TNF receptor 2 (TNFR2) is more limited than that of TNF receptor 1 (TNFR1). New evidence suggests that the TNFR1 drives a predominantly pro-inflammatory program whereas TNFR2 primarily initiates immune modulation and tissue regeneration via boosting T_{regs} and effector T cells to produce IL-2 and promotes the sensitivity of T_{regs} to IL-2 [6]. TNF-α production in the ovarian cancer microenvironment has been recognized for decades, with tumor-infiltrating macrophages likely to be the major source [23]. Numerous reports have demonstrated abnormally high levels of TNF-α in the blood of OC patients. Within groups of patients with the same tumor type, higher levels of TNF-α correlated with more advanced tumor stage and shorter survival time. However, circulating TNF-α is not always detectable in cancer patients and can vary among individual patients over time and the course of disease [5]. Charles et al. demonstrated the importance of TNF-α in promoting tumor growth and in the progression of ovarian malignancies. It was found that in mice TNF-α exerts the effect

markedly in a time-dependent and dose-dependent manner [6]. The initial exposure to TNF transiently abrogates T_{reg} suppressive functions, whereas longer exposures restore their suppressive activity. This means that short-term stimulation of TNF-α mimics the early phases of the inflammatory reaction, thus allowing effector T cells to escape from the inhibition mediated by T_{regs}, presumably favoring elimination of the pathogens. However, long-term exposure to TNF may facilitate the activation and expansion of T_{regs}, restoring their suppressive capabilities, thus limiting excessive inflammation. In the present study, we have found that activity of TNF-α was displaying an initial decrease followed by up wording tendency in ovarian tumor epithelium. The initial decline for TNF-α activity in the I stage of disease indirectly indicates the activation of anti-inflammatory pathways via activation of T_{reg} population, however, further increase of TNF-α levels for II and III stages is more likely to display continuous fluctuations between T_{reg} and Th17 with an increasing prevalence of Th17 with their pro-inflammatory effects. Whether T_{reg}-IL-2-TNF-α-Th17 interactions are prompting T_{reg} activation or favoring Th17 differentiation due to complicacy of interactions in the frame of current research is hard to purpose. Although at this stage of the research existing data are not sufficient to evidence for dysregulation in Ca^{2+}/CaM/CN/NFAT-TNF-α signaling pathway in a tumor microenvironment, however, this may indicate that posttranscriptional regulation most probably could take place.

Thus, further studies aiming at understanding how IL-2 and TNF-α are regulated in tumor context most probably would determine how they could be involved in effective anticancer therapy for OC.

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