Research Article:
Increased Tumor Necrosis Factor-alpha Expression as an Inflammatory Response Indicator and a Cancer Risk in Polycystic Ovarian Syndrome

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ABSTRACT

Background: Polycystic Ovarian Syndrome (PCOS) is a pro-inflammatory condition that supports the development of metabolic aberration and ovarian dysfunction. Chronic inflammation and the increased levels of androgens in this group of patients and their impact on the immune system may increase the risk of developing malignancies, including ovarian cancer. Thus, we interacted ovarian tumor cells with Peripheral Blood Mononuclear Cells (PBMC) to evaluate some of their responses to the tumor microenvironment.

Materials and Methods: PBMC were collected from 25 patients with PCOS and 25 healthy women and isolated by Ficoll density gradient centrifugation. Then, we measured cell proliferation and Tumor Necrosis Factor-alpha (TNF-α) concentration at different time intervals (48 and 72 hours) after co-cultivation of ovarian tumor cell lines (SKOV3, A2780) with PBMC in an indirect contact transwell system.

Results: The proliferative response of effector cells during stimulation with tumor cell lines demonstrated no statistically significant difference between the patients and healthy groups, despite lower mean score in the control group. The proliferation rate after 72 h was significantly higher than that of the 48-h interval (P<0.01). The production of TNF-α in the co-culture of the A2780 cell line significantly increased in the patients at different time intervals compared to the controls (P<0.05).

Conclusion: We observed an increased proliferative response of effector cells and TNF-α production in PCOS patients compared to healthy individuals. This suggests a low grade of chronic inflammation that is the immunological feature of the ovary in PCOS patients. However, an increased risk of cancer in patients with PCOS requires further in vitro investigation of other aspects of anti-tumor responses using diverse sample size. Additional, exploring other immune cytokine profiles could be beneficial.
Introduction

Polycystic Ovarian Syndrome (PCOS) is an endocrine disease with the prevalence rates of 11.1% according to Androgen Excess Society (AES) [1] or 14.6% according to the Rotterdam consensus (Rot) [2, 3]. PCOS is a pro-inflammatory condition. Prior research suggests that chronic low-grade inflammation supports the development of metabolic deviation and ovarian dysfunction in people with PCOS [4-7]. Delayed oocyte competence is among the most causative factors for infertility in a woman with PCOS. Changes in the cytokine profiles and cellular content of ovarian follicular fluid in PCOS patients, including macrophages and T lymphocytes seem to develop many problems leading to hormonal dysregulation. They are also involved in follicular maturation. Additionally, various metabolic and inflammatory complications exist in these patients [8].

Chronic inflammation process is associated with increased proinflammatory chemokines and cytokines, including Interleukin-18 (IL-18), MCP, and MIP. PCOS is also correlated with an increased level of all these factors [9-12]. Meanwhile, the roles of human preovulatory follicles including granulosa cells, leukocytes, T cells, and B cells have been described in mediating diverse ovarian functions. This process is performed by secreting numerous cytokines and chemokines [13-15]. Moreover, they are effective in the hormonal immunoregulation of folliculogenesis [16, 17].

Factors involving inflammation in PCOS patients like oxidative radicals and cytokines have been associated with this period in different inflammatory responses [18]. Furthermore, many reports demonstrate the association between autoimmune markers and chronic mild inflammation in PCOS patients. Women with PCOS are affected by adverse autoimmune reactions and inflammations, which expose many antigens in tissue destruction like Antinuclear Antibodies (ANA). Ovarian antibodies and anti-Glutamic Acid Decarboxylase (GAD), are the most prevalent autoantibodies [19, 20]. Therefore, women with PCOS suffer from immunological disturbances (the simultaneous roles of inflammation and immune regulation), leading to immune system inflammation. As a result, these women could be predisposed to different types of immune system disorders like tumor development [6, 21].

Inflammation is as a risk factor for the development of cancer through providing bioactive molecules from the infiltrating cells into the tumor microenvironment. This process leads to the maintenance of sustained proliferation speed as well as cell survival signals [22-26].

Ovarian cancer is the fourth cause of fatal malignancies in developed countries. It has been investigated in research projects associated with the susceptibility of women with PCOS [27, 28]. The risk of developing ovarian cancer is 2.5 times higher among patients with PCOS compared to the healthy population [29]. Ovarian cancer is categorized among immunogenic tumors [30, 31]. Despite the availability of different modern therapeutic protocols, the majority of the patients with ovarian cancer experience recurrence following the initial treatment. This is usually due to the late diagnosis when the malignancy is disseminated [32-36].

PCOS is a suspect in increasing the risk of ovarian cancer malignancy [2, 27, 37]. The development of a clinically detectable tumor requires the support of tumor microenvironment. Inflammatory cells and immunoregulatory mediators present in the tumor microenvironment evoke host’s immune system responses towards specific phenotypes in tumor development [38]. Therefore, tumor microenvironment plays an important role in the development of ovarian malignancies. Furthermore, ovarian cancers create the most active and strongest molecular microenvironments [39]. Ovarian cancer is among malignancies that due to the presence of inflammatory factors in invasive tumor microenvironment can affect the immune system. Additionally, despite their immunologic nature, they transform the immune responses [40]. Also, the particular precancerous conditions of women with cancer such as lifestyle and nutrition could affect them and transform the tumor growth pathway [22].

Two different mechanisms prevent the development of the tumor. These mechanisms operate based on the cellular and molecular characteristics of the microenvironment of invasive tumors like ovarian cancer. The first group of mechanisms based on the production of T cell inflamed phenotype. This way, T cells with inflammatory phenotype are infiltrated around the tumor with an extensive chemokine profile and interferon type I that refers to innate defenses. In fact, these are a subset of T cells with an innate suppressive function and probably are of either NKT or Tγδ types. The second group of mechanisms is associated with a non-T cell-infiltrated phenotype that forces the immune system to ignore the tumor. Thus, it is resistant to immune system attack [41].

One of the procedures to investigate the immune status of women with PCOS regarding its susceptibility to malignancies is the evaluation of Peripheral Blood
Mononuclear Cells (PBMC) interaction with tumor microenvironment. These are considered as the major cytokine secreting cells of the immune system. Under the laboratory culture conditions, their vicinity with cancer cells in an active co-culture system could demonstrate the events that possibly occur in vivo when immune cells face tumor cells. It could also a response by secreting different cytokines and proliferate responses, including Tumor Necrosis Factor-alpha (TNF-α) release and cytotoxic cells (CD4+/CD8+) response [42-44].

Materials and Methods

Study groups

Twenty-five women with PCOS, who had their medical files in the Reproductive Endocrinology Research Center affiliated to Shahid Beheshti University of Medical Sciences in Tehran, Iran, participated in the study. The subjects were selected according to the inclusion criteria comprising clinical indices and laboratory parameters. The healthy (control) group consisted of 25 age-matched women. The exclusion criteria were histories of cancer, immunodeficiency caused by immunosuppressive medications, the presence of infectious diseases, and systemic disorders. Individuals with a family history of cancer, those with early menopause, and substance dependents were excluded from the study. Blood samples were obtained during the research period.

Tumor cell line culture

Tumor cell lines A2780 and SKOV3 were obtained from the Cell Bank of Institute Pasteur in Karaj City, Iran. The cell lines were cultured in RPMI medium (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin 100 U/mL (Atocel, Austria) and maintained in a humidified 5% CO₂ incubator at 37°C. Following the log phase of growth and propagation, at 80% confluency and cell viability (90-95%), sufficient passages were made and maintained at -80°C for future use or as a backup data.

Isolation of Peripheral Blood Mononuclear Cells

The blood samples were diluted with the culture medium at a ratio of 1:1. Then, the cells were layered onto Ficoll and centrifuged at room temperature. Later, mononuclear cells located between the Ficoll and plasma layers were aspirated and suspended in Complete Tissue Medium (CTM). The suspension was made from the cell pellet, stained with trypan blue. Moreover, the cell count was performed and viability was determined.

Cell co-culture system

Transwell 24-well plates with a pore size of 0.4 µm (SPL, Korea), were used to co-culture tumor cell lines and PBMCs. A day before the experiment, tumor line cells were cultured at 5 × 10⁴ cells per well and incubated at 37°C. Later, cell (PBMCs) counts were performed and a total of 2 × 10⁶ cells per well was added to the upper chamber. The cell ratio considered for the executive cells (PBMCs) and target cells (tumor cell lines) was 1:4. The incubation time continued up to 72 h. A negative control was prepared by adding PBMCs to each well in the absence of tumor cell lines. The samples were separately created from the supernatants of cancer cell lines and PBMCs cultures in Transwell chambers at two time intervals of 48 and 72 h following co-culture.

Here, we explain the activity of ovarian tumor cell line in secreting TNF-α demonstrated by the previous research. TNF-α release by many ovarian cancer cell lines (2021G-IGRov-1) is accompanied by other cytokines and chemokines (IL6-VEGF-MIF and CXCL12). However, there was no significant difference between the levels of TNF-α for SKOV3 cell lines and other co-cultured groups [45]. In addition, another study explored TNF-α production and secretion in the A2780 ovarian cancer cell line. It reported that only during the treatment of sustained culture system with docetaxel and LPS, the release of TNF-α was detected in taxon-induced inflammatory cytokine production from tumor cells required drug accumulation in a time-dependent manner [46].

Evaluation of proliferative response of Peripheral Blood Mononuclear Cells

The proliferation rates of PBMCs during co-culture with cancer cell lines as well as in the absence of tumor cell lines were measured as controls at two time intervals using a commercial cell proliferation assay kit [Cell Proliferation ELISA, BrdU (colorimetric), Roche Diagnostic; Ref:11647229001]. The absorbance was measured at 450 nm with a reference wavelength at 690 nm.

Tumor Necrosis Factor-alpha concentration

The TNF-α level in the supernatants collected from the PBMCs co-cultured with and without tumor cell lines was determined and reported in pg/dL using a commercial sandwich ELISA kit (Quantikine, R&D Systems, USA), for human TNF-α, according to the manufacturer’s instructions. A limitation to this kit is that if samples generate values higher than the highest standard, the samples must be diluted with the appropriate Calibra-
tor Diluent and the assay must be repeated. This assay is
designed to eliminate interference by soluble receptors,
binding proteins, and other factors present in biological
samples. Until all factors have been tested in the Quan-
tikine Immunoassay, the interference was not possible.
The results were calculated using a standard curve.

**Immunophenotyping of Peripheral Blood Mono-
nuclear Cells**

The percentages of total mononuclear cells, as well as
TCD\(^3\)CD\(^8\) lymphocytes in cultured cells, were evaluated
by flow cytometry (FACSCalibur) at two time intervals.
For this reason, we used a combination of phycoerythrin-
conjugated anti-CD\(^6\) (PE, clone SK1), and fluorescein
isothiocyanate-conjugated anti-CD\(^1\) mAbs (FITC; Leu 4
FITC, clone SK7) generated by BD company for the hu-
man cell surface markers CD\(^6\) and CD\(^3\) (BD Biosciences,
Cat No: 340044).

**Statistical analysis**

Data analysis and graph drawing were conducted using
GraphPad Prism. One-way Analysis of Variance (ANO-
VA) and Independent Samples t test were performed to
compare the mean scores and evaluate the normal dis-
tribution of data, respectively. In cases of asymmetric
distribution of variables, nonparametric tests like Krus-
kal-Wallis test or Mann-Whitney U test were used. The
results were expressed as Mean±SEM (Standard Error
of Mean).

**Results**

**Lymphocyte proliferation**

The study revealed significant differences in the pro-
liferation of mononuclear cells in the presence of each
cell line (P<0.01). The mean lymphocyte proliferation
scores of PCOS and control groups in co-cultures of
SKOV3 cell line were higher than that of the A2789
cell line (Figure 1 A). Although the mean lymphocyte
proliferation score in PCOS group was higher than the
healthy group, the difference was insignificant (P>0.05
for both cell lines). Additionally, the impact of time was
significant in both cell lines (P<0.01). The mean lym-
phocyte proliferation score of both cell lines after 72 h
was lower than that of 48 h interval. The optical density
value in respect to the rate of proliferation in the control
group culture was subtracted from the OD reading of co-
cultures as the baseline (Figure 1 B).

**Tumor Necrosis Factor-α concentration**

Figure 2 A, compares the general mean TNF-α concen-
tration score in the case and control groups. This com-
parison suggested a significant effect associated with the
type of specimen, as a variable, only on the co-culture
with A2780 cell line (P=0.03). This was reflected in the
mean TNF-α concentration in PCOS patients following
culture with A2780 cell line which was higher than
the healthy group co-cultured with the same cell line.

As presented in Figure 2 B, the mean TNF-α concen-
tration score between each tumor cell line and the con-
trol group without cell line were compared under 72 h
of co-culture conditions. This comparison revealed a
significant difference between the mean TNF-α con-

**Figure 1.** Comparison of mean lymphocyte proliferation under co-culture conditions

A: Comparing the mean lymphocyte proliferation during co-culture with A2780 and SKOV3 cell lines; B: Comparing the mean
lymphocyte proliferation at two time intervals (48 and 72 h) during co-culture with A2780 and SKOV3 cell lines. The OD value
found for the rate of proliferation in the control culture was subtracted from the OD value of co-cultures as baseline and then
the findings were compared between the two case and control groups.
centration score secreted during the co-cultures (in both groups) with either A2780 or SKOV3 tumor cell lines and the control group without tumor cell line after 72 h (P=0.001). This finding reveals the higher levels of TNF-α in both tumor cell lines compared to the control group. Moreover, the effect of time was only significant in the lymphocyte of both groups in co-culture with A2780 tumor cell line (Figure 3 C) (P=0.04).

Expression of CD3 and CD8 markers on the surface of mononuclear cells

To identify the percentage of this cellular population and evaluate the stimulation status, the flow cytometry diagrams associated with the percentages of cytotoxic lymphocytes culture after 48 and 72 h in the patient group are illustrated in Figure 3. As shown in Figure 4, no significant correlation was observed in the mean percentage of cytotoxic cells between the two groups (P>0.1).

Discussion

Characterization of the tumor microenvironment in people with malignancy provides evidence for different immunological phenotypes based on the presence or absence of inflammation produced by T cells [41, 47]. Researchers assume that the immune system deregulation in PCOS preovulatory follicles contributes to ovarian processes by changing the level of inflammatory cytokines, including Th1 and Th2 [48].

Another issue is the presence of extensive heterogeneity in different types of tumor cell lines and in ovarian malignancies among patients with such conditions. However, it is not associated with the stage of disease and response to treatment [41]. The critical conditions associated with PCOS are reported as tumor-predisposing templates in many studies. Furthermore, the immune system during different stages of tumor malignancy causes extensive changes in invasion, expansion, and the tumor cell metastasis [40]. Additionally, the invasive and inflammatory characteristics of ovarian cancer cells [42], regarding producing IL-1, IL-6, and TNF-α cause numerous changes in the host, indicating an active interaction between the tumor and the host.

The cancer cell lines (SKOV3 and A2780) are enormously used in many research laboratories because of their numerous differences in invasive features [42, 49-51]. These dissimilar pairs of cancer cell lines form the
most common types of ovarian epithelial carcinomas. CA-125 (also known as MUC-16) is among the important factors in differentiating cancer cell lines. This peptide epitope of mucin is responsible for the suppression of the immune system, regeneration, and tolerance factor (pregnancy tolerance factor) [42, 52]. The expression rate of this marker in these two cell lines is different. In SKOV3 cell line it is expressed at higher degrees and this cancer cell line is the more serious form of the disease [53, 54].

Ovarian tumor cell lines produce and contain proinflammatory cytokines including IL-1, IL-6, and TNF-α [55]. The critical role of autocrine IL-6 production, in particular by SKOV3 cancer cell line, leads to tumor growth [56]. A strong correlation between high level of

Figure 3. Flow cytometry diagrams associated with evaluating the percentage of TCD^3^CD^8^+ lymphocytes in cell culture
A. 48-h culture in patient; B. 72-h culture in patient

Following co-culture with both tumor cell lines and the control, the percentage of these cytotoxic cells showed no statistically significant difference between the two healthy and patient groups.

Figure 4. Comparison of mean percentage of TCD^3^CD^8^+ cytotoxic cell in two healthy and patient groups
IL-6 and poor prognosis in patients with ovarian cancer has been reported [57]. In addition, several studies have demonstrated that the tumor cell line SKOV3 in the presence of PBMC causes the production of high concentration of IL-6 released by both SKOV3 cell line and PBMC. They also indicate that the interaction of these two types of cells provokes greater IL-6 production [48, 58].

We also found that the level of TNF-α production, as one of the other proinflammatory cytokines in co-culture with SKOV3 cell line among both the patient and healthy groups, was higher than that of co-culture with A2780 cell line. This was possibly due to the autocrine generation of this cytokine by SKOV3 cell line. According to the previous reports, it was expected that the production level of this cytokine in co-culture with PBMC of women with PCOS and SKOV3 cell line, because of increased inflammatory conditions, be higher than the group without tumor cell lines. However, such a relationship was not observed in this study.

The obtained results reveal that the presence of a chronic but slight inflammation in women with PCOS as well as the effect of such inflammation on immune competent cells like PBMCs [6, 7], provides a specific microenvironment during co-culture with ovarian cancer cells that encourage tumor growth and development. According to our data, the mean TNF-α concentration in the patient group without co-culture was higher than that of the healthy group. Such finding is indicative of a higher level of inflammatory cytokines secretion like TNF-α in patients. This issue disturbs the balance between the innate and acquired immune responses inside microenvironment, leading to actions in favor of the tumor growth. Also, the significant difference in the secretion of this cytokine between the two groups during co-culture with the tumor cell line A2780, confirms the hypothesis of present research. This finding could explain the high prevalence of ovarian cancer in women with PCOS.

It was revealed that the co-culture with SKOV3 cell line increased the stimulation of cellular proliferation, compared to A2780 cell line, in the patient and healthy groups. Moreover, this difference was statistically significant between the two groups. This could be explained by the fact that tumor cell line could increase the stimulation of cellular proliferation in the PBMCs of healthy individuals. This finding means that the proliferation rate is higher than that observed in the patient group, may be due to increased inflammatory conditions of the tumor microenvironment causing the death of competent immune cells.

The impossibility to isolate purified lymphocyte and macrophage populations to detect the major source of cytokine release was a limitation to the current research. That is because of failing to obtain wide-ranging blood samples from the study volunteers.

The potential increased risk of malignancies in patients with PCOS requires further investigations on other anticancer responses using large-scale molecular studies. Also, this method could be beneficial in future studies on other diseases related to immune cytokine imbalance, such as endometriosis, premature ovarian failure, and poor ovarian response. We suggest that future studies explore other immune cytokine profiles, e.g. Th17 and T regulatory cell in cell culture models.

Since chronic inflammation can eventually cause several diseases like malignancies, importance of PCOS as a chronic low-grade inflammation state is undeniable. Generally, considering the results of this study and other information on the occurrence of systemic inflammation in patients with Polycystic Ovarian Syndrome, it can be admitted that the complications that have been observed in many cases in this disease such as metabolic disorders and congestion. Other reproductive organs can provide grounds for increasing the malignancy, especially in the main organ of the target that is the ovary. Control of this inflammation during age in these patients can be significant in creating this disastrous outcome.

**Ethical Considerations**

**Compliance with ethical guidelines**

Upon delivering a comprehensive explanation, written signed consent forms were provided by all of the samples. Meanwhile, medical ethics were observed. The study was approved by the Research Deputy of Faculty of Medicine (code: 6ECRIES).

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**Authors contributions**

Supervision, Project administration and funding acquisition: Nariman Mossafa; Performing and contributing the design of the in-vitro and in-vivo experiments and interpreting results: All authors; Designing, performing...
and interpretation Flowcytometry: Mehrnaz Mesdaghi; Collecting the samples and interpretation of data analysis: Fahimeh Ramezani Tehrani; Directing the study and writing the paper: Mehrji Hajighayi and Nariman Mossaфа; and Writing and critically reviewing the manuscript: All authors.

Conflict of interest

The authors declared no conflict of interest.

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