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Determination of PD-1 expression in peripheral blood cells in patients with endometriosis

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Introduction

Endometriosis is an estrogen-dependent chronic inflammatory disease. It is defined as endometrial stroma and gland growth outside the uterus and includes genetic, environmental, immunological, angiogenic and endocrine processes [1]. While endometriosis affects approximately 10% of reproductive age women, the frequency of detection in women with infertile or chronic pelvic pain can increase to 50% [2–3]. Different theories related to the development of endometriosis have been developed. The first and the most widely accepted one among these theories is the "Retrograde Current Theory" which was put forward by John Sampson in 1927 [4]. Studies have also reported that the incidence of retrograde menstruation is similar between women with and without endometriosis. As a result of these studies, the pathogenesis of endometriosis may be multifactorial.

Endometriosis is described as a benign disease histologically. However, it is similar to malignancy in terms of tissue development, cell attachment to other tissues and infiltration properties [5]. Programmed cell death (apoptosis), plays a major role in the cyclic changes of the endometrium. Ectopic endometrial cells are resistant to apoptosis by various mechanisms and continue their survival and proliferation. In this respect, the biology of tumor and endometriosis are closely related. Consequently, apoptosis-based cancer studies will provide significant guidance in understanding the etiopathogenesis of endometriosis [6].

Human programmed cell death protein 1 (PD-1), a member of the B7/CD28 family, is one of the co-receptors that generate inhibitory signals [7]. They interact with PD-1, human programmed cell death protein ligand 1 (PD-L1 (B7-H1)) and human programmed cell death protein ligand 2 (PD-L2 (B7-DC)) to render T cells anergic [8]. The binding of PD-1 expressed on the surface of activated T cells to the ligand PD-L1 generates an inhibitory signal and reduces cytokine release. As a result of PD-1/PD-L1 interactions, the tumor escapes immune control. Blockage or activation of the PD-1/PD-L1 pathway has become a new therapeutic prospect for autoimmune disease and cancer. Anti-PD-1 agents are used in the treatment of many cancer types [9].

We believe that PD-1 expression may be high in endometriosis patients and therefore, ectopic endometrial structures may escape immune control and survive in other tissues. The aim of this study to determine the PD-1/PD-L1 expression in patients with endometriosis and healthy individuals.

Materials and methods

Study population

In this study, n = 73 cases who underwent surgery or examination at the Obstetrics and Gynecology Clinic of Sivas Cumhuriyet University Faculty of Medicine and diagnosed as endometriosis in the biopsy material taken with the pre-diagnosis...
of endometriosis constituted the patient group. The control group consisted of \( n = 64 \) healthy subjects without concomitant malignancy or chronic inflammatory disease. Venous whole blood samples were obtained from the study groups. The Faculty of Medicine of the Sivas Cumhuriyet University ethics committee approved this study, and all the patients signed informed consent.

**Sample collection**

Blood samples (serum and plasma, 15 ml) were collected. The samples immediately centrifuged 4000 rpm at 10 min and the samples kept at \(-80^\circ\text{C}\) before use for further analysis.

**Enzyme-linked immunosorbent assay (ELISA)**

Enzyme-linked immunosorbent assay was performed to examine serum PD-1 (Cat. No: E4711Hu), and human Programmed Cell Death Protein 1 Ligand 1 PD-L1 (Cat. No: E3680Hu), levels in patients with endometriosis and healthy controls. Samples were tested according to ELISA kit instructions. The concentration range of the PD-L1 and PD-1 standard solution were 20-7000 ng/L, 5-1000 ng/L, respectively. Added 40 \(\mu\)l sample 10 \(\mu\)l anti-PDCD1LG1, and then 50 \(\mu\)l streptavidin-HRP to wells. Plates were incubated at 37 \(^\circ\text{C}\) for 1 h in the dark. After incubation, the plates washed five with buffer solution and were added 50 \(\mu\)l substrate solution A and 50 \(\mu\)l substrate solution B. Then, plates were incubated at 37 \(^\circ\text{C}\) for 10 min in the dark. The reaction was stopped with 50 \(\mu\)l of stop solution, after 10 min, the absorbance value was measured at 450 nm.

**Isolation of RNA and cDNA synthesis**

The total RNA was extracted from whole blood collected by using the RNA Isolation Kit (GeneAll, Cat no:106-101) according to the manufacturer’s recommendations. cDNA synthesis using a HyperScript cDNA synthesis kit (GeneAll Cat no: 601-710), according to manufacturer's protocols. Reaction mixtures were incubated at 25 \(^\circ\text{C}\), 10 min; 55 \(^\circ\text{C}\), 60 min; and 85 \(^\circ\text{C}\), 5 min. cDNAs were measured using a qubit ssDNA Assay Kit (Molecular Probes, Life Technologies).

**Real-time quantitative PCR (qRT-PCR)**

qRT-PCR was performed using REALAMP SYBR Green Master Mix (HIGH ROX DYE) (Cat no:801-051), according to manufacturer’s protocols. About 20 ml PCR reaction included 4 \(\mu\)l RT product, 1 \(\mu\)l (10 pm) forward primer, 1 \(\mu\)l (10 pm) reverse primer, 1 \(\mu\)l ROX, 3 \(\mu\)l sterile water, and 10 \(\mu\)l (2X) SYBR master mix. The following primers were used: PD1-forward primer 5’-ACAGTTTCCCTCCGGTTCAC-3’, PD1 reverse primer 5’-CAGTTTAGACGAGCTCTCC-3’, GAPDH-forward primer 5’-ACGGATTCTGGTGTATTTGGG-3’, GAPDH-revers primer 5’-TGATTTCGGGATCTCGC-3’. PD1 expression levels were normalized to the amount of GAPDH in the same sample. The PCR reaction mixtures were incubated in at 95 \(^\circ\text{C}\) for 10 min, followed by 40 cycles of 95 \(^\circ\text{C}\) for 15 s and 60 \(^\circ\text{C}\) for 40 s. Relative increases in mRNA expression were processed using the 2-\(\Delta\Delta\text{Ct}\) method [10].

**Statistical analysis**

Kolmogorov–Smirnov test was used to compatibility with normal distribution. All statistical analysis was performed by SPSS 22.0, and a significant difference was set at \(p<.05\) \([\sigma = 0.05, \beta = 0.10, (1-\beta)=0.90 \ (R:\text{Sample Allection Ratio}:1.7)\ p = .90064]\).

**Results**

**PD-1 and PD-L1 concentration in the serum**

The concentration of PD-1, PD-L1 in patients with endometriosis against to control group were detected by Elisa Assay. Serum PD-1, PD-L1 that were differentially concentration between the two groups. Serum PD-1 concentration levels were compared in endometriosis patients and control group, it was found PD-1 levels were increased in patients with endometriosis compared to controls (350 ± 150 ng/L, 45 ± 17 ng/L, \(p < .05\), Figure 1). PD-1 value of endometriosis group was approximately 8 fold higher than that of the control group. Serum PD-L1 levels were significantly higher in patients with endometriosis than control group (760 ± 108 ng/L, 140 ± 14 ng/L, \(p < .05\), Figure 2). PD-L1 value of endometriosis group was approximately 4 fold higher than that of the control group.

**Expression of PD-1**

The expression of PD-1 in patients with endometriosis and control group was detected by qRT-PCR. PD-1 expression results are presented in Figure 3. PD-1 value of endometriosis group was approximately 10 fold higher than that of the control group.

**Discussion**

Endometriosis is a chronic inflammatory disease, estrogen-dependent characterized by enlargement outside the uterus of endometrial stroma and glands [1]. Endometriosis is thought to affect approximately 10% of reproductive-age women. Most women in the reproductive age and suffering from endometriosis are asymptomatic. At the same time, the definitive diagnosis of endometriosis is made by histological evaluation of the materials obtained by methods such as laparoscopy or laparotomy. Because of all these conditions, it is very difficult to determine the true prevalence of the disease. Although Sampson theory provides the most accepted explanation for the development of the disease, the cellular and molecular mechanisms responsible for implantation remain unclear. Pathologies such as environmental, immunological, endocrine and genetic play a multifactorial role in the development of the disease [11]. Pathologies such as environmental, immunological, endocrine and genetic play a multifactorial role in the development of the disease [11]. Endometriosis is considered to be a benign disease histologically. Endometriosis is histologically considered to be a benign disease. However, development, infiltration and binding properties to other tissues are similar to malignancy [5]. It is in need of creating new tissue for the development of an endometriotic structure [12]. The differences such as structural, apoptosis mechanism, immune system elements, cell adhesion molecules, proteases and protease inhibitors, gene expression, angiogenesis, protein and cytokine synthesis were found at endometrium’s of patients with endometriosis, compared to the endometrium of healthy women [13]. After retrograde flow include phagocytes in immune...
systems such as endometrial tissues accumulated in the peritoneum, macrophages, and NK. Although NK cells are numerically normal in women with endometriosis, it is suggested that the effect of endometrial cells on phagocytosis is weak [14–15]. Cellular immunity including T lymphocytes is impaired in women with endometriosis. Cytotoxic activity of T lymphocytes against autologous endometrium in women with endometriosis is also impaired [16]. Programmed cell death (apoptosis) plays a major role in the cyclic changes of the endometrium. The decrease in sensitivity to apoptosis in endometrial tissue is increasingly accepted as being involved in the etiopathogenesis of endometriosis as in cancer biology [6]. Ectopic endometrium cells continue to survive and proliferate by resisting apoptosis by various mechanisms. From this perspective, tumor biology and endometriosis biology intersect at many points. Therefore, apoptosis-based cancer studies will provide significant guidance in understanding the etiopathogenesis of endometriosis.

PD-1 that was discovered in 1992 by Tasuku Honjo et al. is a coreceptor producing inhibitory signals [17]. The programmed cell death pathway comes into play in the late stages of inflammation. PD-1 that is found on the surface of activated T cells in this pathway, and PD-L1 (B7-H1) and PD-L2 as its ligands interact to make T cells anergic [8]. PD-1 has two ligands PD-L1 (B7-H1) and PD-L2 (B7-DC). These ligands are expressed in antigen presenting cells such as dendritic cells and monocytes [18]. PD-L1 expression is also detected in non-lymphoid cells, such as endothelial cells in the heart, beta cells in the pancreas, and glial cells in inflamed brain and muscle cells [18–20]. Therefore, it has been assumed that PD-1/PD-L pathway can regulate immune response in both lymphoid and non-lymphoid organs [21–22]. The physiological role of PD-1 is to achieve T cell homeostasis and to balance between the T cell activation and proliferation. Binding of PD-1 expressed on the surface of activated T cells to PD-L1 ligand produces an inhibitory signal and reduces cytokine release. As a result of PD-1/PD-L1 interactions, the tumor escapes immune control [9]. There is evidence that PD-1/PD-L1 binding inhibits the induction of Bcl-xL that is cell survival factor [23]. Binding of PD-1 and PD-L1 limits the effector effects of CD8+ cytotoxic T cells and leads to apoptosis in Tumor Infiltrating Lymphocytes (TIL). High levels of PD-1 production limit TIL functions and reduce the number of tumors around the tumor. Thus, the tumor is freed from the immune response [24].

PD-1/PD-L1 pathway has a very important place in autoimmune diseases and cancer biology. Accordingly, blockade or activation of the PD-1/PD-L1 pathway has become a new therapeutic hope. PD-1/PD-L1-targeted immunotherapies have been found to be effective in many cancer types including non-small cell lung cancer, malignant melanoma and renal cell cancer. These agents are particularly effective in tumors resistant to standard chemotherapy regimens. Expressions of PD-1 and ligands have also been investigated in gynecologic cancers. As a result, a new and promising therapeutic method will be found in the treatment of gynecological cancer cases resistant to standard chemotherapy regimens by blocking this pathway.

When the literature is examined, it is seen that both PD-1 and PD-L1 expression studies are performed by immunohistochemical methods. In our study, PD-1, PD-L1 levels and gene expression were determined in human peripheral blood. In immunohistochemical studies, different results were obtained in PD-1 and PD-L1 levels depending on the selected tumor areas. In this study, PD-1, PD-L1 levels and gene expression in peripheral blood were investigated for the first time in patients with endometriosis. According to our results, gene expression levels
were significantly different between endometriosis patients and control group. The PD-1/PD-L1 immune-control mechanism may be a promising method for the development of new therapeutic agents in endometriosis.

In a study involving 1599 gynecological cancer patients by Thomas J. Herzog et al., PD-1 and PD-L1 expressions were determined according to gynecological cancer types. Increased PD-1 expression was detected in 66.9% of patients with epithelial ovarian cancer, 60% of patients with germ cell ovarian cancer, and 37.5% of patients with sex cord stromal tumors. Although an increase in PD-1 expression was detected in 46.3% and 68.2% of patients with Clear Cell and endometrioid type cancer, the most common ovarian cancer type associated with endometriosis, there was no calculation of gene expression in the study, respectively [25]. In the same study, an increase in PD-L1 expression was detected in 13.5% of patients with epithelial ovarian cancer and 75% of patients with sex cord stromal tumor. PD-L1 increase was not detected in patients with germ cell ovarian cancer. The study was presented as statistical information [25]. Mo et al. that was investigated PD-1 expression in ovarian cancer. The study was presented as statistical information [25].

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Conclusion
This is the first study about PD-1, PD-L1 levels and PD-1 gene expression in peripheral blood cells of endometriosis patients. We have shown that PD-1, PD-L1 levels and PD-1 gene expression increased in endometriosis patients compared the control group. According to the results of this study, PD-1/PD-L1 immune-control mechanism may be a promising method for the development of new therapeutic agents in endometriosis disease.

Disclosure statement
The authors declare no conflict of interest.

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References

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