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ARAŞTIRMA / RESEARCH

Expression of interleukin 6 and tumor necrosis factor alpha in endometriotic tissues: an immunohistochemical study

Endometriotik dokularda interlökin-6 ve tümör nekroz faktör alfa ekspresyonu: bir immünohistokimyasal çalışma

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Abstract

Purpose: The aim of this study is to investigate Tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) expressions in normal and endometriotic human tissue using immunohistochemical methods.
Materials and Methods: This study examined tissue sections obtained from the tissue biopsies of ectopic endometrium taken from 24 women between the ages of 20 to 40 years. Additionally, the tissue sections taken from the normal endometrium obtained from 10 patients without any endometrial dysfunction due to dilation or curettage were also evaluated, as the control group. Tissue sections prepared by light microscopic and immunohistochemical methods were examined under a light microscope.
Results: In the control group TNF-α and IL-6 expressions were identified at varying levels, from weak to moderate, in surface and glandular epithelium; however, there was no evidence of a significant staining in the stromal cells. It is found that TNF-α and IL-6 immunoreactivity in the endometriotic tissues were significantly increased in the epithelial cells, stromal cells and macrophages compared to the control endometrium. Expressions of TNF-α and IL-6 were both strong in the ectopic endometrial tissues.
Conclusion: We conclude that TNF-α and IL-6 are important cytokines involved in the pathogenesis of the endometriosis.

Keywords:. Endometriosis, immunohistochemistry, IL-6, TNF-α.

Öz

Amaç: Bu çalışmada, insanda normal endometriyum ve endometriyotik dokularda Tümör nekroz faktör-α (TNF-α) ve Interlökin-6 (IL-6) ekspresyonlarının immünohistokimyasal yöntemler ile araştırılması amaçlandı.
Gereç ve Yöntem: Çalışmada incelenen doku kesitleri, 20-40 yaşları arasındaki 24 kadından alınan ektopik endometriyum doku biyopsilerinden elde edildi. Ayrıca herhangi bir endometriyal fonksiyon bozukluğu bulunmayan, ancak diletasyon veya küretaj nedeniyle, 10 hastadan elde edilen endometriyum dokularından alınan kesitler de kontrol grubu olarak değerlendirildi. Işık mikroskobik ve immünohistokimyasal yöntemler ile hazırlanan doku kesitleri, ışık mikroskopta incelendi.
Bulgular: TNF-α ve IL-6 ekspresyonunun kontrol grubunda yüzey ve bez epitelinde zayıftan orta dereceye kadar değişen düzeylerde olduğu gözlendi. Buna karşın stromal hücrelerde belirgin bir boyanmaya rastlanmadı. TNF-α ve IL-6'nın endometriyotik dokulardaki immünreaktivitesinin kontrol endometriyum ile karşılaştırıldığında, epitel hücreleri ile stromal hücreler ve makrofajlarda belirgin olarak artış gösterdiği bulundu. TNF-α ve IL-6 ekspresyonlarının her ikisi de ektopik endometriyal dokuda, güçlü olarak eksprese olmuştur.
Sonuç: TNF-α ve IL-6'nın endometriyozis oluşumu ve patogenezinde rol alan önemli sitokinler oldukları kanaatine varıldı.

Anahtar kelimeler: Endometriyozis, İmmünohistokimya, IL-6, TNF-α.

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INTRODUCTION

The endometrium is one of the body tissues which cover the inner surface of the uterus and its structure and function and show significant alterations during the menstrual cycle[1]. The endometrium is composed of the surface epithelium and endometrial stroma, which is known as lamina propria. The surface epithelium is columnar and includes cilial cell groups, creating numerous tubular endometrial glands by penetrating the lamina propria. Endometrium shows cyclic alterations, repeated approximately every 28 days and these alterations are determined by hypothalamic- pituitary- ovarian hormonal activity[2]. Endometriosis is a disease which causes major health problems, such as dysmenorrhea, pelvic pain, and infertility in women at reproductive age and it is characterized by localization of the endometrial tissue outside the uterine cavity in other tissues and organs[3,4,5,6,7]. Although endometriosis is currently one of the most investigated diseases, its pathogenesis is still a question of debate. In recent years, several studies have focused on the relationship between inflammatory responses and the formation of endometriosis[3,7].

The incidence is approximately 10 to 15 % in women of reproductive age, and it has been reported that this ratio can increase for infertile women[6,7]. In addition, the incidence of endometriosis in adolescents with chronic pelvic pain, dysmenorrhea and infertility complaints has increased by up to 50% [8].

It is considered that a genetic predisposition or structural abnormalities in the endometrial tissue and impaired immune responses may also play important roles in the development of endometriosis. Although the roles of disorders in the immune system in the pathogenesis of endometriosis have been examined in various studies, it has not been yet possible to reach a full consensus on this issue[8]. It is thought that the cell population located in the peritoneal fluid, cytokines, and other soluble factors may be responsible for implantation and the progress of endometriotic tissue. Inflammatory responses associated with endometriosis, tissue repair, and neovascularization largely depend on the presence of macrophages and cytokines in the peritoneal fluid[9]. Therefore, cytokines are multi-functional proteins that have a significant role in cell activation, motility, adhesion, chemotaxis, morphogenesis and proliferation. Cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are acknowledged to be effective in endometriosis pathogenesis, as well as interleukin (IL) 1,2,6 and 10[10]. Interleukin 6 (IL-6) is an important cytokine which involves many physiological and pathological events and plays an important role in immune response, acute phase response in the liver, hemopoiesis and the regulation of neuronal functions and osteoclast formation. In endometriotic tissue, IL-6 increases correspondingly and it is thought that IL-6 may have an essential role in the pathogenesis of the disease. Indeed, has been shown that IL-6, a multi-functional cytokine, has been found in high concentrations in the peritoneal fluid of patients with endometriosis[11].

Another cytokine belonging to the TNF superfamily is TNF-α, which is considered to be important in the pathogenesis of endometriosis. It is primarily produced by monocytes and macrophages in response to various inflammatory and immunomodulatory stimulations. TNF-α has a wide range of bioactivity and most of the cells are sensitive to TNF-α.

Under normal physiological conditions TNF-α participates in the formation of immune response, cellular homeostasis, cell survival, cell proliferation, cell migration and differentiation[12]. The presence of this cytokine is also revealed in different cell types (fibroblasts, macrophages, epithelial cells, blood vessels etc.), which are located in endometrium. According to these observations, the expression of TNF-α protein is weak and negative in the early proliferative phase; however, amounts of TNF-α increase in the proliferative phase and the late proliferative phase, reaching a maximum level in the endometrial glands. This expression is high during the secretory phase[12].

In recent research, a presence of TNF-α and IL-6 in patients with endometriosis has been shown using various methods; however, there is no comparative immunohistochemical study in the literature which shows the distribution of the expression of TNF- α and IL-6 in normal endometrium, ectopic endometrial tissue, and the distribution of these cytokines expressions in the cell types. The aim of this study was to investigate TNF-α and IL-6 expressions in normal endometrium and ectopic endometriotic tissues by using immunohistochemical methods.

MATERIALS AND METHODS

Human tissue samples

The ovarian endometriotic tissues in this study were obtained from 24 female patients between the ages of 20 to 40 years, who applied to Çukurova University Faculty of Medicine Department of Obstetrics and Gynecology between January 2012 and December 2014 with complaints including pelvic pain, menstrual irregularities and particularly dysmenorrhea and infertility problems, and obtained from tissue biopsy specimens taken for diagnostic purposes during hysteroscopy and laparoscopy. Additionally, normal endometrium tissue biopsies, obtained from 10 patients without any endometrial dysfunction through dilation and curettage, were also evaluated as a control group. All of the paraffin tissue blocks were prepared at the Department of Pathology of Cukurova University.

All biopsies procedures performed in this study were in accordance with the ethical standards. This study was approved by the Cukurova University, Clinical Researches Ethics Committee, with Decision No:2013/26-4. A written informed consent was obtained from each patient. The study protocol was approved by the Cukurova University, Faculty of Medicine, Non-Invasive Clinical Research Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki.

Histology and immunohistochemistry

For histological analysis, endometrial tissue samples were fixed in 10% formalin overnight at 4^0C and after routine tissue attachments with a Leica TP 1020 autotechnical device, sections taken from paraffin blocks as 5µm thickness stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Also tissue sections were taken and prepared using appropriate methods from these blocks in order to establish TNF-α and IL-6 activity for immunohistochemical studies. After routine tissue attachments with a Leica TP 1020 autotechnical device, paraffin sections with a thickness of 4 µm were taken from the tissue specimens embedded in paraffin. After being dewaxed in xylene and rehydrated in graded alcohol, these sections were performed using antigen retrieval with a sodium citrate buffer (pH=6.0) for 30 min at 95 ^{0}C in a water bath and then incubated at room temperature for 45 minutes. The sections were cooled off, washed in PBS, and incubated with 3% H_2O_2 to block endogenous peroxidase for 15 min at room temperature. After washing three times in PBS, the sections were blocked with blocking (IHC Kit ab93705, Abcam, MA, USA) for 15 minutes. Then incubated with anti-IL-6 (IL-6; anti-human rabbit polyclonal antibody, 1:400 dilution, ab6672, Abcam, USA) and anti-TNF-α (TNF-α; anti-human rabbit polyclonal antibody, 1:2000 dilution, ab66579, Abcam, USA) for overnight at 4 ^{0}C. The slides were rinsed three times with PBS for five minutes. Then, they were further incubated with a secondary antibody (Logan, Utah USA.). Finally, immunohistochemical analysis was performed using an Olympus BX51 light microscope (Olympus BX53, Tokyo, Japan). IL-6 and TNF-α levels was evaluated as semi-quantitatively unpainted, very dense, medium dense and less dense dyed.

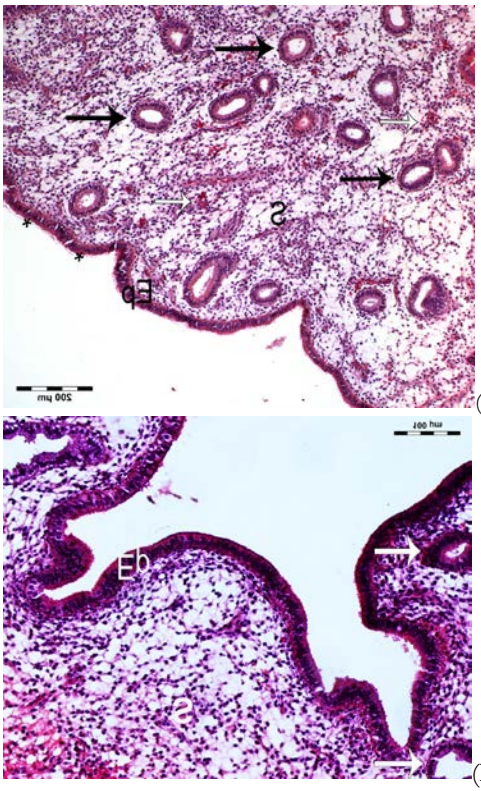
RESULTS

Histological findings

Control endometrium: The light microscopic examinations revealed that in the control group the endometrial tissue consisted of typical endometrial surface epithelium, endometrial glands and stroma. The surface epithelium was columnar. Cells which were located in surface epithelium had microvillus and cilium on their apical surfaces that faced the lumen. Tubular endometrial glands, which reach from the surface to deep into the stroma, were covered with columnar epithelium. Stromal cells, macrophages, connective tissue fibers, and blood vessels could be distinguished in the endometrial stroma (Figures 1A and 1B).

Ectopic endometrium: During light microscopic examination of the endometriotic tissues obtained from the patients with ovarian endometriosis, it was noted that the tissues generally consisted of surface epithelium and stroma located below it. Although it was not clearly distinguished, glandular structures were also found in the endometriotic tissues. Stroma, located in the epithelial and subepithelial area, was surrounded by dense fibrous tissue. Tissue thickness of the endometriotic tissue was less than tissue in the normal endometrium and boundaries of the surrounding tissues were not clear. Stroma contained stromal cells and macrophages with characteristic spherical nuclei. There were a significant increase in the number of macrophages and migrated leukocytes.

In addition, numerous capillary blood vessels and wide edematous areas were also detected in the stroma (Figure 2A and 2B).



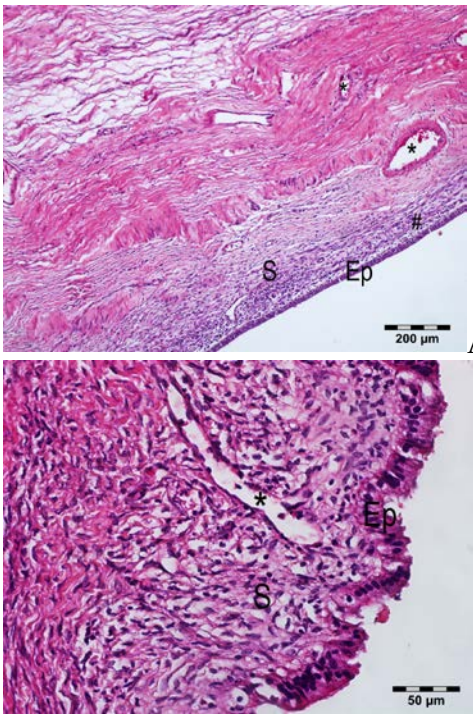
(A)

(B)

Figure 1. (A) Control endometrium. The surface epithelium (Ep), microvilli and cilium in apical surface of epithelial cells (*) are observed in light microscopic examination of the control endometrium. Tubular endometrial glands (black arrows) extend the depth of the stroma (S) and blood vessels are seen (white arrows) (H&E. Bar= 200µm). (B) Endometrium typical surface epithelium (Ep), endometrial glands (arrow) are observed. Stromal cells, macrophages, connective tissue fibers are shown in stroma (S) that are localized under the epithelium.

Immunohistochemical findings

Control endometrium, TNF-α immunoreactivity: TNF-α immunoreactivity was observed in the surface epithelial cells of endometrium tissue sections obtained from control group. Furthermore, TNF-α expression was detected, ranging from weak to moderate, in the glandular epithelial cells. In contrast, there was no significant staining detected in stromal cells. Similarly, the presence of TNF-α immunoreactivity was noted in the cytoplasm of the macrophages, which are located around blood vessels and stroma (Figures 3A and 3B).



A)

(B)

Figure 2. Light microscopic view of the ectopic endometrium. (A) Ectopic endometrium consists of surface epithelium (Ep) and the stroma (S) located below it. Stroma located in subepithelial areas (#), surrounded by dense fibrous tissue. Edematous areas and blood vessels (*) are shown in stroma (H&E. Bar= 200µm). (B) Endometrial surface epithelium including cilium and microvilli (Ep) and stroma(S) are observed. The presence of macrophages, a large number of stromal cells, which is characterized by spherical nuclei and large capillaries (*), are seen in stroma (H&E. Bar= 50µm).

IL-6 immunoreactivity: Examination of IL-6 expression in endometrial surface epithelium revealed that although there were weak expressions in some areas, typically there was no significant staining. Generally, IL-6 immunoreactivity was faintly detected in the basal and apical cytoplasm of cells in the glandular epithelium, similar to the surface epithelium. Furthermore, it was established that IL-6 expression was quite evident in the macrophages located in the stroma; however, there was no IL-6 immunoreactivity in the stromal cells (Figures 4A and 4B).

Ectopic endometrium, TNF-α immunoreactivity: TNF-α immunoreactivity was clearly seen in the cytoplasm of the surface epithelial cells during TNF-α immunohistochemical staining of ectopic endometrium tissue section obtained from patients

with ovarian endometriosis. In addition, TNF-α marking, varying between moderate to strong, was recorded in the macrophages located around the blood vessels and in stromal cells near the epithelium (Figures 5A and 5B).

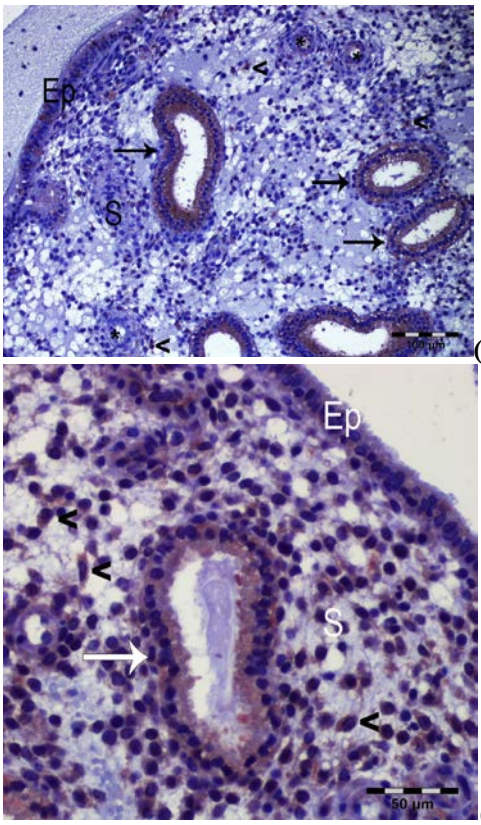


Figure 3. TNF-α immunoreactivity in control endometrium. (A) Although there is distinct TNF-α immunoreactivity in surface epithelium (Ep), glandular epithelium (arrows) and in the cytoplasm of macrophages, there is no significant TNF-α reactivity in stromal cells. Macrophages (arrowheads) located around blood vessels (*) in stroma (S) exhibit significant immunoreactivity (Bar= 100µm). (B) TNF-α immunoreactivity is seen in surface epithelium (Ep) and the cytoplasm of the cells in glandular epithelium (white arrow), however, no distinct immunoreactivity is not seen in stromal cells (S). In contrast, positive immunoreactivity of TNF- α is observed in macrophages (black arrowheads) (Bar = 50µm).

IL-6 immunoreactivity: IL-6 expressions were explicitly seen in stromal cells located under surface epithelium during an IL-6 immunohistochemical examination of the ectopic endometriotic tissue sections obtained from patients with endometriosis. This indicated that IL-6 expression is mostly seen in the basal cytoplasm of epithelial cells. On the other hand, there was no evidence of IL-6 expression in the stromal cells; however, we observed an intense immunoreactivity in macrophages located in stroma. Additionally, there was weak or moderate IL-6 expression detected in endometrial gland structures, which were located in the ectopic endometrium (Figures 6A, 6B, 6C and 6D).

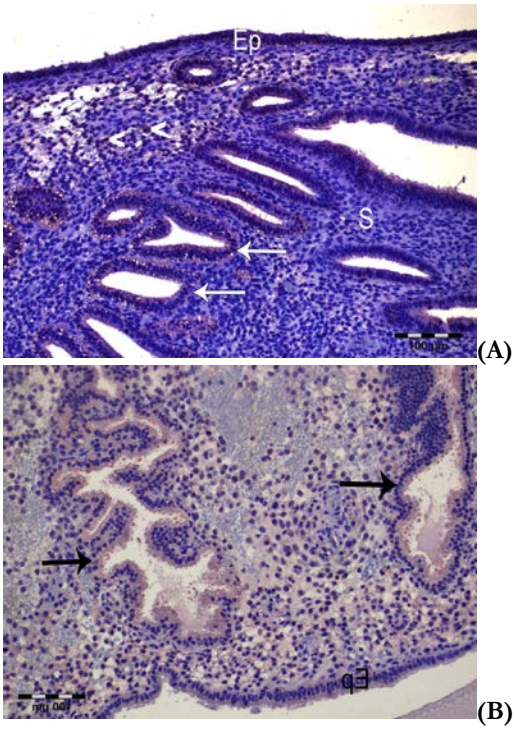


Figure 4. IL-6 immunoreactivity of control endometrium. (A) IL-6 immunoreactivity in the cells of surface epithelium (Ep) and glandular epithelium (arrows). Stromal cells (S) did not show any IL-6 expression (Bar= 100µm). (B) Surface epithelium (Ep) and the glandular epithelium (arrows) exhibit weak IL-6 immunoreactivity (Bar= 100).

DISCUSSION

In the present study, tumor necrosis alpha (TNF-α) and interleukin 6 (IL-6) expressions were investigated in normal and ectopic human endometrium tissues. Compared to the endometriotic tissue, a significant rise in TNF-α and IL-6 immunoreactivity was found in the epithelial and stromal cells. To date, the expression levels of cytokines in peritoneal fluid of healthy women or women with endometriosis have been comparatively researched [4-6, 11,13-16]. Harada *et al.* [17] have found that TNF-α and IL-6 levels in peritoneal fluid were higher in infertile patients with

endometriotic lesion symptoms, compared to healthy women. The authors suggested that increased levels of TNF-α and IL-6 might be associated with the development of endometriosis and associated infertility. In the present study, we investigated both TNF-α and IL-6 expressions in endometriotic tissue, using immunohistochemistry and our study is the first experimental study in the literature to show both TNF-α and IL-6 expressions in endometriotic tissue using immunohistochemistry. It was observed that TNF-α expressions varied from weak to moderate in the surface and glandular epithelium of the control group; however, there was no evidence of significant staining in stromal cells (Figure 3A and 3B). On the contrary, TNF-α expression levels increased significantly in surface epithelium in the ectopic endometrial tissue obtained from patients with endometriosis (Figure 5A and 5B). Correspondingly, it was seen that there was a high level of TNF- α marking in stromal cells and macrophages (Figure 5A).

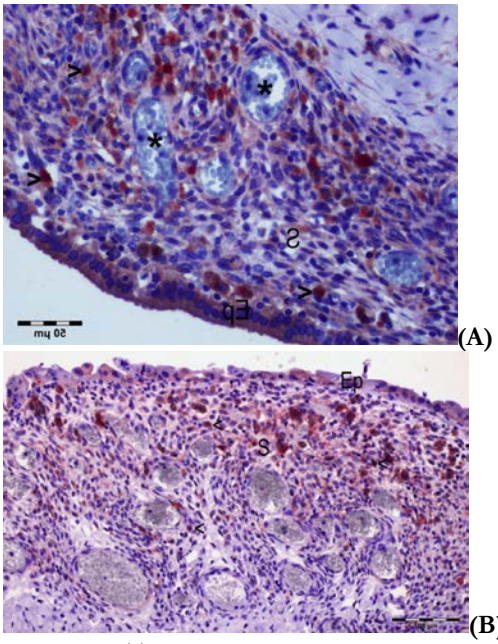


Figure 5. TNF-α immunoreactivity of ectopic endometrium. (A) TNF-α immunoreactivity in cell cytoplasm of surface epithelium (Ep). In addition stromal cells (S) show strong expression. It is noteworthy that the presence of a strong staining in macrophages (arrowheads) located around blood vessels (*) and stroma (S) (Bar = 50 um). (B) TNF-α staining can be traced in the surface epithelium (Ep), stromal cells (S) and macrophages (arrowheads). (Bar= 100μm).

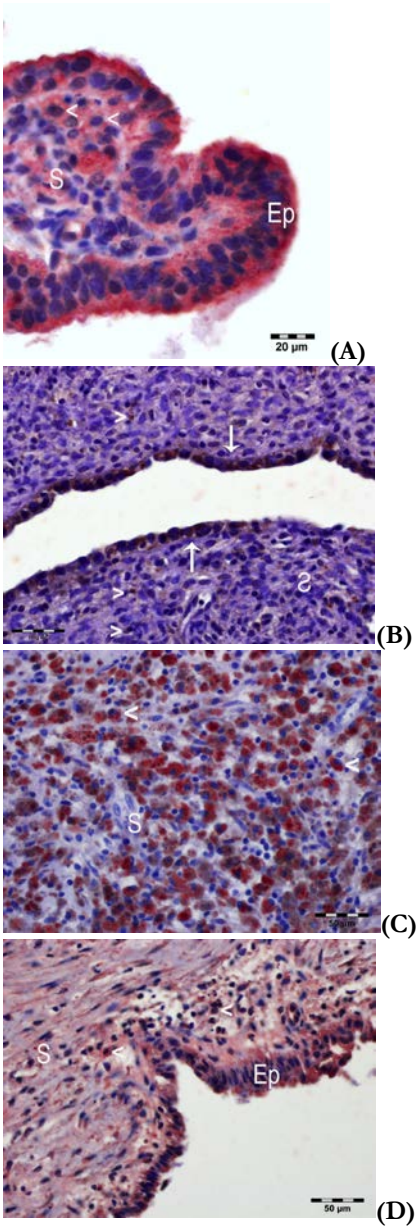


Figure 6. IL-6 immunoreactivity of ectopic endometrium. (A) Strong IL-6 immunostaining in cytoplasm of cells located in the surface epithelium (Ep) and stroma (S). Immunolabeling is stronger in the macrophages (arrowheads) (Bar = 20μm). (B) IL-6 immunostaining is observed in the surface epithelial cells (arrows). Stromal cells (S) and macrophages (arrowheads) are shown (Bar = 50μm). (C) IL-6 immunoreactivity of stromal cells (S) and macrophages (arrowheads) (Bar = 50μm). (D) IL-6 immunoreactivity in the surface epithelium (Ep), stromal cells (S) and macrophages (arrowheads) are evident (Bar = 50μm).

Table 1. IL-6 and TNF-α immunoreactivity in control and ectopic endometrium

	Control Endometrium		**Ectopic Endometrium**	
IL-6	Surface Epithelium	+	Surface Epithelium	++
	Glandular Epithelium	+	Glandular Epithelium	+
	Stromal Cells	-/+	Stromal Cells	++
	Macrophages	++	Macrophages	+++
TNF-α	Surface Epithelium	+	Surface Epithelium	+++
	Glandular Epithelium	+	Glandular Epithelium	+
	Stromal Cells	-/+	Stromal Cells	++
	Macrophages	++	Macrophages	+++

*: None: - , Weak: + , Moderate: ++ , Severe: +++

Endometriosis is an inflammatory disease characterized by the settlement of endometrial epithelium and stroma outside the uterus[18]. Although pathogenesis of the disease is still controversial, it is widely accepted that environmental, genetic, endocrinal, and immunological factors are all applicable in the development and spread of the disease. Endometriosis is known to be an estrogen dependent, chronic inflammatory disease due to the fact that endocrinal and immunological approaches are jointly taken into consideration. Recent studies have shown that there is a significant increase in macrophages and other cells of the immune system, located in the peritoneal fluid of women with endometriosis[3,7,19,20]. It is widely accepted that the pathogenesis of the disease is related to the accumulation of cytokines and various growth factors secreted from macrophages and leukocytes in the peritoneal fluid[8,18]. Undeniably, it is found that, many cytokines and growth factors, such as TNF-α[5,21,22], IL-1[23], IL-6[17], IL-8[24,25], IL-10[26], VEGF[16], MCP-1[25] and MCP-2 increased significantly in peritoneal fluid of women with endometriosis compared to healthy women. It is thought that the presence of a high percentage of peritoneal fluid may have a role in triggering the development of endometriosis; however, the presence of various cytokines and increased levels of these cytokines in body fluids have been shown in several recent studies[5,17,23,25]. It has been reported that endometrial epithelium and stromal cells also express different cytokines. Increases in cytokine levels in body fluids, such as peritoneal fluid and presence of cytokine expressions in endometrial cells, give rise to the thought that there is an interaction between these cases and they can be related to pathogenesis and the spread of the disease. Earlier studies have shown the existence of TNF-α and IL-6 in body fluids of patients with endometriosis[5,17,21,22]. In our study, this is the first time that both TNF-α and IL-6 expressions have been shown immunohistochemically in both normal and ectopic endometrial tissue. It is remarkable that both TNF-α and IL-6 expressions increased significantly in endometriotic tissue, epithelial cells, stromal cells, and macrophages when expression levels were compared to the control endometrium.

TNF-α is an important cytokine secreted by different cell types of the body and involved in immune and inflammatory reactions. TNF-α is responsible for the control of immune system reactions and the body's defense. In addition, TNF-α involves in neurological trauma, homeostasis, cell proliferation, and cell differentiation. It has been claimed that TNF-α may be associated with many pathological events, due to its stimulant effects on the proinflammatory and immune system[27,28,29]. Cytokine is mainly secreted by monocytes and macrophages and also has a broad spectrum of bioactivity[27]. Therefore, TNF-α is regarded as a pluripotent and angiogenic cytokine, and it stimulates expressions of many cytokines and causes cytokine synthesis in endometriotic tissue, such as IL-8. Due to this property it is accepted that TNF-α is a cytokine that has a key role in the induction and stimulation of other cytokines in the peritoneal fluid. Alternatively, it is widely accepted that TNF-α has a dual function in tissue regeneration and degeneration. These stimulating and inhibiting effects of TNF-α are thought to depend on cell types and cytokine concentration. Indeed, some studies have shown that while low dose TNF-α stimulates the angiogenesis, high levels of this cytokine inhibits angiogenesis[30,31].

The role of TNF-α in the development of endometriosis is still not completely clear. It is thought that alterations in the concentrations of TNF-α and other cytokines in peritoneal fluid of patients with endometriosis may be effective in the formation and progression of the disease. It has been reported that the amount of TNF-α in the peritoneal fluid of patients with endometriosis ranges between 5 to 300 pg/mL and it is suggested that low dose

TNF-α stimulates the development of endometriosis[17,32]. Although TNF-α is known to be an important cytokine having roles in the physiology of the normal endometrium, its role in endometriotic tissue is still unclear. In the present study emphasized that TNF-α expression was very apparent in epithelial and stromal cells in ectopic endometrial tissue compared to the control group (Figure 5A and 5B). Moreover, distinct TNF-α reactivity in macrophages located in endometriotic tissue stroma led to the possibility that the relationship between TNF-α and macrophage may be closely related to development of endometriosis. The explicit expressions of TNF-α and the presence of macrophages intensely in these areas was evaluated as evidence of a TNF-α and macrophage relationship that have roles in stimulating or inhibiting the formation and implantation of endometriotic tissue. Indeed, it is widely accepted that growth factors and cytokines, such as IL-6, IL-8 and TNF-α have roles in the implantation of endometriotic tissue[25]. It is also accepted that TNF-α may initiate stages of endometriotic tissue isolation by its stimulating effects on immune system cells and macrophages in areas of implantation[33]. There are very few studies in the literature showing the expression of TNF-α immunohistochemically in normal endometrium and endometriotic tissues. It has been reported that TNF-α expression was very low in glandular epithelium at the early proliferative phase; however, there was an increased immunoreactivity at later stages of the cycle and immunoreactivity reached maximum levels in the proliferative phase. In addition, it has been noted that TNF-α expressions are maintained at high levels during the secretory phase; however, TNF mRNA levels decreased towards the end of the cycle. Moreover, there was a significant increase in TNF-α concentrations in the peritoneal fluid of patients with endometriosis and it has been reported that expression levels of this cytokine can be related to the stage of the disease[34]. In another study[35], a weak TNF-α expression was observed in the epithelium and stroma at early and mid-proliferative phase; however, the expression of this cytokine was more evident in the late proliferative phase. Correspondingly, in our study, TNF-α expressions were very low in the stromal cells and macrophages in normal endometrium. In contrast, expression of this cytokine was more evident in stromal cells and macrophages in ectopic endometrium (Table 1).Recognizing the possible roles of TNF-α in endometrial biology and endometriosis continues to be an important area of research. It has been suggested that there is a close relationship between TNF-α level and the placement and development of endometriotic implants[33]. In *in vitro* settings, TNF-α have been shown to increase the bonding of endometriotic cells to laminin, fibronectin and collagen located in the implantation site. Researchers have also demonstrated that matrix metalloproteinase 3 (MMP3) levels also rise with the presence of TNF-α [36].

In our study, we observed that TNF-α was markedly evidently, specifically in the epithelial cells, as well as stromal cells and macrophages in endometriotic tissue. It is suggested that, due to its proinflammatory effect, TNF-α causes the gathering of macrophages and other cells involved in the immune system of endometriotic implant areas. Moreover, together with the effects of other cytokines and growth factors, TNF-α stimulates fibrosis and the isolation of endometrial tissue and can reduce blood flow by stimulating contractions in stromal cells and in local blood vessels. As a result, it has been suggested that the cellular dynamics of endometriotic implant will be failed[7,28]. Indeed, Philippeaux *at al.*[34] reported that TNF-α mRNA levels increased significantly in the endometrium and there was a strong marking of TNF-α in the epithelium and in the wall of spiral arteries; furthermore, they suggested that an overexpression of TNF-α in the walls of blood vessels may be related to menstruation. It has been shown in our study that IL-6 immunoreactivity was weak in both the surface and gland epithelium and stromal cells in the control group (Figures 4A and 4B). Conversely, it highlighted that there was a distinct IL-6 expression in the epithelial and stromal cells and in the macrophages in endometriotic tissues (Figure 6A- 6D). IL-6 is an important pleiotropic cytokine, which is produced by different cell types in the body and is involved in many physiopathologic events. IL-6 contributes to many biological activities, such as inflammatory reactions, cell differentiation and proliferation, regulation of the immune system, hemopoiesis and tumorogenesis[37]. IL-6 is primarily secreted from T and B lymphocytes, monocytes and macrophages and it has been shown that expression levels of this cytokine increased in patients with endometriosis. Salmassi *et al.*[4] compared the IL-6 levels in the endometrium of healthy women with ectopic and eutopic endometrial tissues of patients with endometriosis and they reported there to be distinct IL-6 mRNA levels in the epithelial and stromal cells of the endometriotic tissue.

Alternatively, it is reported that IL-6 is a cytokine associated with reproductive physiology and it is involved in ovarian steroid hormone synthesis, folliculogenesis and the early stages of implantation[18]. IL-6 expression is also shown in eutopic and ectopic endometrium. In another study, it was noted that IL-6 and INF-γ caused an increase in the secretion of intercellular adhesion molecule-1 from the macrophages and it emphasized that IL-6 plays an important role in the pathogenesis of endometriosis[10]. In another study, it was shown that there is a significant increase in IL-6 and IL-8 concentrations in peritoneal fluid during the later stages of endometriosis. Research has suggested that the increase in the amount of these cytokines may have an effect on the progression of the disease. Furthermore, it is currently widely accepted that cytokines, such as IL-1β, TNF-α, INF-γ and IL-12, are involved in the pathogenesis of endometriosis[16]. However, it is well-established that inflammatory reactions, in particular, lead to endometriotic tissue adhesion and in the interim there is a significant increase in the expressions of major proinflammatory cytokines, such as IL-1, IL-6 ve TNF-α, in endometriosis[5,23].

In our study, the apparent TNF-α and IL-6 expression in epithelial cells of the endometrium and also the presence of IL-6 immunoreactivity in stromal cells and macrophages are considered to be a sign that IL-6 may also be effective in endometriosis development. It could be suggested that with their autocrine, paracrine and endocrine effects, TNF-α and IL-6 may regulate endometriosis development. Indeed, in our study, the presence of overlapping expressions of TNF-α and IL-6 in the endometriotic tissues supports this opinion. Hadisaputra *et al.*[5] indicated in their study that IL-6 and TNF-α were the most important serum markers of endometriosis diagnosis, in which IL-6 and TNF-α levels compared to other cytokines. Harada *et al.*[17] have also reported that there is a positive correlation between the number and size of endometriotic lesions with TNF-α level. It has been recently been determined that the quantities of many cytokines were increased in the peritoneal fluid of patients and the possible effects of these cytokines in pathogenesis of endometriosis have been examined in many studies. Furthermore, it is reported that endometriotic implants synthesize significant amounts of cytokine; however, the relationship between the cellular cytokine expressions of endometriotic tissues and pathogenesis of the disease has only been evaluated in very few studies to date. Altogether, it highlights that there is a close relationship among the excessive cytokine expressions in endometriotic tissue, endometriosis formation and the failure of implantation. On the other hand, the relationship between cytokines and chemokines, which are secreted by several types of cells in endometrium, is still a question of debate. Indeed, Ulukış *et al.*[25] demonstrated that IL-8 and MCP-1 expressions levels altered during the various stages of the menstrual cycle and there were distinct expressions of these cytokines in the epithelial and stromal cells of the endometrium. They also reported that expressions of IL-8 and MCP-1 were more significant in the ectopic endometrium and these cytokines might have a role in the pathogenesis of the disease.

In their study, Boric *et al.*[21] established that the expression level of TNF, TNFR1, TNFR2 and CD45 and their receptors can vary according to the phase of the menstrual cycle. They reported in their immunohistochemical study that high expression levels of these cytokines in the mid-secretory and late-secretory phase might be related to the low implantation ratio in cases of endometriosis. The researchers indicated that different expression levels of cytokines may affect the rate of cell death in eutopic and ectopic endometrium and this situation is very important in the formation and development of ectopic endometrial implants outside the uterus. Varying expression levels of cytokines and chemokines during the menstrual cycle is certainly essential for normal endometrial physiology; however, the excessive increase or decrease of one or more cytokines in the peritoneal fluid, for any reason, will result in the deterioration of the physiological balance and may also affect normal endometrial biology. In our study, although TNF-α and IL-6 immunoreactivity were lower in the control endometrium, there was a strong expression of TNF-α and IL-6 in the epithelial stromal cells and macrophages in ectopic endometrial tissues. These results suggest that these cytokines may have similar effects in the formation and development process of the disease. Indeed, it is widely accepted that the relation of cytokines and chemokines between each other and also with immune system cells, is an important equipoise for normal endometrium biology and endometriotic tissue development[5,10,14,32]

The limiting aspect of this study can also be explored for IL-6and TNF-α expression at the gene level. In

future studies, these cytokines will also be examined at the gene level.

In conclusion, in this study, in which TNF-α and IL-6 expression are shown immunohistochemically in normal and endometriotic human tissues, both TNF-α and IL-6 immunoreactivity significantly increased in the epithelial cells, stromal cells and macrophages in the endometriotic tissues compared to the control endometrium. Based on these results, we conclude that TNF-α and IL-6 play a significant role in the formation and pathogenesis of endometriosis.

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