

Immunohistochemical Evaluation of TGF- β Isoforms in Cases with Ovarian Endometriosis and Follicular Cyst

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OBJECTIVE: To evaluate the distribution of Transforming Growth Factor (TGF)- β 1, β 2 and β 3 in surgical specimens of the cases with endometriosis and follicular cysts.

STUDY DESIGN: This prospective clinical study was carried out at Aegean Obstetrics and Gynecology Training and Research Hospital, a tertiary referral center. A total of 44 reproductive aged women operated because of adnexial masses and revealed pathologic diagnosis' like endometriosis (Group I; n: 22 cases) and follicular cyst (Group II; n: 22 cases) were enrolled into the study. A semi-quantitative examination of immunohistochemical staining was evaluated as light (+), medium (++) and strong (+++). According to these staining levels, each case was scored as having one, two or three points; and mean scores were calculated for the groups.

RESULTS: TGF- β 1, TGF- β 2 and TGF- β 3 staining scores were (48.5 \pm 3.1), (25.4 \pm 2.3), and (43.7 \pm 2.9) in Group I; and (29.2 \pm 2.5), (26.5 \pm 2.2), and (41.1 \pm 3.0) in Group II, respectively.

In contrast to the other two isoforms, TGF- β 1's immunoreactivity was significantly higher in cases with endometriosis (p<0.01).

CONCLUSION: Ovarian tissue TGF- β 1 immunoreactivity was significantly increased in endometriosis cases when compared with follicular cyst. There are needed further studies to elucidate the importance of this finding in the pathogenesis of endometriosis.

(Gynecol Obstet Reprod Med 2007;13:1 34-37)

Key Words: Transforming Growth Factor (TGF)- β isoforms, endometriosis, follicular cyst

Endometriosis, characterized by the presence of viable and abnormally located tissue resembling the endometrium with glands and stroma outside the uterine cavity, is an estrogen-dependent and progressive benign gynecological condition. It affects 2-3% of women of reproductive age,¹ and an evidence of endometriosis during laparoscopies performed due to various causes is found up to 50%.²

Growth factors are chemical messengers being secreted by specific cells of mammals in order to transfer coordinating signals to other cell groups and create specific biologic effects together with coordinating and controlling the cell growth and differentiation.³ Main growth factors studied previously and found to be possible associated with endometriosis pathogenesis are as follows; Transforming Growth Factor (TGF),⁴ Vascular endothelial growth factor (VEGF),⁵ Insulin-like

growth factor (IGF),⁶ Tumor necrosis factor-alpha (TNF- α),⁷ Hepatocyte growth factor (HGF),⁸ Interleukins (IL) (mainly IL-1 and IL-6)^{9,10,11} and Angiogenin.⁶ There is a dynamic equilibrium in between paracrine activities of these substances and macrophage activation,¹² angiogenesis¹³ and apoptosis¹⁴ in peritoneal fluid.

TGF- β family members are multi-functional cytokines that play a key role in cellular growth, proliferation, and differentiation. They also have special roles in embryonic cell migration, carcinogenesis, angiogenesis, apoptosis and tissue repair.^{15, 16} Its most frequently studied isoform was TGF- β 1 in association with endometriosis. But, no study evaluating the association between TGF- β isoforms and follicular cyst was found in the literature.

The objective of this current study is to evaluate the existence of a difference in between levels of TGF- β isoforms in surgical specimens of the cases with endometriosis and follicular cysts.

Material and Methods

Patients

A total of 44 reproductive aged women operated because of adnexial masses and revealed pathologic diagnosis' like endometriosis (Group I; n: 22 cases) and follicular cyst (Group II; n: 22 cases) were enrolled into this prospective clinical study.

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Submitted for Publication: 14.11.2006

Accepted for Publication: 12.01.2007

Scoring of endometriosis cases was done according to American Fertility Society (AFS) Score System and the diagnosis of all endometriosis cases was verified with histopathologic findings.

The study was approved by Institutional Review Board of our hospital. Informed consent was obtained from each patient after the purpose and nature of the study had been fully explained.

Immunohistochemical Evaluation

Specimens of endometriosis cases were obtained by either removing endometriotic cysts in the presence of endometrioma or excising endometriotic implants in the surface of the ovary in the absence of endometrioma. In contrast, all ovarian tissue specimens were obtained by the removal of cyst in cases with follicular cyst.

Tissue specimens fixed in formalin 10% for 24-48 hours at room temperature, were followed-up according to the standard paraffin tissue method and stained with Haematoxyline-Eosine (HE).

Rest of the serial sections was prepared in order to be evaluated by indirect immunoperoxidase. Sections were washed with distilled water after de-paraffinisation and re-hydration procedures. Sections held in trypsin 0.5% solution at 37°C for 15 minutes were surrounded with Dakopen, and H₂O₂ 3% was applied in order to inhibit tissue endogenous peroxidase. Sections washed with phosphate tamponade solution were incubated in a moist environment at +4°C for 18 hours with primary antibodies, anti-TGF-β1 (Santa Cruz SC-146, California, USA), anti-TGF-β2 (Santa Cruz SC-90, California, USA), and anti-TGF-β3 (Santa Cruz SC-82, California, USA). The day after, avidin-biotin-peroxidase kit (Zymed Histostain 85-9043, San Francisco, USA) was applied to the sections re-washed with phosphate tamponade solution after being incubated with hydrogen-peroxide secondary antibody for 30 minutes. After application of streptavidin for 30 minutes, sections were re-washed with phosphate tamponade solution. Background staining of the sections stained with DAB (Diaminobenzidine-Zymed 00-2020, San Francisco, USA) in order to establish the visualization of immunoreactivity was performed with Mayer's haematoxyline. After dehydration with alcohol and transparency procedures, product was coated with entellan. In order to test the specificity of the immunoreactivities, each section is provided for control staining and staining was performed with normal mouse serum in spite of primary antibody.

The preparations were evaluated under light microscope (Olympus® BX-40, Japan). The evaluation of immunoreactivity was carried out in endometrial epithelia and glands for patients with endometriosis and in cyst wall and adjacent connective tissue for patients with follicular cyst.

Staining intensity of the sections was evaluated semi-quantitatively as light (+), medium (++) and severe (+++) by two histologists unaware of the groups and applied primary antibodies. Following this semi-quantitative evaluation mentioned above, each case was scored as 1, 2, or 3 points accordingly and mean scores were estimated for the groups. Figure 1 shows an example of characteristic of strong immunohistochemical staining positivity for TGF-β1 in a case with endometriosis (Figure 1).

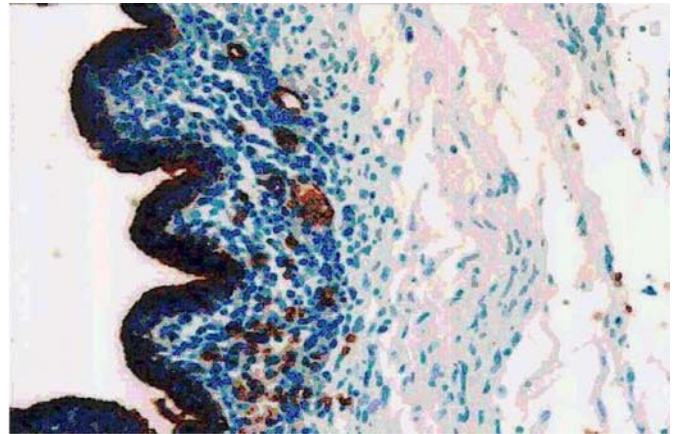


Figure 1. Strong TGF-β1 positivity (+++) using immunohistochemical staining in ovarian endometriotic tissue

Statistical Analysis

According to staining levels, each case was scored as having one, two or three points. Total and mean scores of TGF-β isoforms were estimated for both endometriosis and follicular cyst groups. The groups were compared with respect to demographic and disease-related characteristics and mean TGF-β isoforms scores.

Statistical analyses were carried out using the pocket program of Statistical Program for Social Sciences, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's t test was used in statistical estimations and two tailed P<0.05 was accepted as statistical significance.

Results

The groups were comparable with respect to age and gravidity. In endometriosis group, mean AFS score was 131.91±171.16 (Range 4-600), mean CA-125 level was 48.09±27.3 (Range 18-90) U/mL. The characteristics of the groups were shown in Table I.

Table 1. Characteristics of the cases with ovarian endometriosis and follicular cyst

	Endometriosis Group (n=22)	Follicular Cyst Group (n=22)
Mean age (year)	32.7 (range 21-48)	33.5 (19-50)

Mean gravida	1.2 (range 0-4)	1.3 (0-4)
Mean serum CA 125 level (U/mL)	48.1 (range 18-90)	15.4 (4-38)
Mean AFS score	131.91 (range 4-600)	-

AFS: American Fertility Society

Analysis of immunohistochemical distribution of TGF- β isoforms revealed that, TGF- β 1 immunoreactivity observed in cases with endometriosis was significantly higher than that of cases with follicular cyst ($p < 0.01$). But no statistically significant difference was observed in between the groups when TGF- β 2 and - β 3 immunoreactivities were compared (Table II).

Table 2. Ovarian tissue distribution of TGF- β 1, β 2, and β 3 immunoreactivities

	Group I (n=22)	Group II (n=22)	Significance
TGF- β 1 (Score, mean \pm SD)	48.5 \pm 3.1	29.2 \pm 2.5	P<0.01
TGF- β 2 (Score, mean \pm SD)	25.4 \pm 2.3	26.5 \pm 2.2	NS
TGF- β 3 (Score, mean \pm SD)	43.7 \pm 2.9	41.1 \pm 3.0	NS

NS: non-significant

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Discussion

TGF- β produced by peritoneal macrophages might a crucial role in the pathogenesis of endometriosis.⁴ It is supposed that cytokines originating from macrophages assist in development of endometriosis by stimulating the neovascularization of endometrial cells adhering to the peritoneum.¹⁷ Increased angiogenesis is observed more frequently around the peritoneal explants and increased angiogenetic activity is observed also in peritoneal fluid obtained from women with endometriosis.¹⁸ A previous immunohistochemical study investigating the presence of TGF- β isoforms and receptors in ovarian endometriotic cysts and normal endometrial tissues revealed that the main source of TGF- β was macrophages infiltrating the surrounding of the stromal cells which were stained weakly with TGF- β .¹⁹ Elevated TGF- β 1 may play a role in the establishment of ectopic endometrium in the peritoneal cavity by stimulating matrix metalloproteinases (MMPs) to remodel the mesothelial lining of the peritoneum thus allowing for tissue invasion.²⁰ So that vicious circle formed by TGF- β and peritoneal macrophages seems to be very important in the pathogenesis of endometriosis.

Pizzo et al investigated TGF- β , TNF- α and IL-8 levels in peritoneal fluid and serum of infertile women with endometriosis in a controlled study and consequently reported that peritoneal fluid TGF- β levels of cases with endometriosis were significantly increased when compared with the control group.

Furthermore, peritoneal fluid TGF- β levels of cases with endometriosis were well-correlated with the severity of the disease.²¹ Tamburro et al reported that the physical appearance of endometriotic implants and the severity of dysmenorrhea appeared to be related to the expression of TGF- β 1 in nerve fibers.²² However, it is also reported that increased peritoneal TGF isoforms are associated with not only endometriosis-related adhesions but also surgery,²³ or peritonitis-related adhesions.²⁴

In a recent study Hsieh et al investigated whether the TGF- β 1-509 gene polymorphism could be used as a marker of susceptibility in endometriosis. In their study, women were divided into two groups: endometriosis (n = 150) and non-endometriosis (n = 159). Polymorphisms for TGF- β 1-509 genes were amplified by polymerase chain reaction and detected after restriction enzyme digestion. Genotypes and allelic frequencies in both groups were compared. Proportions of C homozygote, heterozygote, and T homozygote for TGF- β 1 gene polymorphisms were 9.3/61.3/29.4% in the endometriosis group and 41.3/58.5/0% in the non-endometriosis group. Alleles C and T for TGF- β 1 gene polymorphism were 40/60% (endometriosis) and 70.8/29.2% (non-endometriosis). They concluded that T homozygote and T allele for TGF- β 1 are associated with higher susceptibility to endometriosis.²⁵

We established high levels of ovarian tissue TGF- β 1 immunoreactivity in cases with endometriosis when compared with follicular cysts. There are needed further studies to elucidate the importance of this finding in the pathogenesis of endometriosis. However, the evaluation of these data together with the literature reveals that TGF- β especially its β 1 form seems to be associated with developing endometriosis. The immunohistochemical evaluation of some growth factors such as TGF- β 1 in diseased ovary and peritoneal fluid can be used as an adjunctive test in differential diagnosis of suspicious ovarian cystic masses. In the future, targeted therapies such as anti-TGF- β 1 therapy may be an alternative for the treatment of endometriosis.

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