Expression of vascular endothelial growth factor and thrombospondin-1 mRNA in patients with endometriosis

Xian-Jie Tan, Ph.D., Jing-He Lang, M.D., Dong-Yuan Liu, Ph.D., Keng Shen, M.D., Jin-Hua Leng, M.D., and Lan Zhu, M.D.

Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, People’s Republic of China

Objective: To investigate the expression of vascular endothelial growth factor (VEGF) mRNA and thrombospondin-1 (TSP-1) mRNA in endometriosis.

Design: Molecular studies in human tissue.

Setting: Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, P. R. China.

Patient(s): Patients undergoing laparoscopy for infertility or other benign gynecologic conditions.

Intervention(s): Biopsies were taken from endometriotic lesions (red peritoneal lesion, ovarian endometrioma, and uterosacral ligament nodule) and eutopic endometrium during laparoscopy.

Main Outcome Measure(s): mRNA expression from endometriotic lesion and eutopic endometrium was analyzed by reverse transcriptase polymerase chain reaction (PCR) and Northern blotting.

Result(s): Among the endometriotic lesions, red peritoneal lesions expressed higher levels of VEGF mRNA and lower levels of TSP-1 mRNA, whereas ovarian endometrioma expressed lower levels of VEGF mRNA and higher levels of TSP-1 mRNA. Eutopic endometrium of women with endometriosis had higher expression levels of VEGF mRNA and lower expression levels of TSP-1 mRNA than that of women without endometriosis.

Conclusion(s): The expression of VEGF and TSP-1 in endometriotic lesions appears to be associated with the extent of their neovascularization. The imbalance in expression of VEGF and TSP-1 in the endometrium may play a role in the development of endometriosis. (Fertil Steril 2002;78:148–53. ©2002 by American Society for Reproductive Medicine.)

Key Words: Vascular endothelial growth factor, thrombospondin-1, angiogenesis, endometriosis, endometrium

Endometriosis is a common gynecologic disorder, affecting at least 10% of reproductive-age women. Although the pathogenesis of endometriosis remains unknown, it is generally accepted that the establishment of new blood supplies is a key part in the progression of endometriosis (1). Angiogenesis, the formation of new capillaries from preexisting blood vessels, requires the cooperation of a variety of molecules and cells, and depends on the balance between positive and negative regulators (2).

From the many positive regulators known to act on the angiogenesis, the vascular endothelial growth factor (VEGF) has emerged as a pivotal angiogenesis stimulator. The VEGF, also known as vascular permeability factor, is a 23- to 45-kD heparin-binding glycoprotein with potent angiogenic, endothelial cell-specific mitogenic, and vascular permeability activities (3). Compelling evidence indicates that VEGF is a fundamental regulator in many physiological and pathological angiogenic conditions (3). Overexpression of VEGF has been reported in many tumor types and it has been regarded as one of the markers of tumor invasion and metastasis (4, 5).

Thrombospondin-1 (TSP-1), a 450-kD adhesive trimeric glycoprotein, initially identified in platelet α-granules, was later shown to be synthesized and secreted by many normal and transformed cells. Thrombospondin-1 has been
implicated in the regulation of the function of endothelial cells, including inhibiting proliferation, disrupting focal adhesions, diminishing cell spreading, and inhibiting angiogenesis (6). Recently, attention has been focused on TSP-1 and its possible role as a p53-dependent inhibitor of angiogenesis (7). It was reported that TSP-1 could inhibit capillary formation in vitro (8). More recent evidence indicated that TSP-1 could induce endothelial cell apoptosis and block the angiogenesis driven by VEGF (9).

Although endometriosis is a pathologically benign disease, its biological behaviors and clinical presentations are, to some extent, similar to those of malignant tumor, especially with its ability for metastasis and angiogenesis. Although active endometriosis is characterized by hypervascularization both within and around the implant (10), the molecular mechanism responsible for angiogenesis in endometriosis remains to be understood. Several investigators demonstrated higher peritoneal concentrations of VEGF in women with moderate to severe endometriosis than in women without the disease (11–13). Recently, the presence of the VEGF protein was observed in ectopic and eutopic endometrium (14). However, it is still unclear whether the overexpression of VEGF is related to the development of endometriosis and little is known about the expression of TSP-1 and its role in endometriosis.

Therefore, the purpose of this study was to investigate the expression of VEGF and TSP-1 in eutopic and ectopic endometrium and their roles in the pathogenesis of endometriosis.

MATERIALS AND METHODS

Subjects

Women aged 20–44 years were recruited into the study between December 1998 and July 1999 after they provided informed consent for a protocol approved by Peking Union Medical College Hospital’s Ethics Committee on Human Research. The protocol was also reviewed and approved by the Scientific Research Department of the hospital. Women included in the study had regular menstruation without endometrial hyperplasia or neoplasia, and they had not received any anti-inflammatory or hormonal medication during a period of at least 3 months before laparoscopy. Endometriosis was identified at laparoscopy for ovarian endometrioma, infertility, or pelvic pain. The stage of endometriosis was determined according to the revised classification of The American Fertility Society (AFS) (15). Thirty-eight patients with both laparoscopically proven and histologically proven endometriosis were selected as the study group. Control subjects (n = 25) were fertile women requesting tubal ligation or women with benign ovarian tumor and having no visible evidence of endometriosis at laparoscopy. There were no statistically significant differences in the demographic characteristics between the two groups. The cycle phase (proliferative or secretory) was determined according to the menstrual cycle history and histologic criteria. Pathological diagnoses were performed for all samples.

Collection of Eutopic and Ectopic Endometrium and RNA Preparation

In the study group (endometriosis group, n = 38), 32 specimens of ovarian endometrioma, 12 red peritoneal endometriotic lesions, 8 uterosacral ligament nodules, and 30 matched eutopic endometrium were obtained by biopsy during laparoscopy. In the control group, 25 specimens of endometrium were collected by curettage. A small part of each sample was prepared and sent for histologic endometrial dating or pathological confirmation of the clinical endometriosis. The rest of the sample was immediately stored in liquid nitrogen. Total RNA was extracted from eutopic or ectopic endometrium with TRIzol reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer’s protocols.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Four micrograms of total RNA were subjected to reverse transcription with a commercially available kit (the cDNA First Chain Amplification System, GIBCO-BRL) according to the manufacturer’s protocol. Transcribed products were subjected to PCR for VEGF, TSP-1, and β-actin in a 25-μL final reaction volume. For a negative control, the cDNA template was omitted from the reaction. Amplification for VEGF cDNA was started with a 3-minute denaturation at 94°C followed by cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 56°C, and 1 minute of extension at 72°C. The PCR profile for TSP-1 began with the 3-minute initial denaturation at 94°C, followed by cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 62°C, and 1 minute of extension at 72°C. Amplification for β-actin cDNA was started with a 3-minute denaturation at 94°C followed by cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, and 30 seconds of extension at 72°C.

The primers used for VEGF, TSP-1, and β-actin amplification were: [1] VEGF: sense: 5′-CCA TGA ACT TTC TGC TGT CTT-3′, antisense: 5′-TCG ATC GTT CTG TAT CAG TCT-3′; [2] TSP-1: sense: 5′-ACC GCA TTC CAG AGT CTG GC-3′, antisense: 5′-ATG GGG TCG TCC AAC TCA GC-3′; [3] β-actin: sense: 5′-GAA TTC ATT TTT GAG ACC TTC AA-3′, antisense: 5′-CC GGA TCC ATC TCT TGC TCG AAG TTC A-3′.

Final PCR products were subjected to electrophoresis through a 2% agarose gel and stained with ethidium bromide. Optical densities of the electrophoresis bands in ultraviolet (UV)-illuminated gels were analyzed using a UVI scanner (UVItex, Cambridge, England, UK). The intensity of β-actin amplification was used as an internal standard. To
validate target mRNA quantification, the number of amplification cycles required for linearity was tested. Linear range was obtained at 35 cycles for VEGF, 30 cycles for TSP-1, and 30 cycles for β-actin. Then, the relative ratio of VEGF to β-actin products (V/A ratio) or TSP-1 to β-actin (T/A ratio) was calculated with these numbers of cycle. The V/A ratio and T/A ratio were used to represent the relative expression level of VEGF mRNA or TSP-1 mRNA, respectively.

### Northern Blot Analysis

Total RNA (35 μg) from each sample was separated by electrophoresis in 1.2% agarose gel containing 2.2 mol/L formaldehyde. After capillary transferred to Hybond-N+ nylon membrane (Amersham, Buckinghamshire, England, UK) and ultraviolet cross-linking, rRNA was visualized with ethidium bromide staining to verify that equal amounts of RNA had been transferred to the gel.

Hybridization probes were labeled with [α-32P]dCTP (New England Nuclear, Boston, MA) with a random priming labeling kit (Promega, Madison, WI) and purified from the unincorporated nucleotides with a Bio-Spin 6 minicolumn (Bio-Rad, Munich, Germany). Probes had specific activities of $1 \times 10^8$ to $10 \times 10^8$ cpm/μg. The cDNA probes were: [1] for human VEGF165, a 930-bp EcoRI fragment of VEGF plasmid (gift of Genentech Inc. South San Francisco, CA); [2] for human TSP-1, a 1.28-kb XbaI–EcoRI fragment of TSP-1 plasmid (gift of Dr. Jack Lawler, Harvard Medical School, Boston, MA); and [3] for internal standard, β-actin purchased from Zhongshan Biological Technology Co. (Beijing, P. R. China).

Membranes were prehybridized for 4 hours and hybridized for 16 hours at 42°C in buffer containing 5 × standard saline citrate (SSC), 5 × Denhardt’s solution, formamide (50%, vol/vol), and sonicated heat-inactivated salmon sperm DNA (0.5 mg/mL). After hybridization, membranes were washed twice with 1 × SSC and sodium dodecyl sulfate (SDS)(0.1%, wt/vol) for 15 minutes at room temperature, then washed with 0.1 × SSC and SDS (0.1%, wt/vol) for 20 minutes at 60°C for two times.

Autoradiography of the membranes was performed at −70°C using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for 48–72 hours. The densitometry of autoradiographic bands were performed by a laser densitometer (Molecular Dynamic, Sunnyvale, CA). Each VEGF or TSP-1 band was normalized by using the value for corresponding β-actin, thus correcting any variation in amounts of RNA applied to each lane.

### RESULTS

#### VEGF mRNA and TSP-1 mRNA Expression in Endometriosis Detected by RT-PCR

The mRNAs encoding for VEGF and TSP-1 were detected in most eutopic and ectopic endometrium by RT-PCR. The RT-PCR reaction of VEGF mRNA gave rise to two major bands with sizes of 516 bp and 648 bp. The RT-PCR products of TSP-1 mRNA and β-actin mRNA were 492 bp and 326 bp DNA fragments, respectively. The positive rate and relative level of VEGF mRNA and TSP-1 mRNA expression in these samples are shown in Table 1.

Ovarian endometrioma had lower positive rates and relative levels of VEGF mRNA expression, higher positive rates and relative levels of TSP-1 mRNA expression compared to eutopic endometrium. The positive rate and relative level of VEGF expression in red peritoneal lesions were similar to those of eutopic endometrium, whereas the positive rate and relative level of TSP-1 mRNA expression in red peritoneal lesion were slightly lower than those in eutopic endometrium.

The positive expression rate of VEGF mRNA in the eutopic endometrium of patients with endometriosis (study group, n = 30) and without endometriosis (control group, n = 25) were 73.3% and 68.0%, respectively. There was no significant difference in these two groups ($P>.05$). How-

### Table 1

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<th>VEGF mRNA</th>
<th>TSP-1 mRNA</th>
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<tr>
<td></td>
<td>No. of samples</td>
<td>Positive rate</td>
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<tr>
<td>A red peritoneal lesion</td>
<td>12</td>
<td>75.0%</td>
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<tr>
<td>B ovarian endometrioma</td>
<td>32</td>
<td>53.1%</td>
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<tr>
<td>C untersacral ligament nodule</td>
<td>8</td>
<td>62.5%</td>
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<tr>
<td>D eutopic endometrium</td>
<td>30</td>
<td>73.3%</td>
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**Note:** Comparison of the positive expression rate of VEGF mRNA and TSP-1 mRNA between groups were evaluated by χ² test. For VEGF: comparison between any two groups, $P>.05$; for TSP-1: A vs. B, $P<.05$. Comparison of the relative expression level of VEGF mRNA and TSP-1 mRNA between groups were evaluated by Student’s t test. For VEGF: A vs. C, $P<.05$; B vs. D, $P<.05$; for TSP-1: A vs. B, $P<.05$; B vs. D, $P<.05$.

ever, the relative expression level of VEGF mRNA in the study group was higher than in the control group (0.74 ± 0.16 vs. 0.63 ± 0.17; P < 0.05). The study and control groups showed no significant difference in the positive rate of TSP-1 mRNA expression (53.3% vs. 56.0%; P > 0.05). However, the relative expression level of TSP-1 mRNA in the study group was lower than in the control group (0.56 ± 0.14 vs. 0.68 ± 0.16; P < 0.05).

Confirmation and Quantification of the mRNA encoding for VEGF and TSP-1 in Endometriosis by Northern Blot Analysis

In some samples, the difference in levels of VEGF mRNA and TSP-1 mRNA in eutopic endometrium of women with and without endometriosis, and the difference in three different kinds of endometriotic lesion were studied further by Northern blot analysis. Hybridization signals of the transcripts of 4.5-kb VEGF mRNA, 6.0-kb TSP-1 mRNA transcripts, and 2.3-kb β-actin transcripts were detected in almost all samples.

Densitometric analysis of the autoradiographic hybridization bands showed that the red peritoneal lesion had the highest level of VEGF mRNA and the lowest level of TSP-1 mRNA among the three types of endometriotic lesion, whereas ovarian endometrioma had the lowest level of VEGF mRNA and the highest level of TSP-1 mRNA (Fig. 1). In addition, densitometric results showed that eutopic endometrium of women with and without endometriosis had similar low levels of VEGF and TSP-1 mRNA expression during the proliferative phase. However, relatively higher expression levels of VEGF mRNA and lower expression levels of TSP-1 mRNA were observed in the secretory phase of eutopic endometrium of patients with endometriosis compared to that of women without endometriosis (Fig. 2).

DISCUSSION

Angiogenesis is strictly regulated by many factors. The biochemical characterization of many peptides and proteins that stimulate or inhibit angiogenesis has led to the concept of angiogenic balance (16). Among the factors affecting angiogenic balance, VEGF and TSP-1, two important molecules with angiogenic and antiangiogenic activity, respectively, have been extensively studied in the past decade. Overexpression of VEGF and decreased expression of TSP-1 have been shown in many types of tumor cells, and compelling evidence suggests that VEGF and TSP-1 play critical roles in tumor angiogenesis and metastasis (4, 5, 17–20).

The present study showed that the mRNAs encoding for VEGF and TSP-1 were coexpressed in eutopic as well as ectopic endometrium. To our knowledge, the present study investigated for the first time both angiogenesis stimulator and inhibitor in endometrium and endometriosis. The presence of VEGF and TSP-1 in eutopic and ectopic endometrium has important implications for the normal function of the endometrium and the development of endometriosis. During the menstrual cycle, there is a gradual increase in the length, branching, and coiling of spiral arteries. Several researchers demonstrated the presence of VEGF or TSP-1 in human endometrium (6, 12, 14). These findings and the results from our study suggest that VEGF and TSP-1 may act as angiogenesis mediators and are involved in the physiological function of the endometrium. Under certain pathological situations, in women with endometriosis for example, when exfoliated endometrium is attached to the peritoneal layer, the establishment of new blood vessels in endometriotic implants depends also on the angiogenic balance. The presence of VEGF and TSP-1 in ectopic endometrium, therefore, suggests that these molecules may also regulate the angiogenic process of endometriosis.

Nisolle and Donnez (21) recently suggested that peritoneal, ovarian, and rectovaginal endometriotic lesions should be con-
sidered as three separate entities with different pathogenesis. Peritoneal endometriosis could be explained by the transplantation theory, whereas ovarian endometrioma is the result of celomic metaplasia of invaginated ovarian epithelial inclusion, and rectovaginal endometriosis is thought to be a result of metaplasia of mullerian remnants located in the rectovaginal septum.

In the present study, we found that there was some degree of variation in the expression of VEGF and TSP-1 in different types of endometriotic lesions. The red peritoneal lesion is generally regarded as an early and active endometriosis with higher proliferative activity than other types of endometriosis. Our results showed that the expression level of VEGF mRNA in red peritoneal lesions and eutopic endometrium were similar, which was higher than that of ovarian endometrioma. In contrast, the expression level of TSP-1 mRNA in red peritoneal lesions was lower than that of ovarian endometrioma. These findings suggest that an active angiogenic process exists in red peritoneal lesions, like that in eutopic endometrium, which may be regarded as another argument in favor of the transplantation theory. Ovarian endometrioma, on the other hand, presents poor angiogenic appearance under laparoscopy, indicating that its angiogenic activity differs from that of eutopic endometrium. This may not only be related to its lower level of VEGF expression, but also to its higher level of TSP-1 expression. On the basis of our data, we suggest that the expression of VEGF and TSP-1 in endometriotic lesions appears to be associated with the extent of their neovascularization.

More interesting, it was suggested that the eutopic endometrium itself plays a crucial role in the pathogenesis of endometriosis (14). It was demonstrated that the proliferative activity of the endometrium of women with endometriosis was higher than that of women without this disorder (22). Recently, VEGF protein content was found to be significantly higher in the eutopic glandular epithelium of patients with endometriosis (14). In accordance with these findings, we found that the endometrium of patients with endometriosis had increased expression of VEGF mRNA compared to women without endometriosis. Conversely, the expression level of TSP-1 mRNA in eutopic endometriosis was lower than that of women without this disorder.

Overexpression of VEGF or decreased expression of TSP-1 in tumor tissue usually indicates higher proliferative activity and a higher risk of metastasis (4, 5, 17). The microvessel density and the frequency of hepatic recurrence were significantly higher in colorectal carcinomas that were VEGF positive and TSP-1 negative (19). The alterations in VEGF and TSP-1 expression observed in eutopic endometrium, similar to those in malignant tumor, indicate that a change in the angiogenic phenotype accompanied the development of endometriosis. Therefore, our preliminary results suggest that the expression imbalance of the VEGF and TSP-1 in endometrium might have some role in the pathogenesis of endometriosis. This supports the
hypothesis that variation in endometrial angiogenic activity among women may be one determinant of successful development of endometriosis.

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References