

Expression of Osteopontin in Eutopic and Ectopic Endometrial Tissues in Endometriosis

Yun-Hee Koo, Chung-Hoon Kim, Ji-Sun Kim, Young-Jin Lee, Sung-Hoon Kim,
Heedong Chae, Byung-Moon Kang

Department of Obstetrics and Gynecology, College of Medicine, University of Ulsan,
Asan Medical Center, Seoul, Korea

자궁내막증 환자의 정상위치 및 이소성 자궁내막에서의 Osteopontin의 발현

구윤희 · 김정훈 · 김지선 · 이영진 · 김성훈 · 채희동 · 강병문

울산대학교 의과대학 서울아산병원 산부인과학교실

목 적: 본 연구는 세포간 부착과 유착에 관여하는 당단백질의 하나인 osteopontin (OPN)의 mRNA와 단백질의 발현 양상을 자궁내막증 환자의 정상위치 자궁내막조직과 이소성 자궁내막조직, 대조군의 자궁내막조직에서 비교, 분석하기 위해 시행되었다.

연구방법: 개복 또는 복강경 수술을 통하여 자궁내막증으로 확진된 환자 32명을 연구 대상으로 하였고 같은 시기에 자궁경부 상피내암 또는 자궁내막증 이외의 양성 부인과 질환으로 수술적 치료를 받은 환자 34명을 대조군으로 하였다. 수술 시 자궁내막종 또는 복강내 자궁내막 이식물로부터 이소성 자궁내막조직을 얻었고, 동시에 정상위치 자궁내막조직을 생검하였다. 자궁내막조직 내의 OPN mRNA의 발현 정도는 실시간 역전사 중합효소연쇄반응을 이용하여 비교하고 단백질 발현에는 western blot 분석을 사용하였다. 각 군 간의 비교에는 ANOVA와 Kruskal-Wallis test를 사용하였으며 p-value 0.05 미만을 유의한 것으로 판정하였다.

결 과: 월경주기의 증식기와 분비기 모두에서 자궁내막증 환자의 정상위치 및 이소성 자궁내막조직에서의 OPN mRNA의 발현은 대조군의 자궁내막조직에 비해 유의하게 높은 것으로 나타났다. 자궁내막증 환자에서 정상위치 자궁내막조직의 경우 OPN mRNA의 발현은 증식기에 비해 분비기에서 의미 있게 증가하였으나 이소성 자궁내막 조직에서는 분비기에서 뚜렷하게 감소하는 양상으로 나타났다. OPN 단백질의 발현도 mRNA의 발현과 마찬가지로 자궁내막증 환자에서 유의하게 높은 것으로 확인되었다.

결 론: 본 연구의 결과는 자궁내막증이 있는 여성의 정상위치 및 이소성 자궁내막 조직에서의 증가된 OPN mRNA 및 단백질의 발현이 자궁내막 조직의 유착 및 침습에 관여할 수 있음을 시사한다.

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중심단어: 자궁내막증, Osteopontin, 이소성 자궁내막

Endometriosis is a common gynecological disorder, affecting at least 10% of reproductive-aged women.¹ It is defined by the presence and proliferation of endometrial

glands and stroma outside the uterus and is characterized by pelvic pain, dysmenorrhea, and infertility. Despite the identification of endometriosis in the late 1800s, the etiology and pathogenesis of this estrogen-dependent disease remain obscure. Sampson suggested that retrograde menstruation might cause endometriosis; however,

주관책임자: 김정훈, 우) 138-736 서울특별시 송파구 풍납2동 388-1, 울산대학교 의과대학 서울아산병원 산부인과학교실
Tel: (02) 3010-3639, Fax: (02) 3010-6944
e-mail: chnkim@amc.seoul.kr

although clinical evidence supports retrograde menstruation in 90% of women only 10% develop endometriosis.^{2,3} Therefore, some intrinsic factors must be differently expressed for the ectopic implantation and growth of endometrial tissue to take place. The endometrial tissue must attach itself to the host tissue, then invade it and derive the local vasculature to ensure its own blood supply.⁴

Adhesion and invasion involve cell-to-cell and cell-to-matrix interactions and are thought to require adhesive receptors such as $\alpha_v\beta_3$ -integrins. Immunohistochemical evaluation of endometrium throughout the cycle revealed a sudden increase of β_3 -integrin expression in luminal and glandular epithelial cells in the mid secretory phase.^{5,6} The function of the receptor, $\alpha_v\beta_3$ -integrin, essentially depends on binding of certain ligands, which are structurally characterized by the tripeptide arginine-glycine-aspartic acid (RGD) sequence. Osteopontin (OPN), an acidic secreted glycosylated phosphoprotein originally isolated from bone matrix,⁷ contains the RGD sequence and mediates cell adhesion and cellular signaling by binding to integrin $\alpha_v\beta_3$ in osteoclasts.^{8,9}

OPN has been shown to bind to several different integrin receptors, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, and $\alpha_9\beta_1$, through its RGD domain.¹⁰⁻¹³ Interestingly, the occupancy of OPN with these different receptors may have distinct functional consequences. In addition, OPN induces migration of breast cancer cells of high vs. low malignancy has been shown to be mediated through different integrins.¹⁴ Although OPN binds to various integrin receptors, the $\alpha_v\beta_3$ -integrin has been recognized as a primary receptor for osteopontin, promoting cell-to-cell attachment and cell spreading via changes in the cytoskeleton.^{15,16} OPN is expressed in a variety of human tissues, including kidneys, thyroid, gastrointestinal tract, breast, and testis.^{17,18}

Expression of OPN in endometrium and deciduas in several species such as sheep, baboons, pigs, and mice

suggest osteopontin to be involved in the biology of reproduction.¹⁹⁻²¹ In humans, endometrial expression was first described in glandular cells of endometrium and in placenta.^{17,18,21} Recently, von Wolff et al. performed a more detailed analysis of OPN expression in endometrium throughout the menstrual cycle and revealed maximum concentrations in endometrial gland and uterine secretion in the mid to late secretory phase that osteopontin plays a role in implantation.²²

Increasing OPN mRNA and protein expressions around the time of the "implantation window" raised the question of whether OPN might also play a role in ectopic implantation of endometrial tissue in endometriosis. Because retrograde menstruation occurs in nearly all women but endometriosis only develops in minority, we questioned whether eutopic endometrium might be altered or abnormal in women with endometriosis. Therefore, we investigated the expression of OPN mRNA and protein in eutopic and ectopic endometrium of women with endometriosis and in eutopic endometrium of normal women throughout the menstrual cycle.

MATERIALS AND METHODS

1. Patients and tissues preparation

The study group was recruited from women undergoing surgery for endometriosis beginning in October 2004 in Asan Medical Center (Seoul, republic of Korea). All tissue samples were obtained with full and informed consent. The research protocol was approved by Institutional Review Board.

This study group included 32 women aged 20~47 years. All patients did not receive hormonal treatments, such as GnRH agonist or sex steroids, and did not use intrauterine contraception for ≥ 6 months before surgery. Endometriosis was confirmed by pathological examination of biopsied tissue, and the extent of disease was staged according to the revised American Society of

Table 1. Oligonucleotide primer sequences for RT-PCR

Gene	Primer	PCR product
Osteopontin	5'-CTA GCC CCA CAG ACC CTT CC-3'	286 bases
	5'-CCA CAC TAT CAC CTC GGC CA-3'	
Ribosomal protein	5'-AGA TGA TCG AGC CGC GC-3'	163 bases
	5'-GCT ACC AGG GCC TTT GAG ATG GA-3'	

RT-PCR: reverse transcriptase-polymerase reaction

Reproductive Medicine (ASRM) classification of endometriosis.

The control group included 34 women aged 29~48 undergoing surgery for CIN (cervical intraepithelial neoplasia) or other benign gynecological disease not endometriosis. None of these women had a history suggestive of endocrinologic disorders or infertility and all of them had regular menstrual cycles.

In study group, ectopic endometrial tissues were obtained from ovarian endometrioma or peritoneal endometrial implants. Eutopic endometrial tissue biopsies were performed during surgery in study group and after surgery in hysterectomy specimens in control group. Endometrial tissues were flushed with phosphate-buffered saline (PBS) and normal saline until contaminating blood was removed and stored at -70°C until further analysis was performed.

2. Extraction of RNA

The frozen endometrial tissues were ground into powders in liquid nitrogen. 1 ml Triazol solution (Invitrogen, Carlsbad, CA) and 200 µl chloroform were then added, the mixtures were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatants were collected. RNA was isolated by using RNeasy Mini Kit (Qiagen, Valencia, CA) from the supernatants.

3. Quantitative real time reverse transcriptase PCR

Ribosomal protein (RP) known as housekeeping gene

was used as internal control. Complementary DNA PCR primers for human OPN and control RP gene were designed using Primer Express from Genbank. The primer sequences are shown in Table 1.

Total RNA (1 µg) extracted from endometrial tissues was subjected to a reverse-transcription reaction by using 50 pmol random 9-mer primer (Takara, Shifa, Japan), 10X reverse transcriptase buffer, 20 U RNase inhibitor (Takara), 5 mM MgCl₂, 1 mM dNTPs and 5 U AMV (avian myeloblastosis virus) Reverse Transcriptase (Takara).

Quantitative real-time PCR was performed in the ABI PRISM 7700 Sequence Detection System (Applied biosystems, Foster city, CA) by using SYBR Green I kit. In a total volume of 20 µl, each reaction contained 2 µl of chromosomal DNA (cDNA), 10X Master Mix (consisting of SYBR Green I stain, MgCl₂, dNTPs, and Tag DNA polymerase), each primer, with standard or nuclease-free water as negative control.

The parameter for quantification in real-time PCR is the CT value. CT value means threshold cycle number at which a significant increase of signal is first detected. Threshold cycle occurs when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product. Obtained CT values of RP were normalized by RNA concentration (µg/ml) and the CT values of osteopontin were normalized by the compensated CT value of RP. Compensated CT values of osteopontin were converted

Table 2. Patients' characteristics

Group	Endometriosis group	Control group
Patients (n)	32	34
Age (years)		
Mean	36	39
Range	20~47	29~48
% of associated disease		
Leiomyoma	13 (4)	59 (20)
Benign ovarian cyst		15 (5)
CIN		26 (9)
Stage of endometriosis (n)		
I-II	6	
III-IV	26	
Proliferative phase	19	23
Secretory phase	13	11

CIN: cervical intraepithelial neoplasia

into the relative amount of mRNA.

4. Western blot analysis

The frozen endometrial tissues were ground into powders in liquid nitrogen. Whole cell lysate was isolated by adding 200 µg of passive lysis buffer (Promega, Madison, WI). A total of 20 µg of cell lysate was placed on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then the gels were transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) using western transfer apparatus (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose sheets were blocked with Tris-buffered saline (10 mM Tris (pH 7.7), 150 mM NaCl) with 0.1% Tween-20 (Uniqema, Netherlands) (TBS-T) containing 5% non-fat dry milk for 1 hour at room temperature and incubated in a 1:1000 dilution of anti-osteopontin (R&D Systems, Minneapolis, MN) or β-actin (Sigma-aldrich, USA) antibodies for 1 hour at room temperature. After

washing with TBS-T, the membranes were incubated in a 1:1000 dilution of the goat anti-human IgG conjugated to biotin for 1 hour at room temperature. Then the membranes were washed well, incubated with Enhanced Chemical Luminescence (ECL) reagents (Amersham-Biosciences) for 1 minute, and covered in cassette and exposed to X-ray films.

5. Statistical analysis

Statistical analysis was performed using ANOVA, Kruskal-Wallis test. The statistical significance was defined as a P value of <.05.

RESULTS

1. Baseline characteristics

The mean age of women with endometriosis was 36 years (range: 20~47 years), and of control group was 39 years (range: 29~48 years) (Table 2). In women with endometriosis, 6 patients were stage I-II endometriosis and 26 patients were stage III-IV endometriosis according to the revised ASRM classification. Among the 34 women of control group, 59% had leiomyoma, 15% had benign ovarian cyst, and 26% had cervical intraepithelial neoplasia.

2. Expression of OPN mRNA in eutopic and ectopic endometrium

In all 6 kinds of endometrial tissue samples, osteopontin mRNA was detected and PCR products were obtained. To verify the amplification specificity, the PCR products generated by the OPN and control ribosomal protein primers were subjected to the agarose electrophoresis (Figure 1).

The expression of OPN mRNA was significantly higher in both eutopic and ectopic endometrial tissues of women with endometriosis than in endometrial tissues of controls during both proliferative and secretory phase

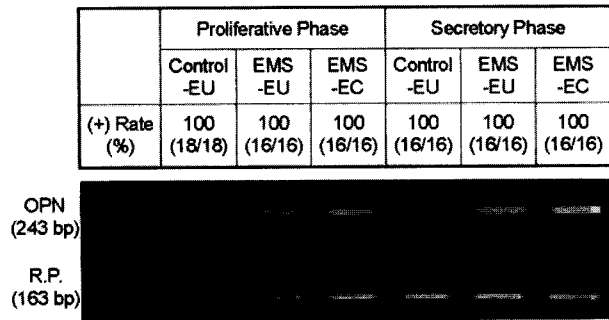


Figure 1. Electrophoretic analysis of the PCR products of OPN and RP mRNAs. Lower lane shows the 163 bp RP bands of RT-PCR RP products and upper lane shows the 243 bp OPN bands. OPN: osteopontin, RP: ribosomal protein, EMS: endometriosis, EU: eutopic, EC: ectopic.

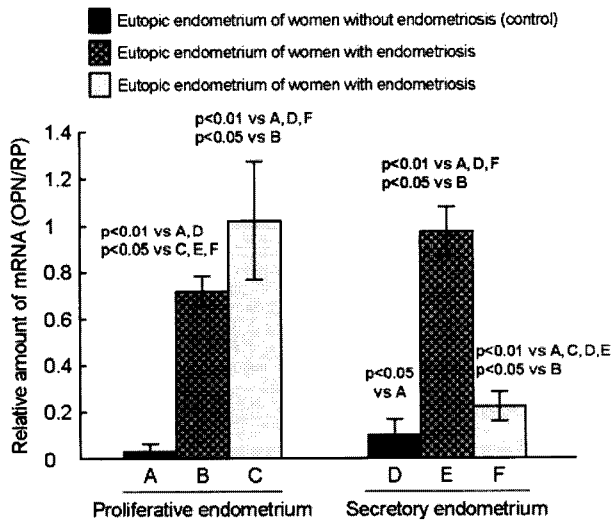


Figure 2. Expression of osteopontin mRNA in eutopic and ectopic endometrium.

(Figure 2). In women with endometriosis, OPN mRNA expression of eutopic endometrial tissue significantly increased during the secretory phase compared to the proliferative phase ($p < 0.05$). In the ectopic endometrial tissue of women with endometriosis, OPN mRNA expression increased compared to eutopic endometrium in proliferative phase ($p < 0.05$), but significantly decreased in secretory phase compared to the proliferative phase ($p < 0.01$) and decreased compared to the eutopic endometrium in secretory phase ($p < 0.01$).

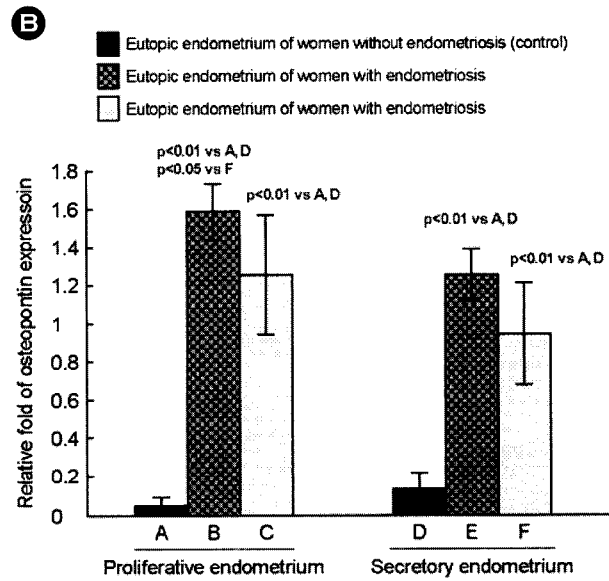
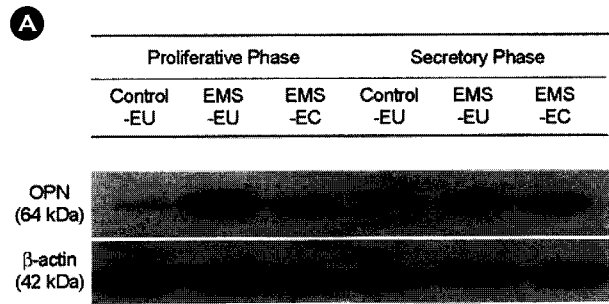


Figure 3. Expression of osteopontin protein in endometrium of controls and eutopic and ectopic endometrium of endometriosis patients. A. Representative western blot analysis. B. Quantification of osteopontin protein levels.

3. Expression of osteopontin protein in eutopic and ectopic endometrium

Figure 3 presents results of western blot analysis of eutopic and ectopic endometrium in endometriosis and controls. The expression of OPN protein was significantly higher in both eutopic and ectopic endometrial tissues of women with endometriosis than in endometrial tissues of controls ($p < 0.01$). This result is similar to that of OPN mRNA. In the eutopic endometrial tissue of women with endometriosis, OPN protein expression increased during the proliferative phase compared to the secretory phase (1.604 ± 0.203 vs. 1.385 ± 0.189) but,

this difference is not statistically significant. OPN protein expression of eutopic endometrium was higher than ectopic endometrium during both proliferative and secretory phases in endometriosis patients. This is not statistically significant as well.

DISCUSSION

Results from the present study demonstrate that expression of OPN mRNA and protein is increased in women with endometriosis. We also demonstrated that OPN mRNA of eutopic and ectopic endometrium in endometriosis expressed differentially during menstrual cycle.

Previous studies have characterized the expression of OPN in human endometrium: OPN is expressed at low concentration during the proliferative and early secretory phase of the menstrual cycle and increase in the mid to late secretory phase.^{22,23} An 8-to 12-fold up-regulation in total endometrium between the early and mid secretory phases has recently been confirmed by microarray screening.^{24,25} The expression pattern correlates with the endometrial epithelial expression of one of the receptors of OPN, the $\alpha_v\beta_3$ -integrin.⁵ The production of OPN in glandular and luminal epithelium, its secretion into the uterine cavity, and the fact that OPN is the ligand of the putative implantation factors, $\alpha_v\beta_3$ -integrin,²⁶ suggest that OPN might play a role during the early stages of implantation.

Both OPN and $\alpha_v\beta_3$ -integrin are expressed by the glandular epithelium and its expression are increased in mid secretory-phase endometrium around the time that implantation occurs, but regulated differentially.²³ The expression of OPN was primarily induced in response to progesterone, whereas the $\alpha_v\beta_3$ -integrin was up-regulated by epidermal growth factor or heparin-binding epidermal growth factor.²³ Although the functional significance of increased OPN expression in secretory-phase endome-

trium remains to be understood, its temporal and spatial patterns of expression suggest an important role during implantation. Several investigators revealed that OPN mRNA and protein are expressed at high concentrations in secretory endometrium in epithelial cells and in decidua in stromal cells.²² And they also demonstrated that expression of OPN in human stromal cells is not directly regulated by short-term stimulation with female steroid hormones and proinflammatory cytokines in vitro but by long-term stimulation with 17 β -estradiol and progesterone, leading to in vitro decidualization of stromal cells. The shift of OPN expression from endometrial epithelial cells to the stromal compartment raises the question of whether OPN might also play a role in the late stages of implantation, the invasion of the trophoblast.

Implantation is a complex process involving proliferation and tissue remodeling in which adhesion molecules, cytokines, and growth factors are believed to play critical roles. In endometriosis, ectopic implantation and growth of endometrial tissue occur and intrinsic factors like adhesion proteins are necessary in these processes as well. In this study, OPN mRNA and protein expression significantly increased in women with endometriosis compared to the control group. These data suggest a role of OPN in the attachment endometrium to peritoneal cavity and furthermore in invasion of endometrium. Indirect evidence for the invasive potential of OPN is given by studies revealing high concentrations of OPN and $\alpha_v\beta_3$ -integrin in malignant tumors.²⁷ Moreover, injection of OPN-transfected benign cell lines into rats resulted in lung metastasis in 55% of the animals.²⁸ These studies raised the question whether binding of OPN to $\alpha_v\beta_3$ -integrins plays a role in adhesive characteristic of endometriosis.

Another role of OPN relates to its effect on the immune system: the sequence of Eta-1 (early T cell activation-1) was found to be identical to OPN. OPN is secreted by activated T-lymphocytes and macrophages

and leads to macrophage migration and enhances immunoglobulin synthesis.²⁹ OPN is an early component of type-1 immunity by potentiating the macrophage IL-12 response through β_3 -integrin and dampens the IL-10 response through engagement of immune cells.³⁰ Endometrium has a high concentration of immune cells.³¹ Several different cytokines are expressed in human endometrium, and the expression of these cytokines is increased during the secretory phase.³² Thus, OPN might play an important role in the regulation of the complex network of immune cell-derived cytokines.^{29,30}

Our study further focused on the differences of OPN mRNA and protein expression between eutopic and ectopic endometrium in endometriosis. In the eutopic endometrial tissue of women with endometriosis, OPN mRNA expression significantly increased during the secretory phase compared to the proliferative phase. In the ectopic endometrial tissue, OPN mRNA expression significantly decreased during the secretory phase compared to the proliferative phase. However, the protein expression of OPN was slightly different from mRNA expression. OPN protein expression of eutopic endometrium was higher than ectopic endometrium during both proliferative and secretory phases in endometriosis patients, but this difference was not statistically significant. Because all expressed mRNA may not be always translated to protein, there may be difference between mRNA expression and protein expression.

Others have demonstrated differences in the endometrium of women with endometriosis as compared to healthy volunteers, leading to the hypothesis that intrinsic abnormalities in the eutopic endometrium may play a role in its implantation in ectopic sites. As recently reviewed, the differences in endometriosis patients include abnormalities in: 1) genes controlling apoptosis, associated with decreased cell death; 2) the regulation of matrix metalloproteinases and their inhibitors, potentially allowing for more efficient implantation of explants;

and 3) integrin expression, possibly enhancing explant-peritoneal interactions and reducing eutopic blastocyst implantation.^{1,33} Hearn-Stokes et al. reported that the spatiotemporal expression of breast cancer nuclear receptor auxiliary factor (Brx) was altered in eutopic endometrium of women with endometriosis.³⁴ The increased expression of Brx supports a possible role for Brx in the augmentation of estrogen signaling and growth of endometrium outside of the uterus.

In conclusion, the present study demonstrated that marked expression of OPN mRNA and protein in endometriosis may be associated with the adhesion and invasion of endometrial explants. Relative amounts of eutopic endometrial OPN mRNA in patients with endometriosis was significantly higher than in controls during both proliferative and secretory phase. It suggests that eutopic endometrium itself might play a crucial role in the pathogenesis of endometriosis. OPN mRNA in eutopic and ectopic endometrial tissue might be differentially expressed and controlled. However, further studies should be necessary to confirm these findings in endometriosis.

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= Abstract =

Objective: This study was performed to compare the expression of osteopontin (OPN) mRNA and protein in the eutopic and ectopic endometrial tissues in women with endometriosis and endometrial tissues in women without endometriosis.

Methods: A total of 32 women with histologically confirmed endometriosis were recruited for study group. For controls, 34 women undergoing operative treatment for cervical intraepithelial neoplasia (CIN) or benign gynecologic condition other than endometriosis were recruited. At the time of laparoscopy or laparotomy, a biopsy specimen was taken from the endometrial cavity and peritoneal endometrial implant or endometrioma whenever appropriate. We employed real time quantitative RT-PCR to quantify OPN mRNA expression of these tissues and performed western blot analysis to measure the quantity of OPN.

Results: The expression of OPN mRNA was significantly higher in both eutopic and ectopic endometrial tissues of women with endometriosis than in endometrial tissues of controls during both proliferative and secretory phase. In the eutopic endometrial tissue of women with endometriosis, OPN mRNA expression significantly increased during the secretory phase compared to the proliferative phase in women with endometriosis as well as controls. However, in the ectopic endometrial tissue, OPN mRNA expression significantly decreased during the secretory phase compared to the proliferative phase. The expression of OPN protein was significantly higher in women with endometriosis than in controls.

Conclusion: This study shows the marked expression of OPN mRNA and protein in eutopic and ectopic endometrial tissues in women with endometriosis may be associated with the adhesion and invasion of endometrial explants.

Key Words: Endometriosis, Osteopontin, Eutopic and ectopic endometrium
