발생과 생식 제 3 권 제 1 호, 29~38 (1999)
Dev. Reprod. Vol. 3, No. 1, 29~38 (1999)

Endometrium from Women with Endometriosis Expresses Decreased Levels of Plasminogen Activator Inhibitor-1 and Tissue Inhibitor of Metalloproteinase-3 Compared to Normal Endometrium

Hye-Won Chung

Department of Obstetrics and Gynecology, Ewha Womans University, College of Medicine. Seoul, Korea 158-070

자궁내막증 환자와 정상 여성의 자궁내막에서 TIMP-3와 PAI-1 mRNA 발현 차이에 관한 연구

정 혜 원

이화여자대학교 의과대학 산부인과학 교실

ABSTRACT : The pathogenesis of endometriosis is unknown, but retrograde menstruation is widely accepted as an etiology. Refluxed endometrium from endometriosis patients is more prone to implant and invade peritoneum possibly through the action of extracellular proteolysis. This proteolytic action may involve plasminogen activators and the collagenase system. Plasminogen activators (PAs) and matrix metalloproteinases (MMPs) play a critical role in the breakdown of extracellular matrix components and basement membrane in the processes of implantation and tumor invasion. PAs are inhibited by plasminogen activator inhibitor (PAI) and MMPs activity is inhibited by tissue inhibitor of metalloproteinase (TIMP). To test the hypothesis that lower expression of PAI-1 and TIMP-3 in endometrium from women with endometriosis, we investigated their PAI-1 and TIMP-3 expression by quantitative competitive RT PCR in endometrium from women with and without endometriosis. Endometrial tissues were obtained from 14 patients with severe endometriosis and 14 patients without endometriosis. Total RNA was extracted and reverse transcribed into cDNA, and quantitative competitive PCR (QC PCR) was performed to evaluate PAI-1 and TIMP-3 mRNA expression. Endometrium from patients with endometriosis showed decreased expression of PAI-1 and TIMP-3 mRNA compared to endometrium from control in luteal phase ($p<0.05$). Our results suggest that endometrium from women with endometriosis expresses lower levels of PAI-1 and TIMP-3 than endometrium from normal women. Endometrium from endometriosis patients may be more invasive and prone to peritoneal implantation than control because of higher PA and MMP enzymatic activity. Thus, increased proteolytic activity may be one of the reasons for the invasive properties of the endometrium resulting in the development of endometriosis.

Key words : PAI-1, TIMP-3, Endometrium, Endometriosis, Quantitative Competitive PCR.

요 약 : 자궁내막증은 흔한 부인과적 질병이며 여성 불임의 한 원인이 되나 그 발생 원인에 대하여서는 아직 논란의 여지가 많다. 최근 월경혈의 역류가 한 원인이며 자궁내막증 환자가 정상여성에서 보다 역류되는 월경혈의 양이 많거나 침습성이 강한 것이 자궁내막증의 발생 원인이 될 수 있다는 이론들이 소개되었다. 종양이나 자궁내막 조직의 침습이나 전이에는 세포막외 기질 및 기저막의 파괴가 일어나야 하는데 이 과정에 plasminogen activators (PAs)나 matrix metalloproteinase (MMPs)같은 proteolytic enzyme이 관여 한다. 이에 자궁 내막증환자와 대조군의 자궁내막에서 PA 나MMP를 억제하는 plasminogen activator inhibitor-1 (PAI-1)나 tissue inhibitor of metalloproteinase (TIMP-3)의 mRNA 발현의 차이를 quantitative competitive RT PCR로 연구하였으며. 그 결과 자궁내막증 환자의 황체기 자궁내막에서는 정상 대조군 환자에서 보다 PAI-1과 TIMP-3 mRNA 발현이 낮음을 알 수 있었다. 따라서 자궁내막증 환자의 자궁내막에서는 PA와 MMP의 활성도가 증가할 수 있으며 이 증가된 proteolytic activity로 인하여 역류된 자궁내막 조직의 복강내 침습이 보다 쉽게 일어날 가능성이 있다.

INTRODUCTION

Endometriosis is a common benign gynecologic disorder. The pathogenesis is controversial, but the theory of retrograde menstruation which postulates reflux of shed endometrial tissue through the tube with implantation on the peritoneal surface is widely accepted (Sampson, 1927). After implantation, this endometrium invades the surrounding tissue with corresponding cell proliferation and

neoangiogenesis. Although retrograde mentsruation is physiologic, such menstrual debris does not result in endometriosis in all women. Recently the volume and characteristics of menstrual debris in women with endometriosis have been examined; menorrhagia or hypotonus of the uterotubal junction resulted in more retrograde menstrual flow than normal (Arumugam and Lim, 1997). In addition to greater volume, refluxed menstrual debris in women with endometriosis may be more prone to implant and invade peritoneum or ovary through the action of extracellular proteolysis. This inherent proteolytic activity may define an important role for matrix metalloproteinase (MMPs) and plasminogen activators.

The MMP family is a group of structurally related proteins, which degrade extracellular matrix (ECM) and basement membrane (BM) components (Hulboy et al., 1997). They are produced as proenzymes and secreted into the ECM, where they are activated through proteolytic cleavage by other MMPs or plasmin. MMPs also play an essential and causal role in the progression of human neoplasm and embryo implantation (Behrendtsen et al., 1992). Degradation of matrix proteins is an important step that allows tumor cells to penetrate the EC, to intravasate into blood vessels, and to extravasate at distant sites (Fishman et al., 1997; Baricos et al., 1995). Consequently downregulation of MMP activity in tumor cells by exogenously added MMP inhibitors can inhibit invasion of tumor cells *in vitro* and metastasis *in vivo* (DeClerck et al., 1992; Khokha, 1992).

In the ECM, the activity of MMPs is tightly regulated by a family of natural inhibitors known as tissue inhibitors of metalloproteinase (TIMPs). TIMP-1, TIMP-2, TIMP-3, and TIMP-4, have been described (Hulboy et al., 1997). TIMP-3, a novel member of the TIMP family, has been shown to have inhibitory activity against stromelysin-1, collagenase-1, and the 92-kDa collagenase (Apte et al., 1996). Both TIMP-1 and TIMP-3 have demonstrated inhibitory activity against the 72- and 92-kDa type IV collagenase. Each MMP can be inhibited by a specific TIMP through the formation of 1:1 complexes (Birkedal-Hansen et al., 1993).

The fibrinolytic system is associated with fibrin removal, the coagulation cascade, tissue remodeling, tumor invasion, ovulation, and embryo implantation (Loskutoff et al., 1989; Ny et al., 1994). The proenzyme plasminogen is transformed into the highly potent plasmin by activating enzymes, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). While the activation by tPA is important for fibrinolysis, uPA triggers localized pericellular proteolysis of ECM. The activity of the plasminogen activators is regulated by specific plasminogen activator inhibitors (PAIs). Type-1 plasminogen activator inhibitor (PAI-1), a 50-kD glycoprotein, is the major physiological inhibitor of both tPA and uPA and plays an important role in determining net fibrinolytic activity in vivo (Booth, 1994). The inappropriate expression of PAI-1 may suppress the normal fibrinolytic activity of the tissues and result in pathological fibrin deposition (Loskutoff et al., 1991).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) can be used to analyze very low abundance mRNAs derived from cells with great sensitivity. Quantitative analysis of mRNA can be achieved by a modification known as quantitative competitive PCR (QC-PCR; Jin et al., 1994; Wang et al., 1989), in which an internal control of specific base sequence is amplified simultaneously with a target sample to give a quantitative measure of mRNA level. In the study reported here, we determined both PAI-1 and TIMP-3 mRNA expression in endometrium from women with and without endometriosis by QC-PCR. We tested our hypothesis that tissue from women with endometriosis would express a lower TIMP-3 and PAI-1 mRNA consistent with increased invasiveness.

MATERIALS AND METHODS

1. Tissue Collection

Endometrial samples were obtained from 28 premenopausal women aged 29~45 yr, undergoing laparoscopic surgery, or hysterectomy for various non-malignant lesions. Patients with pelvic inflammatory disease and dysfunctional uterine bleeding were excluded. Samples from 14 patients with severe endometriosis, diagnosed by both pathology and laparoscopic findings according to the revised American Fertility Society classification of endometriosis

(American Fertility Society, 1985) and samples from 14 patients without endometriosis, also confirmed by laparoscopic surgery were used in the study. Endometrial samples were taken using a curette in the operating room before the laparoscopic procedure; in patients undergoing hysterectomy, the uterine cavity was opened and endometrium obtained immediately after the specimen was removed. Endometriosis tissue was obtained during laparoscopic endometrial cyst enucleation from ovarian tissue. Tissue was fixed and sent to the Pathology Department for histological endometrial dating and confirmation of the endometriosis. Tissue samples were classified by collection and histological dating according to the method of Noyes (Noyes et al ., 1950) into two groups: proliferative phase (n=14), luteal phase (n=14). The tissue was washed in PBS solution in order to remove contaminating blood and was directly processed for RNA extraction.

2. RNA Extraction

The extraction of RNA from the tissue sample was carried out with the RNA-STAT-60 reagent (Tel-Test "B" Inc.). Briefly, tissue samples were washed three times in PBS (Gibco BRL) to remove blood contamination. One hundred milligrams of tissue were homogenized in 1 ml of RNA-STAT-60 reagent. Total RNA was separated from DNA and proteins by adding chloroform and was precipitated using isopropanol. The precipitate was washed two times in 75% ethanol, air-dried and re-diluted in diethylpyrocarbonate (DEPC)-treated dH2O. Amount and purity of extracted RNA was quantitated by spectrophotometry in a GenQuant RNA/DNA calculator (Pharmacia Biotech Ltd) and 10~100 μg of total RNA was routinely obtained.

3. Primers for Reverse Transcription (RT) and PCR

Specific sequences of oligonucleotide primers for detecting ectopic and eutopic endometrium for TIMP-3 (Byrne et al., 1995) and PAI-1 (Ginsberg et al., 1986) were obtained from Gene Bank Database of the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH, Internet address: http://www.2.ncbi.nlm.nih. gov/cgi-bin/genbank). One corresponding set of primers for TIMP-3 and PAI-1 was found with the help of the program OLIGO 5.0 Primer Analysis Software (National Bioscience, Plymouth, MN) and synthesized. The human β-actin primers that were used to amplify an external standard were obtained from Clontech Laboratories Inc., Palo Alto, CA. β-actin mRNA expression was employed as an external positive control, being detected in all the samples studied, confirming the integrity of the RNA and the RT-PCR process. The primer sequences, locations on the mRNA, and sizes of the amplified fragments are listed in Table 1.

4. RT

For RT-PCR, the Gen Amp RNA PCR kit (Perkin-Elmer) was used. Nineteen microliters of RT-mastermix for each sample were prepared containing 5 mmol/L $MgCl_2$, 1X PCR buffer II, 1 mmol/L of each deoxy-NTP, 2.5 μl/L oligo $(deoxythymidine)_{16}$, 20 IU ribonuclease inhibitor (Perkin-Elmer), and 100IU Moloney murine leukemia virus reverse transcriptase (Gibco BRL), and 1μg total RNA diluted in 1 μl DEPC-treated dH_2O and filled into 0.5-ml thin wall PCR tube (Applied Scientific). RT-MasterMix in PCR-tubes was covered with 50 μl of light white mineral oil (Sigma) and kept on ice until the RT. RT was carried out in the DNA Thermal Cycler 480 (Perkin-Elmer) using a program with the following parameters: 42℃, 15 min; 99℃, 5 min; then quenched at 4℃. After the reaction was completed, samples were stored at −20℃ until the PCR. As negative control, 1μl DEPC-treated dH_2O without RNA sample was subjected to the same RT reaction.

5. Construction of the Competitive- and Target-cDNA Fragment for TIMP-3 and PAI-1

A 969 base pair (bp) and 1458 bp fragment of native TIMP-3 and PAI-1 cDNA respectively (the target) were obtained by PCR amplification of reverse-transcribed total RNA from endometrial biopsies with the regular 5′ and 3′ primers (Table 1). The PCR product was visualized by agarose gel elecrophoresis stained with ethidium bromide (EtBr), and the cDNA was extracted from the gel, purified with an agarose gel extraction kit (Amersham Pharmacia Biotech), quantitated by spectrophotometry (Pharmacia

Table 1. Oligonucleotide primers for eutopic and ectopic endometrium mRNA amplification

mRNA		Primers 5'-3'	Size(bp)	Position on mRNA
TIMP-3	Upstream (5'-end)	TGC CAG AAA GAA TGA GGA ACC	969	1167-1187
	Downstream (3'-end)	AGA GAG GGT GCT GAC GGT GTT		2135-2115
	Competitor	AGA GAG GGT GCT GAC GGT GTT CAG ACT CAG GAA CAT	464	2135-2115, 1609-1595
PAI-1	Upstream (5'-end)	GGA TGA GAT CAG CAC CAC AG	1458	408-427
	Downstream (3'-end)	ATG GGG GAC ATT CAC TCT G		1865-1847
	Competitor	ATG GGG GAC ATT CAC TCT GAG GAG AGG CCA AGGT	1067	1865-1847, 1455-1441

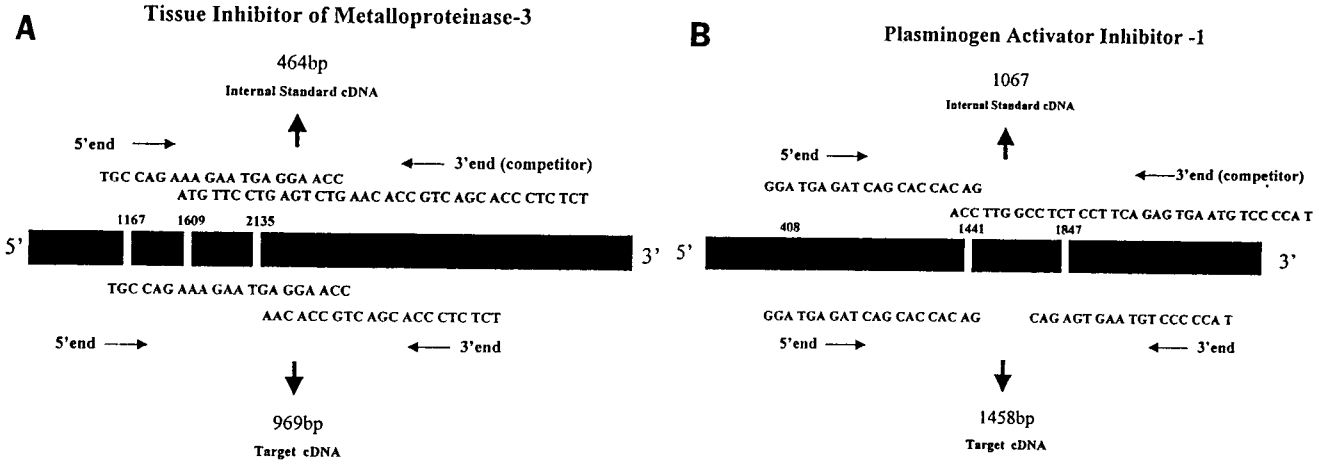

Fig. 1. A representative schematic illustration of construction of internal standard cDNA for TIMP-3(A), and PAI-1(B).

Biotech). To construct a competitive cDNA fragment: a floating primer with a sequence complementary to the cDNA between the 3′ and 5′ primer binding sites was designed by attaching the complementary sequence of the binding site with the original 3′- primer (Fig. 1). After PCR with the regular 5′-primer and the 3′-floating primer, the PCR product was visualized by agarose gel electrophoresis stained with EtBr. cDNA extraction, purification and determination of the concentration were performed as described above. This step resulted in cDNA fragment of 464 bp and 1067 bp with 505 bp and 391 bp deletion compared to the target cDNA and with the 3′-end and 5′-end primer binding sites on its ends.

6. Standard-Curve and Competitive PCR for TIMP-3 and PAI-1

The standard curve for MMP-9 and TIMP-3 was constructed by a co-amplification of a constant amount of competitive cDNA (each 1.25 fmol and 0.5 fmol) with declining amounts of target cDNA obtained by serial dilution. The amounts of target and competitive cDNA added to each PCR for TIMP-3 and PAI-1 are shown in Figure 2. Sixty microliters of the cDNA mix was added to 40 μl PCR-mastermix containing 1.9 mM $MgCl_2$ solution, 10X PCR buffer II, 0.2 mM each dNTP, 2.5 U Taq-polymerase (Perkin-Elmer), corresponding paired primers in a concentration of 0.2 μmol/L of each primer to a total volume of 100 μl and 14.5 μl DEPC-treated dH_2O. The reaction mixture was covered with 50 μl light white mineral oil and put in the Perkin-Elmer DNA Thermal Cycler 480. PCR cycles were composed of 1 cycle of 95℃ for 5 min to denature all proteins, 30 cycles of 45s at 94℃, 45s at 55℃, and 60s at 72℃. The reaction was terminated at 72℃ for 5 min and was quenched at 4℃.

Two percent agarose gel (Life Technologies) electrophoresis was carried out in H5 electrophoresis chamber with 100bp ladder DNA. Gels were stained with ethidium bromide (Sigma). Aliquots (25μL) of each PCR product and dye buffer were analyzed in parallel with a 100-bp DNA ladder (Life Technologies) as a standard. After com-

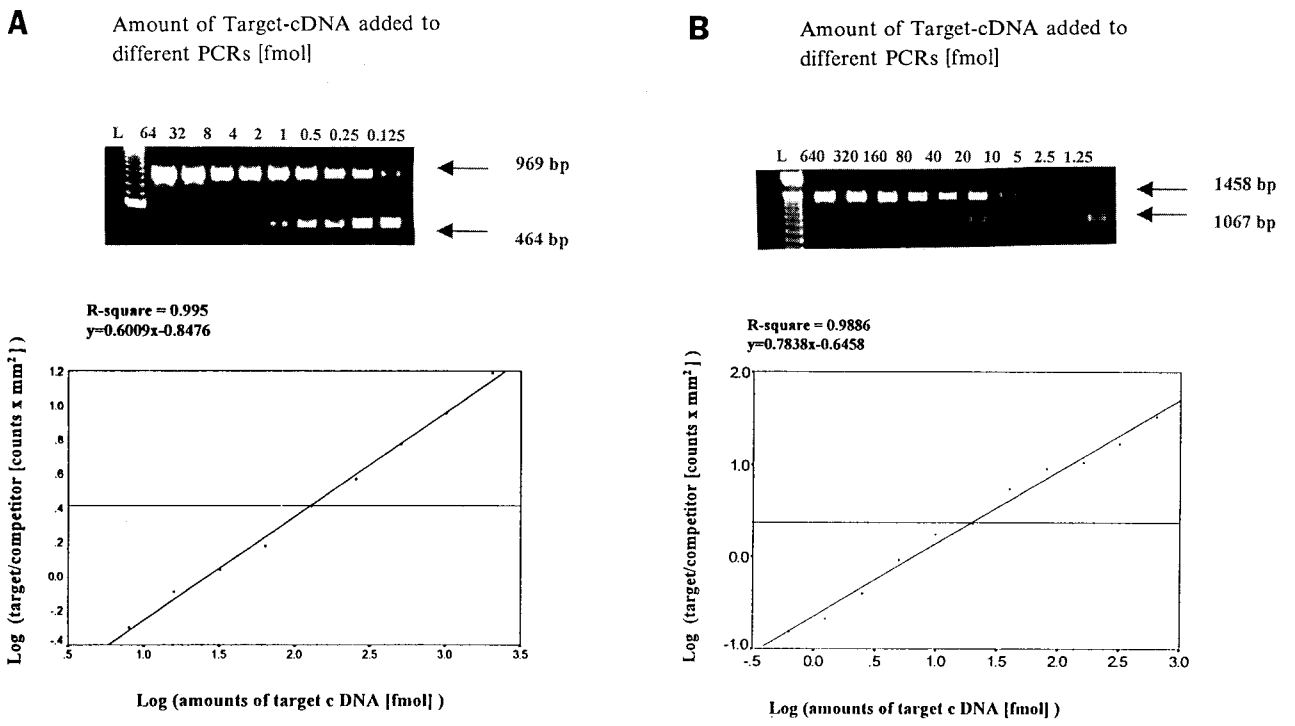

Fig. 2. Standard curves for TIMP-3 (A) and PAI-1 (B) QC PCR. The upper panels show 2% agarose gels stained with ethidium bromide. Declining amounts of target cDNA were coamplified with 1.25 fmol and 0.5 fmol of competitive cDNAs. The lower panels show the standard curves from these gels. The log ratio of target to competitive product density is plotted against the log amount of target initially added to the PCR.

pletion of electrophoresis, the gel blot was analyzed by UV densitometry, and photocopies of the blot were printed (Gel-Doc 1000 system, Bio-Rad Laboratories). The logarithmically transformed ratios of target cDNA to competitive cDNA were plotted against the log amount of initially added target cDNA in each PCR to obtain the standard curves shown in Figures 2A & B. The standard curves were highly reproducible and linear. The values obtained from the regression line of the standard curve ($y = b + mx$) allowed us to calculate the amount of cDNA transcripts in an unknown sample: 1.25 fmol of TIMP-3 and 0.1 fmol of PAI-1 competitive cDNA were added to each unknown sample before PCR. The ratio of the densities of sample target cDNA band (969 bp, 1458 bp) to competitive cDNA (464 bp, 1067 bp) were logarithmically transformed and compared to the values obtained from standard curve. QC PCR was carried out on at least two aliqouts from the RT cDNA of each patient, and the results did not differ more than ±5%.

7. PCR for β-Actin

RT cDNA of each patient was mixed with 78μl of PCR-mastermix described above with 3′-, 5′- primer forβ -actin. PCR program parameters were similar. For this PCR no competitor was added.

8. Data Analysis

Statistical analysis was performed by ANOVA and t-test. The statistical analysis was carried out using the Statistical Package for Social Science Statview Package (SPSS Inc., Chicago, IL) with a p value <0.05 considered statistically significant.

RESULTS

1. RT-PCR of endometrium throughout the menstrual cycle

RT-PCR was employed to increase the sensitivity of detection, and 969 bp sequence of TIMP-3 and the 1458 bp

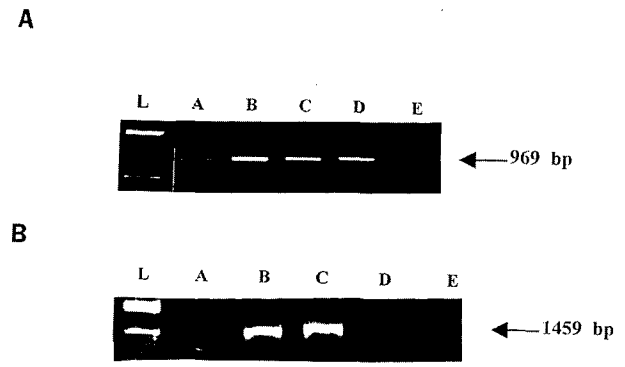

Fig. 3. Agarose gel showing the product of PCR amplification. RT-PCR of total endometrial RNA demonstrates bands corresponding to TIMP-3(A) and PAI-1(B). L; 100 bp ladder DNA. Lane A; Follicular phase endometrium from a normal woman. Lane B; Follicular phase endometrium from an endometriosis patient. Lane C; Luteal phase endometrium from a normal woman. Lane D; Luteal phase endometrium from an endometriosis patient. Lane E; Negative control.

sequence of PAI-1 mRNA were expressed by all endometrial samples from women with and without endometriosis in both the follicular and luteal phases of menstrual cycle (Fig. 3). β-actin mRNA expression was ascertained in all the samples studied, thus confirming the integrity of RNA and the RT-PCR process (data not shown).

2. TIMP-3 mRNA expression in endometrial tissue from women with or without endometriosis

The expression of TIMP-3 mRNA was significantly lower in endometrium from women with endometriosis compared to endometrium from normal women in the luteal phase ($p<0.05$). A similar pattern was seen during follicular phase although the differences in TIMP-3 mRNA expression in endometrium from normal and endometriosis patients did not reach significance (Fig. 4A, B).

3. PAI-1 mRNA expression in endometrial tissue from women with or without endometriosis

The expression of PAI-1 mRNA in endometrial samples from the endometriosis patients throughout the menstrual cycle was compared to that in control patients. Endometrium from endometriosis patients expressed significantly ($p<0.05$) lower PAI-1 mRNA compared to endometrium from women without endometriosis during the luteal phase. A similar pattern was seen during follicular phase although the differences in PAI-1 mRNA expression in endometrium from normal and endometriosis patients did not reach significance (Fig. 5A, B).

DISCUSSION

Endometriosis, a benign gynecologic disorder, occurs in about 10% of women of reproductive age and in up to 50% of women with infertility. Endometriosis is defined as the presence of endometrial glandular and stromal cells outside their normal location in the uterus. Commonly affected areas in the abdominopelvic cavity include the ovaries, the cul-de-sac and other kinds of pelvic peritoneum, bowel and diaphragm.

The pathogenesis of endometriosis is still controversial, but the widely accepted theory is the theory of retrograde menstruation in which the origin of endometriotic tissue is uterine endometrium. This viable and hormonally sensitive tissue reaches the peritoneal cavity by tubal reflux and implants outside of the uterus. However, retrograde menstruation is common to all menstruating women, and cannot by itself explain the pathogenesis of endometriosis.

Recently, invasive proteolysis has been implicated in the development of endometriosis; and the invasion indices of cells from peritoneal endometriosis lesions and a metastatic carcinoma cell line are similar compared with normal endometrium and non metastatic carcinoma cells (Gaetje et al., 1995). Gonadotropin releasing hormone agonist (GnRHa) therapy decreased PA and MMP activities and also increased PAI and TIMP activities, and this GnRHa-induced shift to a less invasive phenotype may alter fibrinolysis and ECM remodeling which resulted in less adhesion formation in a rat model (Sharpe et al., 1998). Compared to uterine endometrium, ectopic endometrium has significantly higher capacity to produce the latent forms (72 kD) of gelatinase A. This leads to the conclusion that endometriotic implants express the protease enabling invasion into surrounding tissue (Wenzl and Heinzl, 1998). The MMP-9 may play an integral role in embryo implantation, placentation, and degra-

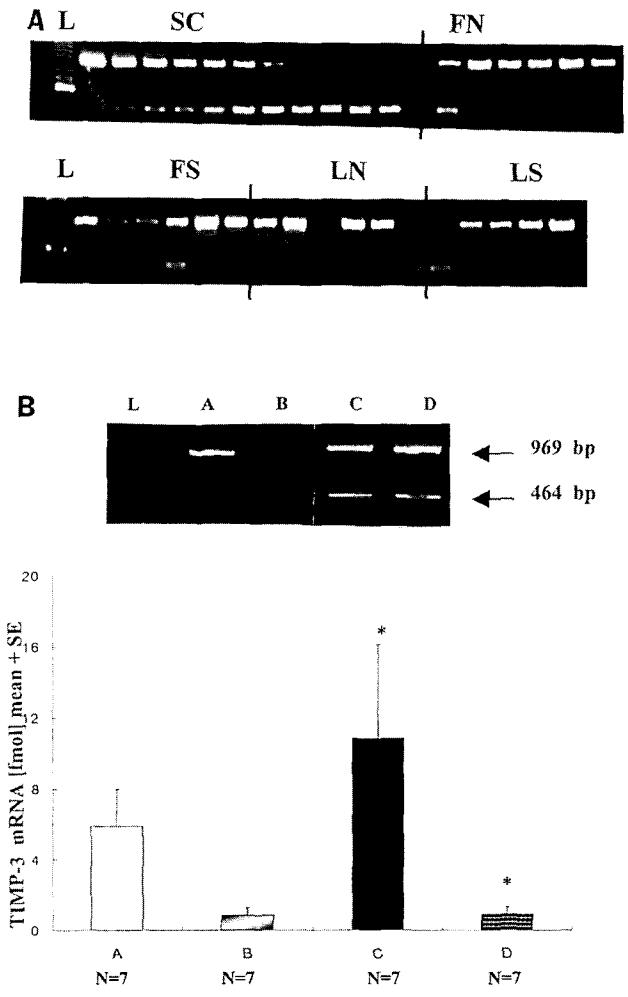

Fig. 4. QC PCR of TIMP-3. (A) QC PCR of TIMP-3 in total endometrium from women with or without endometriosis throughout the menstrual cycle. SC; Standard Curve. FN; Follicular phase endometrium from normal women. FS; Follicular phase endometrium from endometriosis patients. LN; Luteal phase endometrium from normal women. LS; Luteal phase endometrium from endometriosis patients. (B) QC-PCR analysis of TIMP-3 mRNA extracted from total endometrium. Samples were coamplified for 30 cycles in the presence of a defined amount of internal standard cDNA for TIMP-3 (1.25 fmol). A typical gel is shown. L; 100 bp ladder DNA. Lane A; Follicular phase endometrium from a normal woman. Lane B; Follicular phase endometrium from an endometriosis patient. Lane C; Luteal phase endometrium from a normal woman. Lane D; Luteal phase endometrium from an endometriosis patient. * $p<0.05$

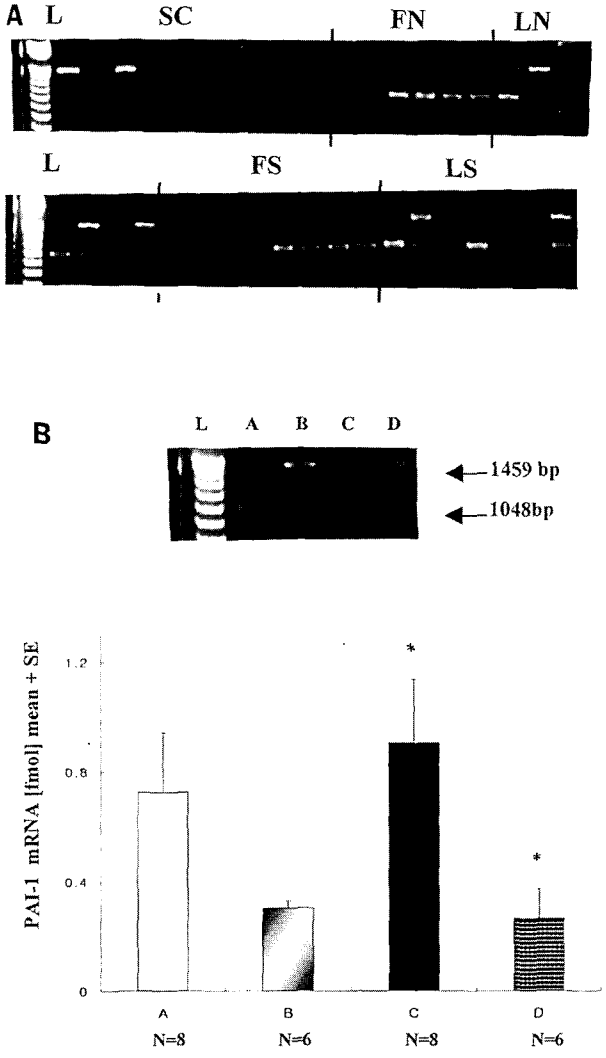

Fig. 5. QC PCR of PAI-1. (A) QC PCR of PAI-1 in total endometrium from with or without endometriosis throughout the menstrual cycle. SC; Standard Curve. FN; Follicular phase endometrium from normal women. FS; Follicular phase endometrium from endometriosis patients. LN; Luteal phase endometrium from normal women. LS; Luteal phase endometrium from endometriosis patients.
(B) QC-PCR analysis of PAI-1 mRNA extracted from total endometrium. Samples were coamplified for 30 cycles in the presence of a defined amount of internal standard cDNA for PAI-1 (0.5fmol). A typical gel is shown. L; 100 bp ladder DNA. Lane A; Follicular phase endometrium from a normal woman. Lane B; Follicular phase endometrium from an endometriosis patient. Lane C; Luteal phase endometrium from a normal woman. Lane D; Luteal phase endometrium from an endometriosis patient. * $p<0.05$

dation of the basement membrane (Shiminovitz et al, 1994). It is inhibited by TIMP-1 and 3 which are originated from decidual or trophoblast cell. uPA has been strongly implicated in the progression of several malignancies including breast and prostate cancers (Yu and Kim, 1997). PAI-1 plays an important role in determining net fibrinolytic ac-

tivity. There is no attempt to compare PAI-1 and TIMP-3 mRNA expression differences in endometrium from endometriosis patient and control women. In this study, we have shown that endometrium from women with endometriosis expresses lower levels of TIMP-3 and PAI-1 expression when compared with normal endometrium. The results of this study suggest that endometrium from women with endometriosis may be inherently more invasive because of decreased TIMP-3 and PAI-1 mRNA expression allowing higher MMP and PA proteolytic activity which permits easy peritoneal invasion after ectopic implantation. Thus, TIMP-3 and PAI-1 may have crucial roles in the invasive pathogenesis of endometriosis.

If the origin of endometriosis tissue is the uterine endometrium, then endometrium from endometriosis patients may be biologically different from those of normal patients. In this study, endometrium from women with endometriosis expressed lower levels of TIMP-3 and PAI-1 mRNA. Thus, TIMP-3 and PAI-1 may play an important role for endometriosis pathogenesis and be a critical differentiating factor responsible for invasiveness.

The MMP family is a group of structurally related proteins that degrade ECM and BM components (Edwards et al., 1996). The overexpression of members of the MMP family results in pathological conditions characterized by connective tissue destruction, including diseases such as arthritis, periodontitis, and cancer suggesting that tight regulation of MMP genes is critical for normal tissue homeostasis. The positive correlation between the level of MMP detected in tumor tissues and the degree of local recurrence, lymph node metastasis, distant metastasis, and adverse clinical outcome has been documented (Gray et al., 1993). Thus, MMPs play an important and active part in the invasive and metastatic behavior of human cancer (Powell and Matrisian., 1996). In ECM the activity of MMPs is tightly regulated by a family of natural inhibitors known as TIMPs. TIMPs, therefore, also play an important role in maintaining the integrity and substrate specificity of MMPs. TIMP-3, a novel member of the TIMP family, has been shown to have inhibitory activity against stromelysin-1, collagenase-1, and the 92-kDa collagenase.

The plasmin/PA cascade is a key component in the tissue remodeling that accompanies repair processes. PA converts plasminogen to plasmin, a potent serine proteinase that solubilizes fibrin, but can also degrade various basement -membrane components. There are two types of PAs: tPA and uPA. These are serine proteinases that cleave the proenzyme plasminogen into the highly potent serine protease plasmin. Plasmin is able to dissolve fibrin clots, to digest ECM proteins, and to activate procollagenase. There are two members of the serine family of inhibitors, the endothelial-type inhibitor PAI-1 and the placental-type inhibitor PAI-2, which bind to and inactivate both PAs with particularly high affinity (Loskutoff et al., 1989). PAI-1 is secreted as an active PA inhibitor, but rapidly undergoes a unique conformational change to its latent form. PAI-1 is a 50-54kDa, acid-stable glycoprotein found in plasma, platelet, endothelial cells, and hepatoma cells. PAI-1 appears to be the primary physiologic inhibitor of both PAs (tissue plasminogen activator and urokinase plasminogen activator). Stabilization of PAI-1 in its active form, by binding to circulating vitronectin (Loskutoff et al., 1989) and to ECM-sequestered vitonectin (Salonen et al., 1989), is integral to the regulation of fibrinolysis and of proteolysis of ECM components, respectively.

Due to its extraordinarily high sensitivity, polymerase chain reaction (PCR) has been used to amplify cDNA copies of low abundance mRNA (Sarkar and Sommer., 1989). However, quantitation is unreliable because the amount of PCR product increases exponentially with each cycle of amplification; therefore, minute differences in any of the variables that affect the efficiency of amplification can dramatically alter product yield. Rather than analyzing a different reporter gene product (Frye et al., 1989), we constructed an internal standard with a defined deletion fragment from the target cDNA, and used the same primers to coamplify the unknown and the competitor allowing us to quantify the amount of specific target cDNA available. In addition, because the efficiency of amplification of the internal control molecules is identical to that of the target template, QC PCR can avoid the discrepancies associated with tube-to-tube or sample-to-sample variations in the kinetics of the RT reaction (Uberla et al., 1991). For RNA quantitation Nothern blot technique is widely used. How-

ever, the Northern blot technique requires at least 10 μg of total RNA for semiquantitation. For mRNA localization in situ hybridization has been widely used, but it lacks enough quantitative information. Thus, we have investigated mRNA expression differences between normal and endoemtriosis patient's endometrium using QC-PCR technology and quantified the amount of specific target cDNA available for both TIMP-3 and PAI-1.

Our results suggest that uterine endometrium from women with endometriosis expresses lower levels of TIMP-3 and PAI-1 than endometrium from normal women, which may be the critical factor in the initial peritoneal invasion of endometrial tissue. Thus, increased proteolytic activity may be one of several reasons for the invasive properties of the endometrium resulting in the development of endometriosis. This is the first report on PAI-1 and TIMP-3 mRNA expression report in diffferences between endometrium from women with endometriosis and without endometriosis.

REFERENCES

Apte SS, Olsen BR, Murphy G (1996) The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem 271:2874-2881.

American Fertility Society (1985) Revised American Fertility Society classification of endoemtriosis. Fertil Steril 43:351.

Arumugam K, Lim JM (1997) Menstrual characteristics associated with endometriosis. Br J Obstet Gynaecol 104:948-950.

Baricos WH, Cortez SL, el-Dahr SS, Schnaper HW (1995) ECM degradation by cultured human mesangial cells is mediated by a PA/plasmin/MMP-2 cascade. Kidney Int 47:1039-1047.

Behrendtsen O, Alexander CM, Werb Z (1992) Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development 114:447-456.

Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA (1993) Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 4:197-250.

Booth NA (1994) Natural inhibitors of fibrinolysis. In: Haemostasis and Thrombosis, 3rd edition. Bloom AL, Forbes CD, Thomas DP, Tuddenham EGD eds., Churchill Livingstone, Edinburgh, 699-717.

Byrne JA, Tomasetto C, Rouyer N, Bellocq JP, Rio MC, Basset P (1995) The tissue inhibitor of metalloproteinases-3 gene in breast carcinoma: identification of multiple polyadenylation sites and a stromal pattern of expression. Mol Med 1:418-427.

DeClerck YA, Perez N, Shimada H, Boone TC, Langley KE, Taylor SM (1992) Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res 52:701-708.

Edwards DR, Beaudry PP, Laing TD, Kowal V, Leco KJ, Leco PA, Lim MS (1996) The roles of tissue inhibitors of metalloproteinases in tissue remodelling and cell growth. Int J Obes Relat Metab Disord 20 Suppl. 1996; 3:S9-15.

Fishman DA, Bafetti LM, Banionis S, Kearns AS, Chilukuri K, Stack MS (1997) Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. Cancer 75:1457-1463.

Frye RA, Benz CC, Liu E (1989) Detection of amplified oncogenes by differential polymerase chain reaction. Oncogene 4:1153-1157.

Gaetje R, Kotzian S, Herrmann G, Baumann R, Starzinski-Powitz A (1995) Invasiveness of endometriotic cells *in vitro*. Lancet 150:1463-1464.

Ginsburg D, Zeheb R, Yang AY (1986) cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. J Clin Invest 78: 1673-1680.

Gray ST, Yun T, Motoori T, Kuys YM (1993) Interstitial collagenase gene expression in colonic neoplasia. Am J Pathol 143:663-671.

Hulboy DL, Rudolph LA, Matrisian LM (1997) Matrix metalloproteinase as mediators of reproductive function. Mol Hum Reprod 3:27-45.

Jin CF, Mata M, Fink DJ (1994) Rapid construction of deleted DNA fragments for use as internal standards in

competitive PCR. PCR Methods Appl 3:252-255.

Khokha R, Zimmer MJ, Wilson SM, Chambers AF (1992) Up-regulation of TIMP-1 expression in B16-F10 melanoma cells suppresses their metastatic ability in chick embryo. Clin Exp Metastasis 10:365-370.

Loskutoff DJ (1991) Regulation of PAI-1 gene expression. Fibrinolysis 5: 197-206.

Loskutoff DJ, Sawdey M, and Mimuro J (1989) Type 1 plasminogen activator inhibitor. Prog Hemost Thromb 9: 87-115.

Noyes RW, Hertig AT, Rock J (1950) Dating the endoemtrial biopsy. Fertil Steril 1:3-25.

Ny T, Leonardsson G, Peng XR (1994) Role of plasminogen activators and their inhibitors in ovulation and reproduction. In Aznar J, Gilabert J and Estelles A. (eds), Fibrinolytic Inhibitors. Cellular, Biological and Clinical Aspects. Garsi-Masson, Madrid, pp.32-42.

Powell WC, Matrisian LM (1996) Complex roles of matrix metalloproteinases in tumor progression. Curr Top Microbiol Immunol 213 (Pt 1):1-21.

Salonen EM, Vaheri A, Pollanen J, Stephens R, Andreasen P, Mayer M, Dano K, Gailit J, Ruoslahti E (1989) Interaction of plasminogen activator inhibitor (PAI-1) with vitronectin. J Biol Chem 264:6339-6343.

Sampson JA (1927) Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 14:422-469.

Sarkar G, Sommer SS (1989) Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science 244:331-334.

Sharpe-Timms KL, Zimmer RL, Jolliff WJ, Wright JA, Nothnick WB, Curry T (1998) Gonadotropin-releasing hormone agonist (GnRH-a) therapy alters activity of plasminogen activators, matrix metalloproteinases, and their inhibitors in rat models for adhesion formation and endometriosis: potential GnRH-a-regulated mechanisms reducing adhesion formation. Fertil Steril 69(5) :916-23 29.

Shimonovitz S, Hurwitz A, Dushnik M, Anteby E, Geva-Eldar T, Yagel S (1994) Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. Am J Obstet Gynecol 171(3):832-838.

Uberla K, Platzer C, Diamantstein T, Blankenstein T (1991) Generation of competitor DNA fragments for quantitative PCR. PCR Methods Appl 1:136-139.

Wang AM, Doyle MV, Mark DF (1989) Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci USA 86:9717-9721.

Wenzl R, Heinzl H (1998) Localization of matrix metalloproteinase-2 in uterine endometrium and ectopic implants. Gynecol Obstet Invest 45:253-257.

Yu W, Kim J, Ossowski L (1997) Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. J Cell Biol 137(3):767-77.