Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 mRNA expression in ectopic and eutopic endometrium in women with endometriosis: a rationale for endometriotic invasiveness

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Objective: To investigate mRNA expression of metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-3 (TIMP-3) in ectopic endometriosis tissue and uterine endometrium from women with and without endometriosis throughout the menstrual cycle.

Design: Molecular studies in human tissue.

Setting: Department of Gynecology and Obstetrics, Reproductive Immunology Laboratory, Stanford University Medical Center.

Patient(s): Fifty-three premenopausal woman (23 women with endometriosis and 30 women without endometriosis undergoing laparoscopic surgery). Endometrium and ectopic endometriosis tissue were obtained at the time of surgery.

Intervention(s): None.

Main Outcome Measure(s): mRNA expression from eutopic and ectopic endometrium was analyzed by quantitative, competitive PCR.

Result(s): Both uterine endometrium and ectopic endometriotic tissue from women with endometriosis expressed significantly ($P<.05$) lower levels of TIMP-3 than endometrium from normal women. Also, ectopic endometrium expressed higher levels of MMP-9 and a higher ratio of MMP-9/TIMP-3 than eutopic endometrium from normal and endometriosis patients.

Conclusion(s): These results suggest that ectopic and eutopic endometrium from endometriosis patients may be more invasive and prone to peritoneal implantation because of greater MMP and less TIMP-3 mRNA expression than endometrium from women without endometriosis. Thus, increased proteolytic activity may be one of the reasons for the invasive properties of the endometrium, resulting in the development of endometriosis. (Fertil Steril® 2001;75:152–9. ©2001 by American Society for Reproductive Medicine.)

Key Words: MMP-9; TIMP-3; endometrium; endometriosis; quantitative, competitive PCR

Endometriosis is a common benign gynecologic disorder. The pathogenesis is controversial, but the theory of retrograde menstruation that postulates reflux of shed endometrial tissue through the tube with implantation on the peritoneal surface is widely accepted (1). After implantation, this endometrium invades the surrounding tissue with corresponding cell proliferation and neoangiogenesis. Although retrograde menstruation 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 (2). In addition, menstrual debris in women with endometriosis may be more prone to implant and invade peritoneum or ovary through the action of extracellular proteolysis.

The metalloproteinase (MMP) family is a group of structurally related proteins that degrade extracellular matrix (ECM) and basement membrane (BM) components (3). The 92-kD type IV collagenase (MMP-9), an important enzyme for degradation of the basement membrane (primarily collagen type IV), is crucial for the invasive ability of trophoblast cells (4, 5) and in the progression of human neoplasm (6). A decrease in MMP activity inhibits the invasion of tumor cells both in vivo and in vitro (7).

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 (3). TIMP-3, a novel member of the TIMP family, has been shown to have inhibitory activity against stromelysin-1, collagenase-1, and MMP-9 (8) through the formation of 1:1 complexes (9).

The balance between the inhibitors and MMPs is important because invasion depends on the MMP/TIMP ratio (10), which is a critical determinant in maintaining the homeostasis and the integrity of the ECM. Ectopic endometrium has a significantly higher capacity to produce the latent MMP-2 (11). MMP-1 expression correlates with activity of endometriotic tissue (12), and TIMP-1 concentrations were significantly lower in peritoneal fluid and sera of women with endometriosis (13). Subtle changes in the MMP-TIMP balance occur during many physiologic processes, such as trophoblast implantation and neoplastic invasion (14), and may be important in the peritoneal invasion of endometriosis.

Reverse transcription-polymerase chain reaction (RT-PCR) can be used to analyze very low abundance mRNAs derived from cells with great sensitivity. In the study reported here, we determined both MMP-9 and TIMP-3 mRNA expression in eutopic and ectopic endometrium from women with and without endometriosis by using quantitative, competitive PCR (QC-PCR) (15). We tested our hypothesis that tissue from women with endometriosis would express a higher MMP-9/TIMP-3 ratio consistent with higher proteolytic activity and increased invasiveness.

MATERIALS AND METHODS

Tissue Collection

Endometrial samples were obtained from 53 premenopausal women aged 29–45 years who were undergoing laparoscopic surgery or hysterectomy for nonmalignant lesions such as fibroids and stress incontinence. Patients with pelvic inflammatory disease, adenomyosis, and dysfunctional uterine bleeding were excluded. Sufficient paired eutopic and ectopic endometrial tissue was available from 23 patients with severe endometriosis, stages III and IV, diagnosed by both pathology and laparoscopic findings according to the revised American Fertility Society classification of endometriosis (16). Endometrial tissue from 30 control patients without endometriosis confirmed by laparoscopic surgery was also collected. Written consent from the patients and approval by the Institutional Committee on the Use of Human Subjects in Research at Stanford University were obtained for this study.

Endometrial samples were taken with a curette in the operating room before the laparoscopic procedure; in patients undergoing hysterectomy, the uterine cavity was opened and the endometrium obtained immediately after the specimen was removed. Sufficient endometriosis tissue for analysis was obtained during laparoscopic endometrial cyst enucleation from ovarian tissue but not from excised peritoneal implants. Tissue was fixed and sent to the pathology department for histologic endometrial dating and confirmation of endometriosis. Tissue samples were classified into two groups by histologic dating according to the method of Noyes et al. (17): proliferative phase ($n = 30$; 13 samples from women with endometriosis and 17 samples from control women) and luteal phase ($n = 23$; 10 samples from women with endometriosis and 13 samples from control women). A total of 21 endometriosis samples were obtained: 12 from follicular-phase women and 9 from women in the luteal phase. Tissue was washed in phosphate-buffered saline (PBS) solution in order to remove contaminating blood, and RNA was immediately extracted.

RNA Extraction

The extraction of RNA from the tissue samples was carried out with the RNA-STAT-60 reagent (Tel-Test "B" Inc., Friendswood, TX). Briefly, tissue samples were washed three times in PBS (Gibco BRL, Grand Island, NY) to remove blood contamination. One hundred milligrams of tissue was 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 rediluted in diethylpycocarbonate (DEPC)-treated dH_2O. Amount and purity of extracted RNA were quantitated by spectrophotometry in a GenQuant RNA/DNA calculator (Pharmacia Biotech Ltd., Cambridge, UK), and 10–100 μg of total RNA was routinely obtained.

Primers for Reverse Transcription and PCR

Specific sequences of oligonucleotide primers for detecting ectopic and eutopic endometrium for MMP-9 (18) and TIMP-3 (19) were obtained from GenBank. One corresponding set of primers for MMP-9 and TIMP-3 was found with the help of OLIGO Primer Analysis Software, version 5.0 (National Bioscience, Plymouth, MN) and synthesized by the Protein, Aminoacid and Nucleic Acid Facility at the Beckman Center, Stanford University. 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 and was detected in all the samples studied, confirming the integrity of the RNA and the RT-PCR pro-

Oligonucleotide primers for mRNA amplification in eutopic and ectopic endometrium.

mRNA		Primers 5′-3′	Size (bp)	Position on mRNA
β-Actin	Upstream (5′-end)	ATC TGG CAC CAC ACC TTC TAC AAT GAC ATG CG	838	294–325
	Downstream (3′-end)	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC		1131–1100
92-kDA type IV collagenase	Upstream (5′-end)	GCC TGC CAC TTC CCC TTC ATC	473	704–724
	Downstream (3′-end)	CCC CAC TTC TTG TCG CTG TCA		1176–1156
	Competitor	CCC CAC TTC TTG TCG CTG TCA GTT TCC CAT CAG C	196	1176–1156, 878–866
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

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cess. The primer sequences, locations on the mRNA, and sizes of the amplified fragments are listed in Table 1.

Reverse Transcription

For RT-PCR, the Gen Amp RNA PCR kit (Perkin-Elmer, Foster City, CA) was used. Nineteen microliters of RT-Mastermix for each sample was prepared, containing 5 mmol/L $MgCl_2$; 1× PCR buffer II; 1 mmol/L each of deoxy-NTP, 2.5 μL/L oligo (deoxythymidine)$_{16}$, 20 IU ribonuclease inhibitor (all Perkin-Elmer), and 100 IU Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY); and 1 μg total RNA diluted in 1 μL DEPC-treated H_2O and placed into 0.5-mL thin-wall PCR tubes (Applied Scientific, South San Francisco, CA). RT-Mastermix in PCR tubes was covered with 50 μL of light white mineral oil (Sigma, St. Louis, MO) and kept on ice until the reverse transcription (RT). RT was carried out in the DNA Thermal Cycler 480 (Perkin-Elmer) using a program with the following parameters: 42°C, 15 minutes; 99°C, 5 minutes; then quenching at 4°C. After the reaction was completed, samples were stored at −20°C until the PCR. As negative control, 1 μL of DEPC-treated H_2O without RNA sample was subjected to the same RT reaction.

Construction of the Competitive- and Target-cDNA Fragment for MMP-9 and TIMP-3

A 473–base pair (bp) and 969-bp fragment of native MMP-9 and TIMP-3 c-DNA (the target), respectively, were obtained by PCR amplification of reverse-transcribed total RNA from endometrial biopsies with the regular 3′ and 5′ primers (Table 1). The PCR product was visualized by agarose gel electrophoresis stained with ethidium bromide (ETB), and the cDNA was extracted from the gel, purified with an agarose gel extraction kit (Amersham Pharmacia Biotech Ltd., Piscataway, NJ), and quantitated by spectrophotometry (Pharmacia Biotech Ltd., Cambridge, UK). 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 of the original 3′- MMP-9 and TIMP-3 primer. After PCR with the regular 5′-primer and the 3′-floating primer, the PCR product was visualized by agarose gel electrophoresis stained with ETB and by cDNA extraction, purification, and determination of the concentration performed as described earlier. This step resulted in cDNA fragments of 196 and 464 bp with 277- and 505-bp deletion compared with the target cDNA and with the 3′-end and 5′-end primer-binding sites on its ends.

Standard Curve and Competitive PCR for MMP-9 and TIMP-3

The standard curve for MMP-9 and TIMP-3 was constructed by a coamplification of a constant amount of competitive cDNA (0.3 fmol for MMP-9 and 1.25 fmol for TIMP-3) with declining amounts of target cDNA (2.4–0.0023 fmol for MMP-9 and 6.4–0.125 fmol for TIMP-3) obtained by serial dilution. Sixty microliters of the cDNA mix were added to 40 μL of PCR-Mastermix containing 1.9 mM of $MgCl_2$ solution; 10× PCR buffer II; 0.2 mM each of dNTP, 2.5 U Taq-polymerase (all Perkin-Elmer), and 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 of DEPC-treated H_2O. The reaction was covered with 50 μL of light white mineral oil and put in the Perkin-Elmer DNA Thermal Cycler 480. PCR cycles were composed of 1 cycle at 95°C for 5 minutes to denature all proteins, 30 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 60 seconds at 72°C. The reaction was terminated at 72°C for 5 minutes and was quenched at 4°C. Two percent agarose gel (Life Technologies, Grand Island, NY) electrophoresis was carried out in an H5 electrophoresis chamber. Gels were stained with ethidium bromide (Sigma). Aliquots (25 μL) of each PCR product and dye buffer were analyzed in parallel with a 100-bp DNA ladder (Life Technologies, Grand Island, NY) as a standard.

After completion of electrophoresis, the gel blot was analyzed, and photocopies of the blot were printed by UV

densitometry (Gel-Doc 1000 system, Bio-Rad Laboratories, Hercules, CA). 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 linear and reproducible standard curve (data not shown). 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: 0.3 fmol of MMP-9 and 1.25 fmol of TIMP-3 competitive cDNA were added to each unknown sample before PCR. The ratio of the densities of the sample target cDNA band (473 and 969 bp) to competitive cDNA (196 and 464 bp) were logarithmically transformed and compared with the values obtained from standard curve. QC-PCR was carried out on at least two aliquots from the RT cDNA of each patient, and the results did not differ by more than ±5%.

PCR for β-Actin

RT cDNA of each patient was mixed with 78 μL of PCR-Mastermix as described earlier with 3′,5′ primer for β-actin. PCR program parameters were similar. For this PCR, no competitor was added.

Data Analysis

Statistical analysis was performed by ANOVA and t-test. The statistical analysis was carried out by using the Statistical Package for Social Science Statview Package (SPSS Inc., Chicago, IL) with a P value of <.05 considered statistically significant.

RESULTS

RT-PCR of Eutopic and Ectopic Endometrium Throughout the Menstrual Cycle

RT-PCR was employed to increase the sensitivity of detection, and the 473-bp sequence of MMP-9 and the 969-bp sequence of TIMP-3 mRNA were expressed by all eutopic and ectopic endometrial samples from women with and without endometriosis in both the follicular and luteal phases of the menstrual cycle (data not shown). β-Actin mRNA expression was also ascertained in all the samples studied, thus confirming the integrity of RNA and the RT-PCR process.

Quantitative MMP-9 mRNA Expression in Eutopic and Ectopic Endometrial Tissue

Quantitative expression of MMP-9 mRNA in paired eutopic and ectopic endometrial samples from the same endometriosis patient was examined throughout the menstrual cycle and was compared with eutopic endometrial expression in control patients. Endometrial expression of MMP-9 from endometriosis patients and normal patients was similar. Ectopic endometrium from endometriosis patients expressed significantly (P<.05) more MMP-9 mRNA compared with eutopic endometrium from women with and without endometriosis during the luteal phase. During the follicular phase,

ectopic endometrium also showed increased MMP-9 mRNA expression compared with eutopic endometrium from control and endometriosis patients, although the differences did not reach statistical significance (Fig. 1).

Quantitative TIMP-3 mRNA Expression in Eutopic and Ectopic Endometrial Tissue

Quantitative expression of TIMP-3 mRNA was significantly lower in both eutopic and ectopic endometrium from women with endometriosis compared with eutopic endometrium from normal women in the luteal phase (P<.05; Fig. 2). A similar pattern was seen during the follicular phase, although the decrement in TIMP-3 mRNA expression in eutopic endometrium from normal and endometriosis patients did not reach significance.

MMP-9/TIMP-3 mRNA in Eutopic and Ectopic Endometrial Tissue

The ratio of MMP-9/TIMP-3 in ectopic endometrium from endometriosis patients was significantly higher compared with eutopic endometrium from women with and without endometriosis in both follicular and luteal phases (P<.05; Fig. 3).

DISCUSSION

Endometriosis, a benign gynecologic disorder, occurs in about 10% of women of reproductive age and in up to 50% of women with infertility and is defined as the presence of endometrial glandular and stromal cells outside their normal location in the uterus. Retrograde menstruation, the most accepted theory of etiology, is common to all menstruating women and cannot by itself explain the pathogenesis of endometriosis.

Increased MMP activity in peritoneal fluid after manipulation of uterine and/or peritoneal tissue resulted in adhesion formation in a rat model (20). In addition, TIMP-1 concentrations were significantly lower in peritoneal fluid and sera of women with endometriosis (13). Recently, invasive proteolysis has been implicated in development of endometriosis; invasion indices of cells from peritoneal endometriosis lesions and a metastatic carcinoma cell line were similarly high compared with normal endometrium and nonmetastatic carcinoma cells (21). Compared with uterine endometrium, ectopic endometrium had a significantly higher capacity to produce the latent forms (72 kD) of gelatinase A (11), and MMP-1 expression correlated with the activity of endometriotic tissue (12). This leads to the conclusion that endometriotic implants express the protease, enabling invasion into surrounding tissue (11, 22)

In this study, we hypothesized that aberrant MMP-9 proteolytic activity might be important in the pathogenesis of endometriosis. We have shown that endometrium from women with endometriosis expresses lower levels of TIMP-3 mRNA and a higher ratio of MMP-9/TIMP-3 in endome-

QC-PCR analysis of MMP-9 RNA extracted from total ectopic and eutopic endometrium. Samples were coamplified for 30 cycles in the presence of a defined amount of internal standard cDNA for 92-kD type IV collagenase (0.3 fmol). A typical gel is shown. *Lane A*: Follicular phase endometrium from a normal patient. *Lane B*: Follicular phase endometrium from an endometriosis patient. *Lane C*: Follicular phase ectopic endometrium. *Lane D*: Luteal phase endometrium from a normal patient. *Lane E*: Luteal phase endometrium from an endometriosis patient. *Lane F*: Luteal phase ectopic endometrium. *Lane L*: 100-bp ladder DNA. Quantitative MMP-9 levels from all patients were correlated and analyzed. †*$P < .05$.
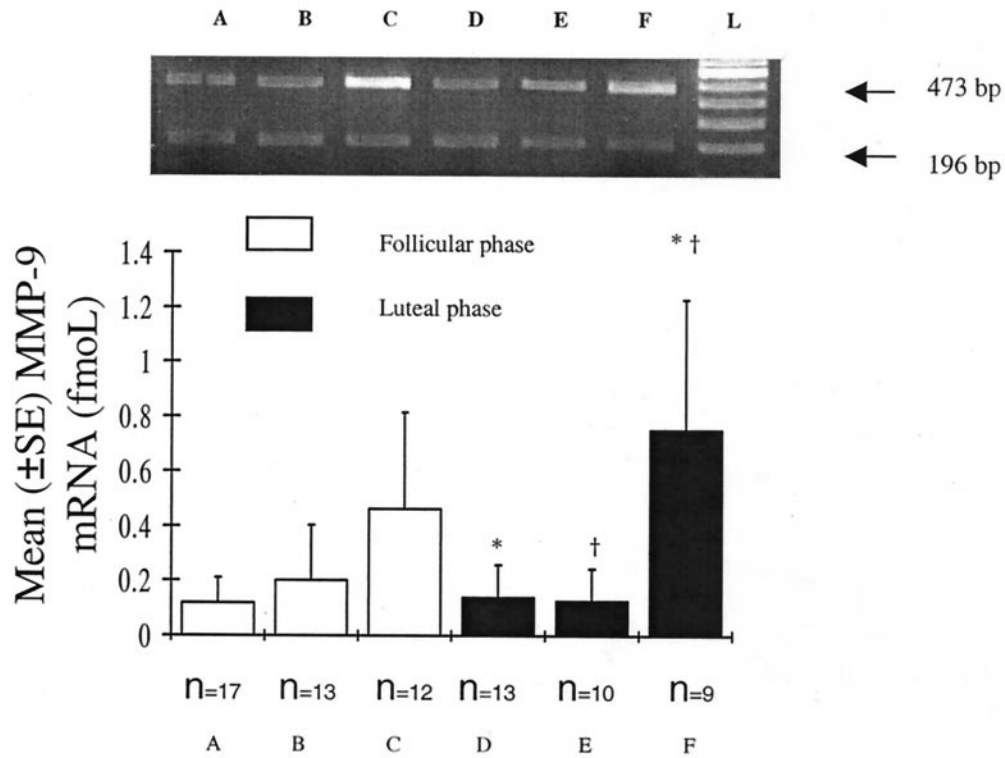
Chung. MMP-9 and TIMP-3 mRNA in endometriosis. Fertil Steril 2001.

trium when compared with endometrium from normal women who do not have endometriosis. In addition, ovarian endometrioma tissue exhibits greater expression of MMP-9 mRNA, lower expression of TIMP-3 mRNA, and a higher ratio of MMP-9/TIMP-3 than eutopic endometrium from control patients. More important, ectopic endometrium shows higher expression of MMP-9 mRNA and a higher ratio of MMP-9/TIMP-3 than eutopic endometrium from endometriosis patients. The results of this study suggest that endometrium from women with endometriosis may be inherently more invasive because of decreased TIMP-3 mRNA expression allowing higher MMP proteolytic activity that favors peritoneal invasion.

The MMP family is a group of structurally related proteins that degrade ECM and BM components (23). In ECM, the activity of MMPs is tightly regulated by a family of natural inhibitors known as TIMPs. Thus, the ratio of activated MMP to TIMP plays an essential role in regulating MMP proteolytic activity. Subtle changes in the MMP/TIMP

ratio occur during many physiologic processes associated with tissue penetration by normal migratory cells and ECM remodeling. For example, proteolytic degradation of matrix proteins occurs during trophoblast implantation (22), neovascularization (23), endometrial proliferation (24), and embryogenesis.

Recent studies have demonstrated a requirement for a balance between the expression of MMPs and TIMPs in the processes of growth, differentiation, and destruction of the endometrium that occur throughout the menstrual cycle (25, 26). In addition, several efforts have begun to define the involvement of MMPs and TIMPs in the progression and pathophysiology of endometriosis (25, 27). We hypothesized that endometrium from endometriosis patients may be biologically different from normal patients. In this study, endometrium from women with endometriosis expressed higher levels of MMP-9, lower levels of TIMP-3 mRNA, and a higher ratio of MMP-9/TIMP-3 in endometrium from women with endometriosis and in endometriotic tissue, sug-

QC-PCR analysis of TIMP-3 mRNA extracted from total ectopic and eutopic 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. *Lane A*: Follicular phase endometrium from a normal patient. *Lane B*: Follicular phase endometrium from an endometriosis patient. *Lane C*: Follicular phase ectopic endometrium. *Lane D*: Luteal phase endometrium from a normal patient. *Lane E*: Luteal phase endometrium from an endometriosis patient. *Lane F*: Luteal phase ectopic endometrium. *Lane L*: 100-bp ladder DNA. Quantitative TIMP-3 levels from all patients were correlated and analyzed. †*$P<.05$.
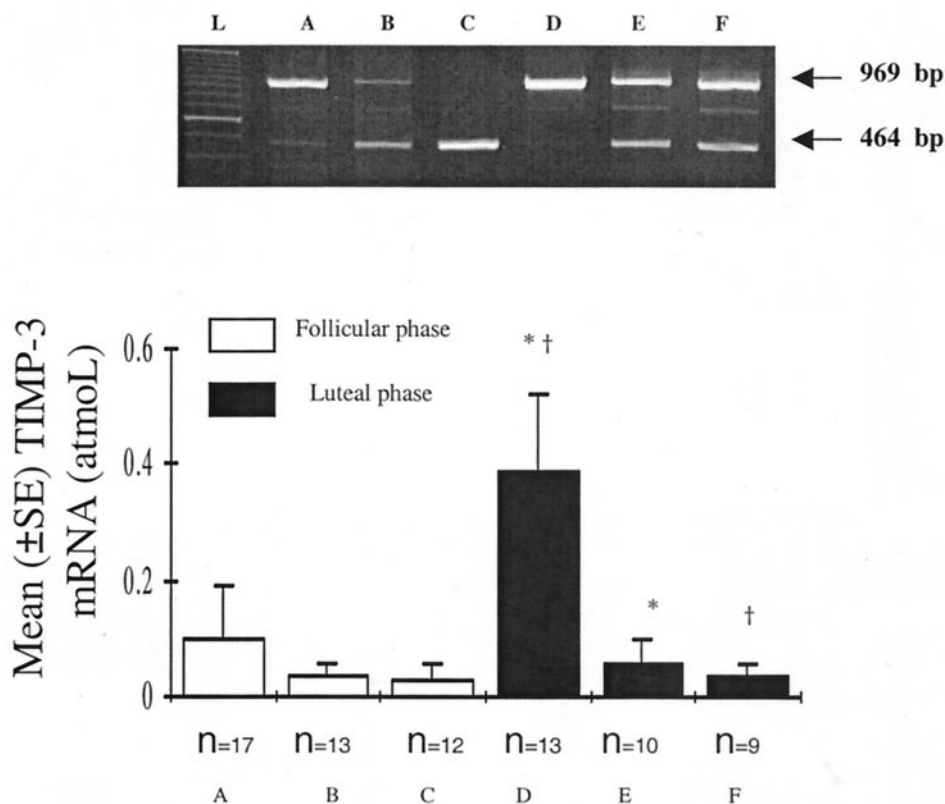
Chung. MMP-3 and TIMP-3 mRNA in endometriosis. Fertil Steril 2001.

gesting increased proteolytic enzyme activity favoring subsequent endometriotic invasion. Because TIMP-1 also binds to and inhibits MMP-9, it would be interesting and important to examine TIMP-1 expression in paired eutopic and ectopic endometrium from endometriosis patients to determine the overall extent of MMP/TIMP aberration.

Because of its extraordinarily high sensitivity, polymerase chain reaction (PCR) has been used to amplify cDNA copies of low-abundance mRNA (28). We constructed an internal standard with a defined deletion fragment from the target cDNA (29) 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, quantitative PCR can avoid the discrepancies associated with tube-to-tube or sample-to-sample variations in the kinetics of the RT reaction (15). For RNA quantitation, Northern blots are widely used. However, the Northern blot technique requires at least 10 μg of total RNA for semiquantitation, more than is obtainable from endometriosis tissue. Because of limiting amounts of tissue, we employed QC-PCR technology to quantify the amount of specific target cDNA available for both MMP-9 and TIMP-3.

Our results suggest that uterine endometrium from women with endometriosis may be biologically different from endometrium in normal women, explaining a critical factor in the initial peritoneal invasion of endometrial tissue. This may indicate either biologic or perhaps genetic differences in the peritoneal milieu that in some way modulate MMP-9 and TIMP-3 expression. In either situation, the eutopic endometrium from endometriosis patients appears to be more invasive and prone to peritoneal implantation than that from women without endometriosis because of decreased TIMP-3 expression. Once implanted on the peritoneal surface, ectopic en-

MMP-9/TIMP-3 concentration ratio from ectopic and eutopic endometrium. *Lane A*: Follicular phase endometrium from normal patients. *Lane B*: Follicular phase endometrium from endometriosis patients. *Lane C*: Follicular phase ectopic endometrium. *Lane D*: Luteal phase endometrium from normal patients. *Lane E*: Luteal phase endometrium from endometriosis patients. *Lane F*: Luteal phase ectopic endometrium. †*$P < .05$.
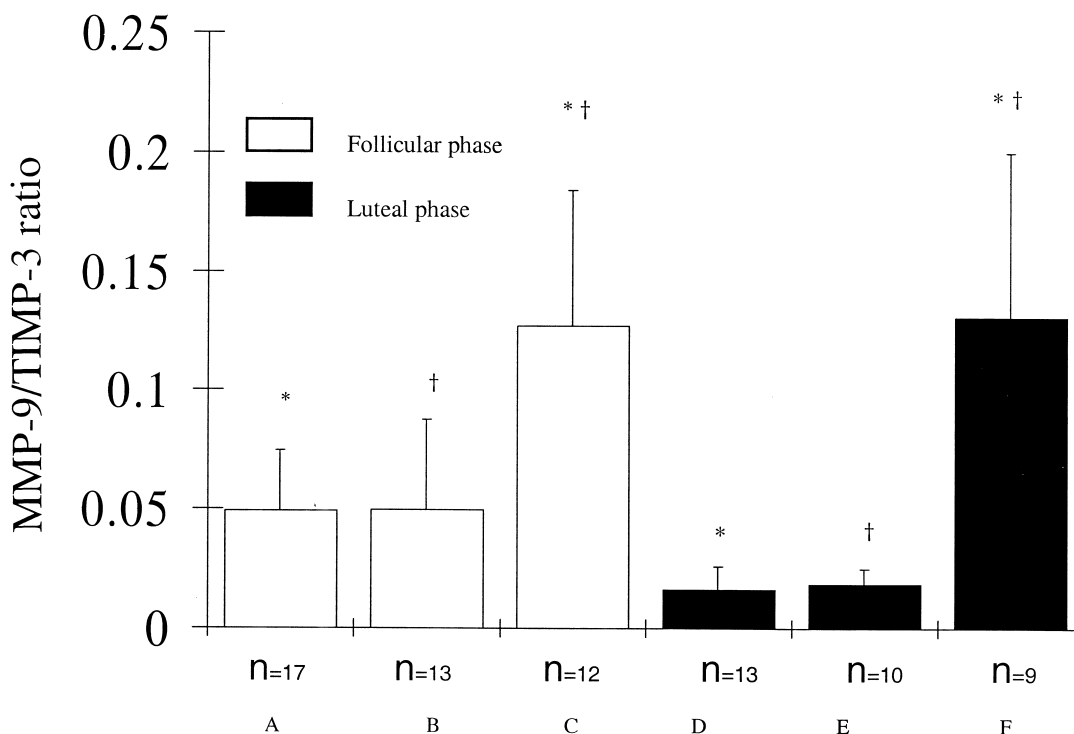
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dometrium may respond to this changed milieu with increased MMP-9 expression, rendering it even more invasive.

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