RESEARCH ARTICLE

MMP-9 and TIMP-1 Promote Extracellular Matrix Remodeling in the Formation of Ovarian Endometrioma: *in vitro* Study on Chicken Chorioallantoic Membrane

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Abstract

BACKGROUND: The invasion of endometrial tissue in the peritoneal layers is an important precursor to the formation of endometriosis. However, the mechanisms of the initial development of endometrioma remain unclear. This study aimed to explore the correlation between matrix metalloproteinase (MMP)-9 activity and tissue inhibitors of metalloproteinase (TIMP)-1 expression in the initial development of endometriosis through the invasion process of endometrial lesions in extracellular matrix (ECM) remodeling.

METHODS: A total of 30 samples of endometrioma tissue were obtained from 24 women with endometriosis and examined at Dr. Sardjito General Hospital, Yogyakarta, Indonesia. The tissue was then implanted into a chicken chorioallantoic membrane (CAM) for five days to examine its invasion ability. The invasion scores were assessed using hematoxylin-eosin staining. MMP-9 activity and TIMP-1 expression were analyzed using gelatin zymography and western blot, respectively. The correlations between MMP-9 activity and TIMP-1 expressions with the invasion scores were analyzed using Spearman correlation tests with significance set as $p<0.05$.

RESULTS: The results showed that there was a strong positive correlation between MMP-9 activity and invasion scores of endometriomas tissue (r=0.656; p=0.000). Meanwhile, expression levels of TIMP-1 had a weak negative correlation with the increase of invasion scores of endometrioma (r=-0.388; p=0.034). These results indicate the involvement of MMP-9 and TIMP-1 in the initial development of endometriosis during the ECM remodeling.

CONCLUSION: MMP-9 activity and TIMP-1 expression were correlated with the invasion score of endometrioma tissue. This study provides evidence for understanding the mechanisms involved in ECM remodeling in the early process of endometriosis.

KEYWORDS: endometrioma, extracellular matrix remodeling, MMP-9, TIMP-1

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Introduction

Endometriosis is a chronic inflammatory disease caused by the presence of endometrial-like tissue outside the uterus, which is predicted to afflict up to 5-20% of women at reproductive age and 35-50% of women who suffer from this disease seek treatment for infertility and pain relief. (1,2) Endometrioma, a benign characterized by the presence of endometrial tissue in the ovary, is the most common type of endometriosis disease, afflicting up to 17-44% of women at reproductive age, yet its pathogenesis is still unclear.(3,4)

Retrograde menstruation is the earliest and most commonly recognized explanation for endometriosis development, as well as for the pathogenesis of endometrioma. This theory proposes that endometriosis is caused by endometrial cells moving retrogradely via the fallopian tubes into the pelvic peritoneum or ovaries during the normal menstrual cycle.(5) Endometrial cells within the peritoneal cavity must attach, implant, and invade the peritoneal layers while evading the immune system to develop a lesion.(6-8) According to this theory, the invasion potential of endometrial cells is an essential factor for endometriosis development.

Endometrial cells invade the pelvic peritoneum through the process of remodeling the extracellular matrix (ECM).(1,6,8) In endometriosis, there is an abnormal ECM remodeling process that is influenced by proteolytic enzymes, which its proteolytic activity is regulated by steroid hormones, growth factors, and cytokines.(1,8-10) Matrix metalloproteinases (MMPs) are the proteolytic enzymes involved in the invasion of endometrial cells on the outer surface of ovarium.(1,9,11) Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of metalloproteinases and are crucial regulators of MMPs in the process of ECM turnover, tissue remodeling, and cellular activity.(12,13) MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are the most significant MMPs in the process of invasion and angiogenesis that specifically degrade collagen type IV as their substrate.(14) These enzymes are regulated by their natural inhibitors, TIMP-1 and TIMP-2, which are also involved in the regulation of angiogenesis.(15,16) The initial formation of endometriosis is preceded by the reflux of endometrial tissue in the pelvic peritoneal cavity, followed by adhesion and invasion on the mesothelium layer through the proteolysis of the ECM.(2) Both mesothelial and endometrial ECMs contain collagen type I, IV, laminin, and fibronectin, which can be degraded by MMPs.(8) The activated MMP-9 that binds to the receptor on the cell surface degrades the ECM, particularly collagen type IV (17), which triggers the tissue remodeling process.

Previous studies demonstrate that MMP-9 and TIMP-1, a natural inhibitor of MMP-9, play a pivotal role in endometriosis. The expression level of MMP-9 and TIMP-1 in the menstrual blood of endometriosis patients is related to the presence of endometriosis.(18) The imbalance between MMP-9 and TIMP-1 enhance the ectopic endometrium capability to invade and breakdown extracelullar matrix component in the process of implantation and development of endometriosis lesions.(15) However, the study about the correlation between MMP-9 activity and TIMP-1 expression with the invasion potential of endometrioma during the initial development of endometriosis has not been well explained.

Hence this study was conducted to investigate the development of endometrioma tissue using chicken chorioallantoic membrane (CAM) as a model that provides suitable conditions similar to the human peritoneum and stimulates implantation, MMPs expression, and angiogenesis.(19-21) The present study evaluated the correlation between MMP-9 activity and TIMP-1 expression with invasion scores of endometrioma in CAM through ECM remodeling.

Methods

Endometrioma Tissue Samples Collection

Thirty samples of endometrioma tissue were obtained from 24 women diagnosed with ovarian endometrioma at Dr. Sardjito General Hospital, Yogyakarta, from April to December 2021. The clinical diagnosis was confirmed by ultrasonography (USG). Inclusion criteria for subjects involved in this study were women with ovarian endometrioma at reproductive age of 15-49 years, who were not in a menstruation phase, did not consume hormonal drugs in the last three months, and did not have abnormal uterine bleeding and or pelvic inflammatory disease. The study protocol was approved by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia (No. KE/FK/0239/EC/2021), and informed written consent was obtained from all subjects.

Laparoscopy or laparotomy was used to excise the endometrioma tissue. Immediately after collection, samples were carefully sectioned into small fragments (±3 mm) using a scissor in a cold Dulbecco's Modified Eagle Medium (DMEM) containing antibiotics (Penicillin-Streptomycin, Gibco, Grand Island, NY, USA; Amphotericin B, Gibco). The time allowed to elapse between transporting the samples and transplantation to the CAM was three hours.

CAM Assay

Endometrioma CAM assay was performed according to the previously described method.(22,23) Fertilized eggs were obtained from Veterinary Public Health Laboratory (VPHL), Bantul, Yogyakarta, Indonesia. The eggs were laid blunt end down in an incubator at humidity ≥44% and temperature 37.5±0.5°C. On day five post-fertilization, a circular

window 1 cm in diameter was made at the sharp end using a mini electric drill and scissors. The window was covered with non-woven adhesive tape (leukoplast). Then, the eggs were kept in the incubator under sterile conditions prior to use. Endometrioma tissues were transplanted on the CAM near the blood vessel (2 fragments) at the different polar regions, each for histology and protein assay. Implants were incubated for five days. After incubation, the CAM was cut in circular near the implants and placed in cold phosphate buffered saline (PBS) solution. The implant was placed in a microtube under a cold temperature for protein assay. For histology assay, the implant was stitched in a simple knot to distinguish the chorion and allantoic epithelium and then placed in a sample tube filled with 4% paraformaldehyde (PFA). Only implants on viable CAM were used for the assay.

Hematoxylin & Eosin Staining Histology Assay

Histology assay was performed according to the previously described method.(24) Briefly, harvested implants were fixated in PFA for 24 h, then washed using alcohol 70%. The implants were dehydrated in ascending alcohol series and submerged in toluol for 15 min. Then, the implants were embedded in paraffin wax and sectioned at 5 µm thickness, approximately. Afterward, the implants were deparaffinized in xylol I and II for 10 min, rehydrated in descending alcohol series, and stained with hematoxylin and eosin. Distilled water-washed implants were dehydrated in ascending alcohol series and xylol I, II, and III for 1 min. Then, the implants were covered with Canada balsam and observed under a microscope at 400× magnification. The invasion scores of endometrioma on CAM were observed by two medical physicians and indexed according to previous literature (Supplementary 1).(25)

Protein Extraction

Protein extraction was done according to the previously described method.(24) Briefly, harvested implants were washed with PBS solution 1x and sectioned into smaller fragments. Then, the samples were kept in a sterile microtube at -80°C overnight. After that, the samples were put in cold buffer lysis (0.5 M Tris-HCl, pH 8.5, 0.05 M NaCl, Triton X-100 1%, and protein inhibitor). The samples were sonicated for 1 min and 10-sec break, 4 times, then incubated for 20 min at 4°C, subsequently were centrifuged for 10 min at 10,000 g and 4°C. The supernatant was moved into a new microtube and stored at -80°C prior to use. Total protein concentration was measured by the Bradford method using Pierce Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) was used as standard.

Gelatin Zymography

MMP-9 activity was measured by gelatin zymography. (26,27) Briefly, protein lysate (100 µg) was mixed with 5x loading buffer and loaded into 10% SDS-polyacrylamide gel containing 20 mg/mL gelatin (25 µL/lane). Electrophoresis was done using running buffer (0.02 mM Tris-base, 0.19 M glycine, SDS 0.1%, pH 8.3) for 90 min at 120V. Then, the gel was incubated in renaturing buffer (Triton X-100 2.5%) with mild agitation for 30 min at room temperature and then incubated in incubation buffer (0.2 M NaCl, 5 mM CaCl, 50 mM Tris-base, NaN3, pH 7.6) for 30 min at room temperature and 20 h at 37°C. The gel was stained with Coomassie Brilliant Blue G-250 (Thermo Fisher Scientific) 0.05% for 30 min, followed by destaining. The zone of gelatinolytic activity of MMP-9 appeared as a negative staining band (clear zone). The bands were quantified by densitometry method using ImageJ 1.53e software (NIH, Bethesda, MD, USA).

Western Blotting

TIMP-1 expression was measured by western blot.(24) Briefly, protein lysate (100 µg) was mixed with 5x loading buffer containing β-mercaptoethanol and denaturized for 5 min 95°C. Then, samples (25 µL/lane) were loaded into 10% SDS-polyacrylamide gel. Electrophoresis was done using running buffer (0.02 mM Tris-base, 0.19 M glycine, SDS 0.1%, pH 8.3) for 90 min at 120V. The bands were transferred to activated polyvinylidene fluoride (PVDF) membrane (Merck, Carrigtwohill, Ireland) in transfer buffer (25 mM Tris-base, 2 M glycine, 1% methanol) for 10 min at 25V and 1.3A. After that, the membrane was blocked in 5% BSA for 30 min with mild agitation at room temperature, followed by overnight incubation in the primary antibody of anti-TIMP-1 (Invitrogen, Waltham, MA, USA) and anti- β-actin (Invitrogen) (1:1,000 and 1:5,000 dilution in 5% BSA, respectively) at 4°C. Membrane was washed with PBST (Tween 20 0.05%) 4 times followed by incubation in the secondary antibody of anti-mouse (IgG-HRP) (Invitrogen) (1:5,000 dilution in 5% BSA) for 60 min at room temperature. Chemiluminescence (ECL) (Tanon, Woburn, MA, USA) was used as the substrate for visualization. The bands were detected using G:Box Chem (Syngene, Frederick, MD, USA). TIMP-1 and β-actin appeared as black color bands. The bands were quantified by densitometry method using ImageJ 1.53e software (NIH).

Statistical Analysis

Data were analyzed using SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA). The level of invasion scores of endometrioma on CAM between two observers were analyses by Intra-Class Correlation (ICC) test. Correlation analyses between groups were performed using Spearman tests, followed by Kruskal-Wallis tests. The MMP-9 activity and TIMP-1 expression in each invasion level were analyzed by Mann-Whitney tests. Data were presented as median and percentages with significance set as $p<0.05$.

Results

Subjects Characteristics

A total of 30 samples of endometrioma tissue from 24 women were analyzed during the study. The clinical characteristics of the subjects involved in this study were presented in Table 1. Briefly, subjects were at reproductive age with median age of 32 (19-45) years old, and 62.5% of the subjects had normoweight body mass index (BMI). Most of the subjects were married, with 78.3% having primary infertility. Samples were collected during the proliferative phase and secretory phase (45.8% and 54.2%, respectively), in which 83.3% of the samples were taken at a severe stage of endometriosis and had a median revised American Society for Reproductive Medicine (rASRM) score of 77 (21-178). The cysts had diameter with median size of 4.4 (1.50-13.18) cm.

Table 1. Patients characteristics and samples histological examination of invasion scores.

Characteristic	n	Value
Age (year) (Median (min-max))	24	32 (19–45)
Body Mass Index (%)		
Underweight	1	4.2
Normoweight	15	62.5
Overweight	8	33.3
Marital status (%)		
Married	23	95.8
Unmarried	1	4.2
Fertility (%)		
Primary infertility	18	78.3
Secondary infertility	2	8.7
Fertile	3	13.0
Menstrual phase (%)		
Proliferative	11	45.8
Secretory	13	54.2
Endometriosis stage (%)		
Moderate	4	16.7
Severe	20	83.3
rASRM score (Median (min-max))	24	77 (21–178)
Invasion Score (%)		
1	7	23.3
2	6	20.0
3	11	36.7
4	6	20.0

Invasion of Endometrioma on CAM

The successful development of endometrioma tissue on CAM was indicated by a strong attachment of the tissue with good vascularity (Figure 1). We determined the invasion scores based on the ability of endometrioma tissue to invade the CAM shown by the destruction of the chorion epithelium and remodeling of the ECM (Figure 2) and described the parameters in Supplementary 1. The Intra-Class Correlation (ICC) coefficient between two observers on the invasion scores of endometrioma was 0.97 (95% CI=0.94–0.97, indicating good reliability) which denotes a very good agreement ($p<0.01$). Based on the histological examination, the invasion scores 1, 2, 3, and 4 of the samples were 23.3%, 20%, 36.7%, and 20%, respectively (Table 1).

MMP-9 and Invasion Score of Endometrioma

Our results showed the endometrioma tissue contains both pro and active forms of MMP-9 protein with molecular weights 92 and 82 kDa, respectively (Figure 3A). The highest MMP-9 activity was in invasion score 4, followed by invasion scores 3, 2, and 1 (Figure 3B). Overall, MMP-9 activity had median value of 0.52 (0.03-1.88) arbitrary unit (AU) (Table 2). Moreover, the Spearman correlation test showed a significant strong positive correlation between MMP-9 activity and invasion score ($p=0.000$; r=0.656). This result indicated that an increase in MMP-9 activity was strongly correlated with an increase in invasion scores. In addition, the Kruskal-Wallis test showed there was a significant difference between MMP-9 activity and each

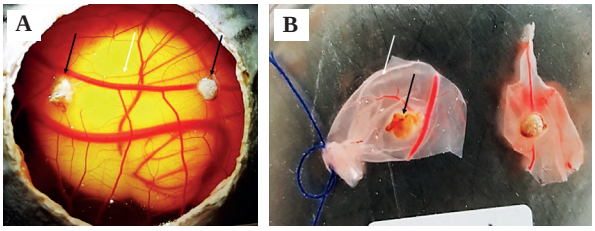

Figure 1. Endometrioma tissue on CAM at harvest day. A: Vascularization around endometrioma tissue; B: Harvested endometrioma tissue surrounded by CAM, with one simple knot as a marker, for histology assay. Black arrow: endometrioma tissue; White arrow: CAM.

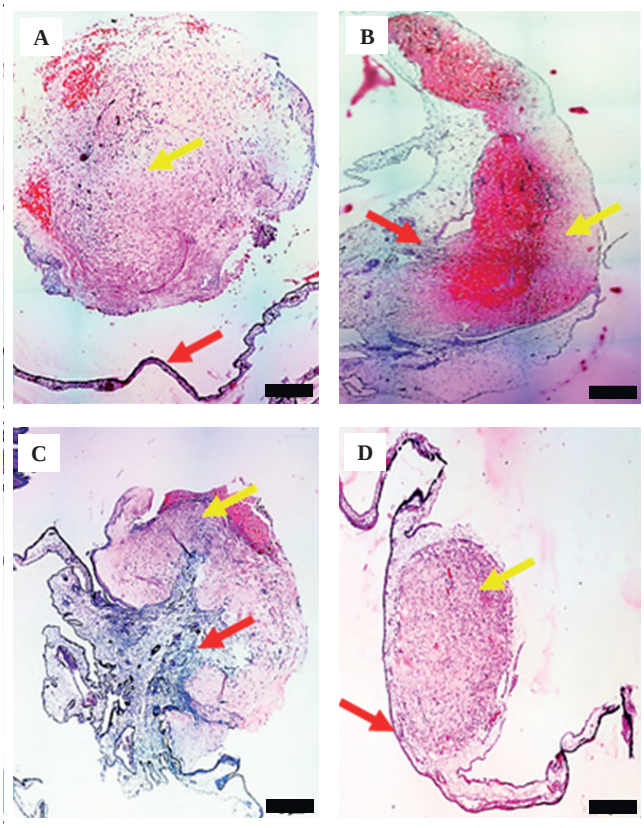

Figure 2. Histological examination of endometrioma tissue invasion on CAM. A: Invasion score 1; B: Invasion score 2; C: Invasion score 3; D: Invasion score 4. Red arrow: Destroyed chorion epithelium of CAM; Yellow arrow: endometrioma tissue; Black bar: 5 µm.

invasion score of endometrioma (p=0.005) (Supplementary 2), and further analysis by Mann-Whitney post hoc tests revealed that the significant differences were between invasion score 1 and 3 (p=0.008), invasion score 1 and 4 (p=0.002), and between invasion score 2 and 4 (p=0.015) (Supplementary 3).

TIMP-1 and Invasion Score of Endometrioma

Expressions of TIMP-1 protein were also detected from our samples by western blotting with a molecular weight of 28 kDa (Figure 4A). The highest TIMP-1 expression was in invasion score 1, followed by invasion scores 2, 3, and 4 (Figure 4B). Overall, TIMP-1 expression had a median value of 0.50 (0.04-1.75) AU (Table 2). The Spearman correlation test showed a significant weak negative correlation between TIMP-1 and invasion scores (p=0.034; r=-0.388). This result indicated that a decrease in TIMP-1 expression was weakly correlated with an increase in invasion scores. Moreover, The Kruskal-Wallis tests confirmed that there were no significant differences between TIMP-1 expression and each invasion score of endometrioma (p=0.148) (Supplementary 4).

Discussion

The initial development of endometriosis involves several complex mechanisms including ECM remodeling, during which the process includes proteolysis, synthesis, and deposition of ECM components. Various enzymes simultaneously promote the proteolysis of the ECM, particularly MMPs which regulate the deposition of ECM components.(28) In endometriosis, the ECM remodeling is abnormal due to the imbalance of MMPs and TIMPs, in which MMPs increase and TIMPs decrease in the ectopic endometrium compared to normal endometrium.(1) Our results showed that MMP-9 activity significantly increased with the increase of invasion score (Figure 3C). This pattern indicated that MMP-9 promotes the invasion process of endometrioma into the CAM. Moreover, MMP-9 activity

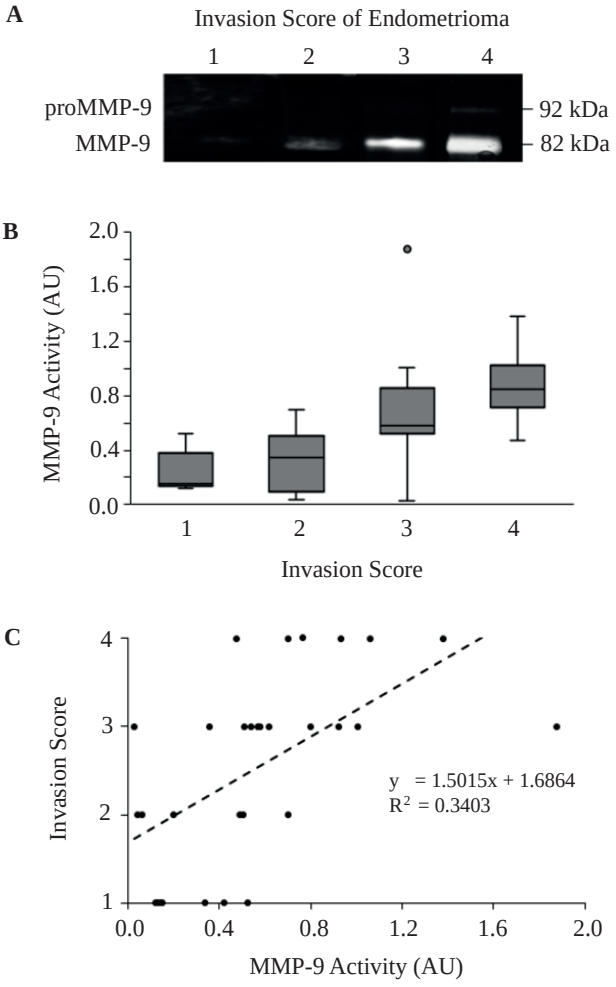

Figure 3. MMP-9 activity of endometrioma on CAM. A: Zymography analysis (representative result); B: Median MMP-9 activity on each invasion score; C: Scatter graph between MMP-9 activity and invasion scores.

Table 2. MMP-9 activity and TIMP-1 expression of endometrioma tissue on CAM.

Invasion Score	n	Median (min-max)	
		MMP-9 Activity (AU)	TIMP-1 Expression (AU)
1	7	0.15 (0.12–0.52)	0.84 (0.23–1.75)
2	6	0.35 (0.04–0.70)	0.73 (0.18–1.31)
3	11	0.58 (0.03–1.88)	0.37 (0.04–1.31)
4	6	0.85 (0.48–1.38)	0.28 (0.06–1.32)
Total	30	0.52 (0.03–1.88)	0.50 (0.04–1.75)

increased significantly between the non-invasion and partial invasion levels, indicated by damage to the entire chorion epithelium of CAM. MMP-9 also increased significantly between the non-invasion and total invasion levels, indicated by the complete reorganization of the chorion epithelium of CAM. These results confirmed that MMP-9 activity contributes significantly to the ECM remodeling, during which MMP-9 activity can degrade the ECM membrane leading to the invasion of endometrioma. This phenomenon demonstrates the potential of MMP-9 activity to predict the formation of endometrial lesions. In accordance with a previous study, the increase of MMP-9 expression in endometriosis is associated with an increased risk of endometrial lesions.(18) MMP-9 expression also increases in endometrial endometriosis, peritoneal endometriosis, and endometrioma compared to normal endometrium. (13) Moreover, we found that TIMP-1 expressions were significantly decreased with the increase in invasion scores (Figure 4C). This result revealed that low TIMP-1 expression might promote the invasion of ectopic endometrial lesions. Decreased TIMP-1 expression in endometriosis was also found in the previous study, in which TIMP-1 expression was detected in endometrial endometriosis 20-50% lower than in controls.(18)

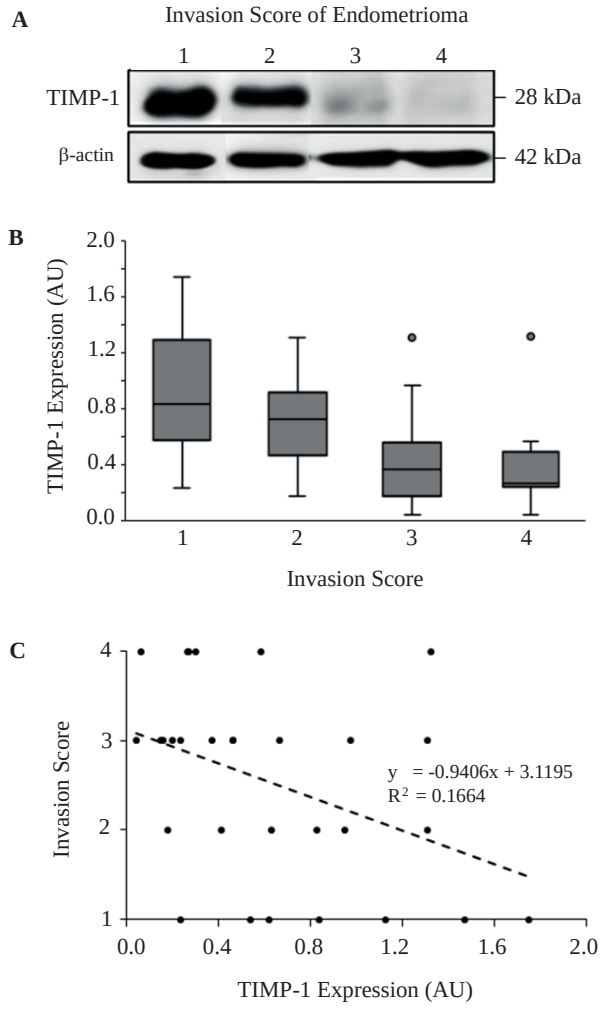

Figure 4. TIMP-1 expression of endometrioma on CAM. A: Western blot analysis (representative result); B: Median TIMP-1 expression on each invasion score; C: Scatter graph between TIMP-1 expression and invasion scores.

In this study, we found that the decrease in TIMP-1 expression was correlated with the increase in the invasion scores. Meanwhile, increased MMP-9 activity was correlated with an increase in the invasion scores. These results illustrated that in the ECM remodeling process, there is a disturbance in the regulation of the inhibitory system or an imbalance between TIMP-1 and MMP-9. Studies have shown that if the ratio of MMP-9/TIMP-1 expression is significantly higher, it is strongly associated with the development of endometriosis.(29,30) Moreover, a previous study that detected MMP-9 and TIMP-1 in endometrial endometriosis found an imbalance expression between MMP-9 and TIMP-1 which increases the ability of this tissue to invade and break down the ECM.(15) Our study successfully demonstrated that an imbalance of MMP-9 and TIMP-1 contributes to an increase in endometrioma invasion in CAM as a model of the human peritoneum, which reflects the initial development of endometriosis lesions.

In addition, we found that there were no significant differences in both MMP-9 activity and TIMP-1 expressions between the proliferative phase and secretory phase of the

menstrual cycle in endometrioma patients (Supplementary 5). These results indicated that the menstrual phases may not interfere with the enzymatic activity of the samples. Our findings were similar to a previous study that compared MMP-9 expression between normal endometrium and endometrium endometriosis.(31) In normal endometrium, MMP-9 expression was higher in the proliferative phase than in the secretory phase, whereas in endometrium endometriosis, there is no significant difference between the proliferative phase and secretory phase. MMP-9 is highly expressed throughout the menstrual phase due to the ability of endometriosis lesions to produce estrogen. The estrogenic environment tends to increase the expression of MMP-9 through its influence on the c-fos regulatory protein, which plays a role in increasing the MMP-9 expression.(31) The TIMP-1 result is also similar to the findings of a previous study that measured TIMP-1 expression in endometrioma using ELISA methods in which the women did not experience a significant change throughout the menstrual phase even though there was an increase in days 1–5 of their menstrual cycles.(32) The increasing of MMP-9 activity, which was unfollowed by increasing TIMP-1 expression, reduced the ability of TIMP-1 to control MMP-9 in remodeling ECM, resulting in non-significant differences of TIMP-1 expression among invasion levels of endometrioma in this research.

Conclusion

Our findings show a correlation between the increased activity of MMP-9 with increased scores of endometrioma tissue invasion in CAM and between decreased TIMP-1 expression level with increased endometrioma tissue invasion scores in CAM. These results show that MMP-9 and TIMP-1 have a significant role in ECM remodeling during the invasion process of endometriosis. This study may contribute to the understanding of endometriosis development to develop new therapy for endometriosis.

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Authors Contribution

VS performed the data collection, data interpretation, and writing of the manuscript. RIJ was involved in designing and collecting the experimental data of gelatin zymography and western blot analysis, supervising the writing of the manuscript. SW took part in sample collection and gave critical ideas concerning the experiment. AD was involved in concepting, planning, and supervising the research.

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