The activity of Matrix Metalloproteinase-2 (MMP-2) and Tissue Inhibitors of Metalloproteinase-2 (TIMP-2) that are associated with the degree of endometrioma tissue invasion transplanted onto the Chorioallantoic Membrane (CAM)

Tri Agung Wahyu Cahyono1*, Shofwal Widad1, Cahyono Hadi1, Agung Dewanto1, Riris Jenie2

ABSTRACT

Background: One of the fundamental pathogenesis of endometriosis to grow and develop is to carry out a cellular invasion of the surrounding tissue where it attaches. The invasion process of endometriotic focus is linked with the activity of matrix metalloproteinases-2 (MMP-2) and tissue inhibitors of metalloproteinases-2 (TIMP-2). In this study, we used endometrioma tissue to be implanted in chick chorioallantoic membrane (CAM) as a medium culture model to reconstruct and evaluate the invasion process of endometriosis. Then the MMP-2, TIMP-2 and invasion pattern can be in vitro observed.

Methods: Endometrioma tissue fragments were taken from ovarian endometrioma patients, reproductive age, no medication and no malignancy who underwent laparoscopic surgery. Sample research was taken in consecutive and non-probability sampling. The tissue fragments were divided into 4-8 strips, each dimension of 2 x 2 mm2. Two strips were assessed for the activity of MMP-2. Two remaining strips were transplanted onto the CAM. After incubation for five days, the transplanted tissues were harvested for assessment of MMP-2 by gelatin zymography, TIMP-2 by western blot and histological assessment by hematoxylin-eosin staining. The assessment was carried out by two qualified biologists as an observer.

Result: Twenty-four fragments of endometrioma have been collected from 24 endometrioma patients who underwent laparoscopy surgery. The average age was 31±5 years, the BMI was 22±3 kg/m2, the duration of infertility was 58±3 months, and r-ASRM was 67±35. In CAM of transplanted endometriomas tissue, MMP-2 activity was positively correlated with TIMP-2 in a moderate relationship (r = 0.56, p-value < 0.05), very weak positive correlation with invasion level (r = 0.08, p-value > 0.05). TIMP-2 expression was negatively correlated with invasion level in a strong relationship (r = -0.35, p-value < 0.05). The ratio between MMP-2/TIMP-2 was positively correlated with invasion level in a very strong relationship (r = 0.72, p-value < 0.05). ICC (intraclass correlation coefficient) value was 0.96, and p-value= 0.01, CI 95%. It shows that there was high reliability between observers.

Conclusion: The invasion level of endometrioma transplanted onto CAM was determined by the balance between MMP-2 and TIMP-2.

Keywords: CAM, Endometrioma, Invasion, MMP-2, TIMP-2.

Cite This Article: Cahyono, T.A.W., Widad, S., Hadi, C., Dewanto, A., Jenie, R. 2023. The activity of Matrix Metalloproteinase-2 (MMP-2) and Tissue Inhibitors of Metalloproteinase-2 (TIMP-2) that are associated with the degree of endometrioma tissue invasion transplanted onto the Chorioallantoic Membrane (CAM). Bali Medical Journal 12(2): 2271-2277. DOI: 10.15562/bmj.v12i2.3845

INTRODUCTION

Endometriosis is a common invasive gynecological disorder of reproductive women characterized by the growth of endometrial glands and stroma outside the uterus.1 An Endometrioma is the localization of endometriosis in the ovary, often developing as a cyst.2 Endometriosis is associated with chronic pelvic pain, severe dysmenorrhea, dyspareunia and infertility. The disease affects almost 10–15% of women of reproductive age and 50% of women with infertility.3 The mechanism underlying its initiation and progression has still not been fully clarified. However, it is thought to be caused by retrograde menstruation followed by the ectopic establishment of endometrium-like cells, which acquire the ability to survive, attach, invade and proliferate to the host tissue.4 Endometriotic foci growths are supported by the local hormonal and inflammatory microenvironment and are further spread over multiple locations.5

Invasion of the host tissue is one of the fundamental pathogenesis of
endometriosis. The early step of host tissue invasion is the degradation of the extracellular matrix (ECM) and basement membrane (BM). Endometriotic cells, using proteolytic enzymes, can destroy ECM and create space for cells to invade the tissue where it attaches. MMP-2 is a group of zinc-dependent proteolytic enzymes mainly involved in ECM and BM degradation to promote cellular invasion and migration of endometriotic cells. The ECM degradation, operated by the MMP, is closely regulated and naturally inhibited by tissue inhibitors of metalloproteinases (TIMP). MMP-2 is a specific natural inhibitor for MMP-2. The imbalance between these proteolytic enzymes and their tissue inhibitors leads to excessive BM and ECM degradation, facilitating the spread of endometriotic cells and neoangiogenesis. The role of MMP-2 and tissue inhibitor of metalloproteinases-2 (TIMP-2) in the invasive processes of endometriosis and their functional interrelating effects are the subject of a growing number of studies. Chung HW et al. has examined mRNA expression of MMP-2, MT1-MMP, and TIMP-2 in endometriosis tissue in the endometrium of healthy women and endometriosis patients. They found that the eutopic endometrium of women with endometriosis is more invasive and likely to cause peritoneal implantation in accordance with the high expression of MMP-2 and MT1-MMP mRNA the low expression of TIMP-2. Other researchers also found an association between MMP-2 and endometriosis invasion.

The study on the activity of MMP-2 and TIMP-2 at the early phases in invasive endometriosis formation is challenging to study in humans in vivo. In this study, we used endometrioma tissue to be implanted in chick chorioallantoic membrane (CAM) as a culture medium to reconstruct the invasion process of endometriosis. Then the MMP-2, TIMP-2, and invasion pattern can be invitro observed. We used CAM as a culture medium for endometrioma tissue transplantation because CAM provides an excellent model for studying the initial process of tissue invasion. The extracellular matrix of CAM is similar to the peritoneum. Collagen types I and IV, laminin and fibronectin are present in the ECM of the CAM, which is similar to the ECM of the human peritoneum.

**METHODS**

**Tissue collection and culture**

The use of human tissue was approved by The Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada–DR Sardjito General Hospital Yogyakarta (No: KE/FK/0115/EC/2021), and patients gave their written informed consent. Endometrioma tissue was obtained from 24 patients of their reproductive age (18-40) who had undergone laparoscopic surgery at Permata Hati, DR Sardjito General Hospital. According to the American Society for Reproductive Medicine (ASRM) revised guidelines, endometriosis was staged surgically. Medical records were reviewed to collect relevant clinical information. All subjects were confirmed to be premenopausal with regular menstrual cycles. None of the subjects received medical therapy for endometriosis nor hormonal contraception for at least 3 months before laparotomy or laparoscopy.

Endometrioma samples immediately, within 1 hour, were transported from the operating room to the Laboratory of Human Physiology at the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, using a sterile transport box filled with ice to maintain a stable temperature between 5°C and 8°C. The transport medium consisted of dulbecco's modified eagle medium (DMEM) F12 culture medium supplemented with gentamycin 100 μg/mL (Thermo Fisher Scientific). Fresh endometrioma tissue samples were washed with phosphate-buffered saline (PBS) and dissected into 4-8 strips, each dimension of each 2 x 2 mm² within medium DMEM, 2% penicillin-streptomyacin (10,000 U/mL), and fungizone (Thermo Fisher Scientific)

**Experimental design**

Each endometrioma sample was divided into 4-8 strips; 1 strip was sent to Molecular Biology Laboratory to assess the activity of MMP-2. Two remaining strips were transplanted onto the CAM. After incubation for 5 days, the transplanted tissues were harvested for assessment of MMP-2, TIMP-2 and histological assessment. Histological assessment for the invasion score was carried out by hematoxylin-eosin staining, MMP-2 was assessed by gelatine zymography, and TIMP-2 was assessed by western blot.

**Chorioallantoic membrane (CAM) culture**

Fertilized eggs of Lohman selected White Leghorn chickens, purchased at Laboratory of Veterinary Public Health, Yogyakarta, and incubated at 37 °C with 60% relative humidity, were prepared for implantation on days 5-6 of incubation. A Standard microbiology assessment was performed to exclude subclinical infections. Each egg was washed with 40% Na-citrate, after which a window was drilled through the pointed pole of the shell with dimension 2 x 2 cm. Part of the CAM of the embryo was exposed by peeling a 2.0 cm window in the shell. Then, the 3 endometrioma strips were transplanted onto CAM and covered with tape, and the egg was replaced in the incubator for 5 days. After 5 days of transplantation and incubation, the endometrioma strips were retrieved for the next step. In the first retrieved strip, the tissue was sent to the Histology and Cell Biology Laboratory to assess the invasion score, while the other was sent to the Molecular Biology Laboratory to assess the levels of MMP-2 and TIMP-2.

**Gelatin zymography**

MMP-2 activity was analyzed by gelatin zymography. Frozen samples were homogenized in lysis buffer containing 100 mmol/L Tris-Cl, pH 7.6, 20 mmol/L NaCl, and 1% Triton X-100. The lysates were incubated on ice for 30 min, and insoluble materials were removed by centrifugation. Samples containing 100 μg of protein were mixed with 5×sample buffer (4:1) and electrophoresed (120 V) for 2-3 h at 4 °C. When the tracking dye at the front reached the bottom of the gel, the gel was removed and shaken gently for 30 min in 2.5% Triton X-100 to remove SDS. Then, the gel was incubated in 50 mmol/L Tris-HCl, pH 8.0, 50 mmol/L NaCl, 10 mmol/L CaCl2, and 1% Triton X-100 for 20 h at 37 °C. Last, following staining with 0.5% Coomassie brilliant blue R250 for 1.5-2 h and decolorized for 1 h. The clear band
against a blue background representing the activity of MMP-2 was measured by using a gel image system (Image-J analysis software). Quantification can be achieved by computer-aided densitometry.

**Western blot analysis**

LisaT (100 μg) sample (protein TIMP-2) was mixed with the SDS electrophoresis sample buffer (10 mmol/L Tris-HCl, pH 7.8, 1 mmol/l EDTA, 3% sodium dodecyl sulfate, 5% glycerol, 10% mercaptoethanol), heated for 5 min at 95°C, run on a 9% polyacrylamide electrophoresis gel line (Mini-Protein II, Bio-Rad) and then inserted into the polyvinylidene difluoride membrane (Bio-Rad, CA). The filter is blocked in 5% dry milk in PBS and incubated for 1 hour at room temperature with anti-TIMP 2 with a dilution of 1:500 in PBS. After four washes with 0.1% Tween di PBS were incubated then for 1 hour at room temperature with an anti-mouse antibody diluted in a ratio of 1:2000 with PBS. The sample was washed and added with chemiluminescence western blotting detection reagents (Amersham, Little Chalfont, Bucks) and given a blue light-sensitive autoradiography film (Hyperfilm-ECL, Amersham) for negative control used serum of normal rats as a primary antibody. Positive band density is measured using Image-J. The coloring results on the gel are converted into values using the software.

**Histologic processing**

For histological examination, CAM and in vitro cultured endometrioma tissues were fixed with 4% (w/v) paraformaldehyde, embedded in paraffin wax, serially sectioned at 5 μm thickness, stained with hematoxylin and eosin, and examined under a light microscope with x 100 and x 400 magnification. The invasion score was determined microscopically based on the condition of regularity chorionic epithelium of CAM, where endometrioma tissue was attached and grew. The levels of invasion were grouped into 4 levels based on the invasion score, non-invasion or invasion level 1 for an invasion score 1, partial invasion or invasion level 2 for an invasion score 2, a total invasion or invasion level 3 for invasion score 3, and invasion score 4 for reorganization.

**Statistical analysis**

Statistical analysis was conducted using SPSS (Statistical package for social service) version 16.0. Univariate analysis of qualitative data was carried out by calculating percentages, and quantitative data were reported as mean and median. Bivariate analysis of the qualitative data of MMP-2 activity, TIMP-2 expression, and quantitative data of endometrioma invasion levels in CAM was carried out by determining the correlation of these data with the spearman formula of correlation. A P-value < 0.05 was considered significant for all statistical analyses. The data test of normality is calculated using the Shapiro-Wilk formula.

**Table 1. Clinical characteristics of endometrioma patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>%</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td>31±5</td>
<td>31.5</td>
<td>22-40</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td></td>
<td></td>
<td>22±3</td>
<td>23.3</td>
<td>16-28</td>
</tr>
<tr>
<td>Underweight</td>
<td>4</td>
<td>16.6</td>
<td>16.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>62.5</td>
<td>62.50</td>
<td>62.50</td>
<td></td>
</tr>
<tr>
<td>Overweight</td>
<td>5</td>
<td>20.8</td>
<td>20.83</td>
<td>20.83</td>
<td></td>
</tr>
<tr>
<td>Menstrual Phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>11</td>
<td>46.0</td>
<td>46.00</td>
<td>46.00</td>
<td></td>
</tr>
<tr>
<td>Secretory</td>
<td>13</td>
<td>54.0</td>
<td>54.00</td>
<td>54.00</td>
<td></td>
</tr>
<tr>
<td>Duration of Infertility (month)</td>
<td></td>
<td></td>
<td>59±3</td>
<td>49</td>
<td>24-102</td>
</tr>
<tr>
<td>r- ASRM</td>
<td></td>
<td></td>
<td>67±36</td>
<td>61</td>
<td>23-168</td>
</tr>
<tr>
<td>Stage of endometriosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>21.0</td>
<td>21.00</td>
<td>21.00</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>19</td>
<td>79.0</td>
<td>79.00</td>
<td>79.00</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Endometrioma tissue samples were taken through laparoscopic surgery from June 2021 to December 2021. The number of samples involved in this study was 24 endometrioma tissue samples obtained from 24 patients. The activity of MMP-2 was evaluated using the gelatin zymography method, and TIMP-2 expression was assessed using the western blot method. The activity of the MMP-2 and TIMP-2 can be seen as color bands. Then quantification was carried out by measuring the intensity of the color band using Image-J software.

Histological assessment for the invasion level of endometrioma tissue into CAM was carried out by experienced observers under a light microscope with x 200 and x 400 magnification. The assessment of the r-ASRM score is carried out based on the results of the view at the time of laparoscopic surgery by the operator.

The description of the study subjects is listed in Table 1. The average age was 31 ± 5 years old, the reproductive age. The average body mass index was 22 ± 3 kg / m², and 62.5% of patients had a normal BMI. Endometrioma tissue was taken 45% in the proliferation phase and 54% in the secretion phase of menstruation. Patients experience infertility for a long time before laparoscopy, with an average of 4 years. The stage of the disease, according to the r-ASRM score, showed that the subjects were almost 80% at a severe stage and 20% at a moderate stage.

Table 2 shows the character of endometrioma tissue samples. The average MMP-2 activity in the sample was 0.8 ± 0.4 AU while the average expression of TIMP-2 was 0.9 ± 0.6 AU.

Histological Analysis

The endometrioma tissue invasion scores on CAM were assessed under a light microscope with x 200 and x 400 magnification. The invasion score was assessed by adapting the chorion epithelial invasion score (Annemiek, 2004). The observation and assessment of the invasion score based on staining are subjective, so the assessment was carried out by two biologically qualified observers and experienced in making the assessment. Interobserver reliability was performed by intra-class correlation (ICC) test, with p = 0.01 and CI 95% ICC value of 0.96 (0.94-0.96), indicating high reliability between observers.

Correlation Analysis Between MMP-2 and Invasion Level

Based on figures 3 and 4, the strongest activity of MMP-2 expression was 1.8 AU, while the weakest activity was 0.4 AU. This value was positively correlated with the invasion score ($r = 0.08$, P-value >0.05, but with a very weak level of relationship. It suggests that an increase in MMP-2 activity will not raise the level of endometrioma tissue invasion in CAM.

Correlation Analysis Between TIMP-2 and Invasion Level

The strongest TIMP-2 expression was 2.5 AU, with the weakest expression of 0.2 AU. Expression of TIMP-2 was negatively correlated with endometrioma invasion score ($r = -0.35$, P-value <0.05) and has a strong level of relationship.
Correlation Analysis between MMP-2 and TIMP-2

The activity of MMP-2 is positively correlated with TIMP-2 expression ($r_\tau = 0.56$, p-value < 0.05) with a moderate level of relationship. MMP-2 is produced as a pro-hormone in carrying out its activities before it becomes an active MMP-2 requiring TIMP-2 as an activator, so TIMP-2 not only acts as an inhibitor in...
DISCUSSION

The characteristics of patients in this study are presented in Table 1. The study subjects had ages between 22-40 years with an average of 31 ± 5 years. It suggests that most of the incidence of endometrioma occurs at reproductive age. A Body mass index with an average of 22 ± 3 indicates that the study subjects were in normal constitutional conditions. The duration of infertility ranges from 23 months to 102 months. It is a sufficiently long duration before a laparoscopic procedure is performed. Several studies have shown that delay in diagnosis will increase the degree of endometrioma and clinical complaints. The subjects of this study had an average r-ASRM of 67 ± 36, which means most of the patients were classified as moderate to severe (79%).

One of the fundamental characteristics of endometriosis development is the occurrence of tissue invasions, which are mainly responsible for an increase in the level of severity and clinical complaints. Therefore, it is necessary to understand the cellular and molecular mechanisms of endometriosis invasion. Endometriosis invasion is considered a dynamic, complex, and gradual process, but the essential step is the degradation of the extracellular matrix (ECM) and basement membrane (BM). It is known that MMP-2 is one of the enzymes that play a role in the degradation of ECM, especially collagen type-4. TIMP-2 is a specific inhibitor for MMP-2 with the ability to form non-covalent bonds with MMP-2 to suppress MMP-2 activity degrading the ECM so that TIMP-2 has anti-tissue invasion activity.

Some reports have stated the correlation between the expression MMP-2, TIMP-2 and the development of endometriosis. However, no research reports were found on the correlation between MMP-2, TIMP-2 activity and the clinicopathological of endometriosis. Research by Salata N et al., using the ELISA method, reported that the expression of MMP-2 and TIMP-2 in endometriosis of mild and severe levels did not have any significant differences. Research by Sotnikova NY et al. showed that MMP-2 expression in endometriosis patients increased significantly, while TIMP-2 levels did not differ from the control group. Jana S et al. showed different results where MMP-2 expression strongly correlated with the level of endometriosis. Research by Barbe A et al. also showed an increase in MMP-2 expression in endometriosis patients.

Our data showed that in the activity of MMP-2 endometrioma, there was an increase in activity after being transplanted on CAM, but this increase in activity had a very weak relationship, even with a p-value > 0.05, indicating that the relationship was not meaningful. This study’s results align with those reported by Salata N et al. and Szymanowski K et al. Several other studies showed different results that MMP-2 expression in endometrioma tissue increased, further mentioned that the increase was associated with an increase in the severity of endometriosis. Although there are differences in the analysis method of MMP-2 and TIMP-2, this study showed that the activity of MMP-2 in invasion was still determined by other factors. In addition, the role of MMP-2 is more active in the initiation phase of the development of endometriosis. Jana S et al. also expressed that an increase in MMP-2 occurs in the initiation phase, and TIMP-2 expression will increase concurrently. In this phase, the function of TIMP-2 was as an inhibitor after previously functioning as an MMP-2 activator.

TIMP-2 is an inhibitor of gelatinase-A MMP-2. In addition to serving as an inhibitor, it also functions as an activator to change the pro-hormone form of MMP-2 into active MMP-2. Correlation analysis of TIMP-2 with the level of invasion was a negative correlation with a strong relationship. This finding illustrates that proteolytic activity induced by MMP-2 inhibited TIMP-2 activity at a certain level after previously carrying out MMP-2 activity. High TIMP-2 expression from the beginning indicates that control of the proteolytic activity of MMP-2 occurs from the initial phase until the invasive process.

Correlation analysis of the ratio MMP-2/TIMP-2 with the level of invasion showed a positive correlation with a strong relationship. It indicated that the balance of MMP-2 and TIMP-2 maintains the stability of the extracellular matrix to remain solid. During the invasion process, there was a possibility of disturbance to the balance of the two enzymes resulting in the degradation of ECM. The author believes the invasion will occur due to the imbalance between MMP-2 and TIMP-2.

Some limitations in this study are very likely to cause interference with the activities of MMP-2 and TIMP-2. Estrogenic, progestogenic, and contaminant activities that can cause micro-inflammatory greatly affect the activity of MMP-2 and TIMP-2. The process in this study was not controlled. Measuring estrogen and progesterone levels in samples or grouping samples by phase of menstruation will provide more uniform sample data.

CONCLUSION

The balance of MMP-2/TIMP-2 is the decisive factor for maintaining ECM steadiness and integrity, and the roles of MMP-2 in invasive endometriosis do not depend on the absolute concentration of MMP-2 in the local area but depend on the MMP-2/TIMP-2 ratio. During the invasive process, the balance of MMP-2/TIMP-2 is broken, favoring ECM degradation without regulation. The concentration of MMP-2 in the local area had no significance and could not reflect the invasive potential of endometriosis cells.

CONFLICT OF INTEREST

All authors declare that they have no competing interests.

ETHICAL CONSIDERATIONS

The Medical and Health Research Ethics Committee from the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Indonesia, approved all procedures in this study with registration number No: KE/FK/0115/EC/2021.

FUNDING

This study received no funding from public, commercial, or non-profit sectors.
AUTHORS CONTRIBUTIONS

AW was involved in the study design, laboratory analysis, manuscript writing, data analysis, and editing; SW was involved in the study design, data interpretation, manuscript writing and review; CH was involved in the study design and manuscript review; RJ was involved in the laboratory analysis and data interpretation; AD was involved in the study design, data interpretation, and manuscript review. All authors have read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors thank the Department of Physiology and Department of Histology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, for technical assistance.

REFERENCES