ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2016, Vol 23, No 4, 649–653
www.aaem.pl

Matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of matrix metalloproteinases (TIMP-1) and transforming growth factor-β2 (TGF-β2) expression in eutopic endometrium of women with peritoneal endometriosis

Krzysztof Szymanowski[1], Mateusz Mikołajczyk[2], Przemysław Wirstlein[2], Anna Dera-Szymanowska[3]

[1] *Department of Mother's and Child's Health, Poznań University of Medical Sciences, Poland*
[2] *Department of Obstetrics and Gynecology, Division of Reproduction, Poznań University of Medical Sciences, Poland*
[3] *Department of Perinatology and Gynecology, Poznań University of Medical Sciences, Poland*

Szymanowski K, Mikołajczyk M, Wirstlein P, Dera-Szymanowska A. Matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of matrix metalloproteinases (TIMP-1) and transforming growth factor-β2 (TGF-β2) expression in eutopic endometrium of women with peritoneal endometriosis. Ann Agric Environ Med. 2016; 23(4): 649–653. doi: 10.5604/12321966.1226861

Abstract

Introduction. The prevalence of endometriosis among reproductive age women is 7–17%; however, these figures reach 20–50% in patients suffering from infertility. Matrix metalloproteinases (MMPs) activity is thought to be particularly essential in the early phases of endometriosis development. Any changes in the equilibrium between MMPs activity and their tissue inhibitors (TIMPs) could be potentially harmful, promoting endometriosis development. The aim of this study was to investigate whether the MMP-2, MMP-9, TIMP-1 or TGF-B2 expression in eutopic endometrium from women with early endometriosis differ when compared with healthy subjects. The results were referred to the serum progesterone levels.
Materials and method. Endometrial biopsy was taken from 42 patients (18 in the study group, 22 in thecontrol group) at the time of hysteroscopy for routine histology and for RT-PCR procedures. Comparison of the quantity of gene products was performed with a programme for densitometry and compared to GADPH product, which was a reference value.
Results. The obtained results did not reveal any statistical difference in endometrial expression of MMP-2, MMp-9, TIMP-1, and TGF-β2 or serum progesterone level between women with endometriosis and without visible signs of this illness.
Conclusion. Despite the lack of statistical differences, it was observed that both examined metalloproteinases expressed a tendency to higher gene expression in the eutopic endometrium of women with endometriosis. However, both TIMP-1 and TGF-β2 expressions had the same tendency – higher values in endometriosis patients.

Key words

Endometriosis, matrix metalloproteinases, tissue inhibitor of matrix metalloproteinases

INTRODUCTION

Endometriosis is defined as the presence of functioning endometrial glands and stroma outside the uterus, predominantly within the peritoneal cavity [1]. The prevalence of endometriosis is 7–17% among reproductive age women [2, 3, 4]; however these figures reach 20–50% in patients suffering from infertility [5]. Despite intensive investigations, the pathogenesis of endometriosis remains unclear. The adhesion of the endometrium refluxed through the fallopian tubes is one of the first necessary stages of the implantation theory. Matrix metalloproteinases (MMPs) activity is thought to be particularly essential in the early phases of endometriosis development. Extracellular matrix remodeling is a common phenomenon in normal development, growth and tissue repair. Any change in the equilibrium between MMPs activity and their tissue inhibitors (TIMPs) could be potentially harmful.

Address for correspondence: Anna Dera-Szymanowska, Department of Perinatology and Gynecology, Poznań University of Medical Sciences, Górki 15D/15, 60–204 Poznań Poland
E-mail: annaszerszen@wp.pl

Received: 03 March 2013; accepted: 22 November 2013

It may be assumed that aberrant MMP-2, MMP-9, TIMP-1 or TGF-β2 activity in eutopic endometrium could contribute to the endometriosis development. As might have been expected, Ueda et al. [6] have shown higher MMP-2, MMP-9 and MT1-MMP (membranous type-1 matrix metalloproteinase) expression in clinically-active endometriotic foci, compared with eutopic endometrium of endometriosis free patients. On the other hand, Chung et al. [7] found for the very first time that the expression of MMP-2 and MT1- MMP was higher, and TIMP-2 was lower in the eutopic endometrium of women with endometriosis. They also found a higher MMP-9:TIMP-3 ratio in the eutopic endometrium of women with endometriosis. However,these studies were performed exclusively on advanced endometriosis patients.

OBJECTIVE

To the knowledge of the authors of the presented study, there is no clinical study comparing MMPs and tissue inhibitors expression in eutopic endometrium of women with peritoneal endometriosis. Therefore, the aim of the present work was to investigate whether the MMP-2, MMP-9, TIMP-1 or

TGF-β2 expression in eutopic endometrium from women with early endometriosis differ when compared to healthy subjects. Furthermore, these results are to be referred to serum progesterone levels.

MATERIALS AND METHOD

The study was performed on endometrium obtained from 42 women treated in the Division of Reproduction at the K. Marcinkowski University of Medical Sciences in Poznań, Poland. All patients had laparoscopy and hysteroscopy within 7–9 days after ovulation, as confirmed by ultrasonography. The Human Research Committee at the University approved acquisition of the endometrial tissue (No: 1058/98), and the patients signed an informed consent. All patients had regular menstrual cycles and had not taken any hormonal treatment for at least 3 months prior to the endometrial biopsy. Endometriosis was diagnosed by direct visualization of the endometrial lesions at laparoscopy, with subsequent histological confirmation. Staging of the endometriosis was performed according to the revised American Fertility Society classification [8]. Only peritoneal endometriosis (I^0 and II^0) was qualified for further study.

The study group consisted of 18 patients with endometriosis (I° – 4, II° – 14). Mean age of the patients was 29.2 years (20–37). The indications for laparoscopy were as follows (in some patients more than one): primary or secondary infertility (n=14; primary – 11, secondary – 3), suspicion of endometriosis (n=7), recurrent miscarriages (n=4) or bilateral occlusion of the fallopian tubes (n=2). Endometriosis treatment was performed due to the protocol used at the Division of Reproduction. Twenty-four patients without visible endometriotic foci at laparoscopy were qualified as the control group. Mean age of women in this group was 29.6 years (24–39). The indications for laparoscopy were (in some women more than one): infertility (n=16; primary – 13, secondary – 3; among them, male factor – 3), occlusion of the fallopian tubes (n=11; bilateral – 5, unilateral – 6), uterine fibroids (n=5), recurrent miscarriages (n=3) or suspicion of endometriosis (n=1).

In all patients, endometrial biopsy was taken at the time of hysteroscopy for routine histology and for RT-PCR procedures. Immediately after specimen acquisition, 30 mg of the endometrium was placed in 300 µl (10 µl/1 mg of the tissue) of RNA later Stabilization Reagent (Qiagen, Hilden, Germany) and frozen at -20 °C until RNA extraction. The RNeasy Protect Mini Kit (Qiagen, Hilden, Germany) was used for isolation of the total RNA. The QIAshredder columns (Qiagen, Hilden, Germany) were used for homogenization of the endometrium. Isolated total RNA was in the form of suspension in 50 µl free of RNA-ases water. This suspension was frozen until analysis in -20 °C. Qiagen OneStep Reverse transcription and Polymerase Chain Reaction (RT-PCR) Kit (Qiagen, Hilden, Germany) was used for RT-PCR reaction. This allowed reversed transcription and cDNA amplification in one thermal profile. PTC-200 thermocycler (MJ Research, USA) was used for reaction.

Starters used are listed in the table below (Tab. 1)

The thermal profile applied in the experiment was as follows:

50 deg C – 30 min
95 deg C – 15 min

94 deg C – 1 min
55 deg C – 1 min × 25 cycles
72 deg C – 1 min

72 deg C – 10 min

Table 1. Primers for the examined gene (9, 10, 11, 12)

Gene	Primer 5′	Primer 3′	Size of product (bp)
MMP2	CCACGTGACAAGCCCATGG	GCAGCCTAGCCAGTCGG	486
MMP9	TGGGCAAGGGCGTCGTGG-TTC	TGGTGCAGGCGGAGTAG-GATT	276
TIMP1	TGCACCTGTGTCCCACCC	GGCTATCTGGGACCGCAG	552
TGF-β2	TCCGCACCCGAGACTGAC	AGGCTGAGCGCGACCGTG	441
GAPDH (control)	TGAAGGTCGGAGTCAACGG-ATTTGGT	CATGTGGGCCATGAGGTC-CACCAC	983

Reactive mixture was prepared according to manufacturer's instructions. In the next step, 12 µl of the 6x Mass Loading Dye Solution (Fermentas, Vilnius, Lithuania) was added to the reaction products. Final products were plated in 10 µl aliquots on 0.8% agarose gel (*Prona Agarose*, Spain) with 5 µl/100 ml of ethidine bromide (Sigma, USA). To one of the pockets 10 µl of MassRuller DNA ladder, Low Range (Fermentas, Vilnius, Lithuania) was added. Finally, gel electrophoresis was performed in TBE buffer 1x. Gel visualization was performed with UV light. Comparison of the quantity of gene products was performed with a programme for densitometry. This quantity was compared to GADPH which was the reference value.

Prior to the anaesthesia induction, a blood sample was taken for progesterone assessment (ELISA). The values were expressed in ng/ml.

For statistical analysis, the students T-test and Pearson`s test were used. A $p<0.05$ was considered significant.

RESULTS

Results of the MMP-2, MMP-9, TIMP-1, TGF-β2, and GADPH (as a reference) are presented in Figures 1 and 2.

No statistical differences were found in the expression of MMP-2, MMP-9, TIMP-1 and TGF-β2 gene products in endometrium between the group of women with and without endometriosis. Mean values of the gene products are presented in the Table 2.

As a next step, a search was made for possible correlations between the assessed genes. For the entire group of 42 women following positive correlations were found:

1. MMP-2 and TIMP-1 ($p<0.001$);
2. MMP-9 and TIMP-1 ($p<0.05$);
3. MMP-9 and MMP-2 ($p<0.05$);
4. TGF-β2 and MMP-9 ($p<0.01$).

In the group of women with endometriosis (n=18) the following correlations were found:

1. MMP-2 and TIMP-1 ($p<0.001$);
2. MMP-9 and TIMP-1 ($p<0.05$);
3. MMP-9 and MMP-2 ($p<0.05$).

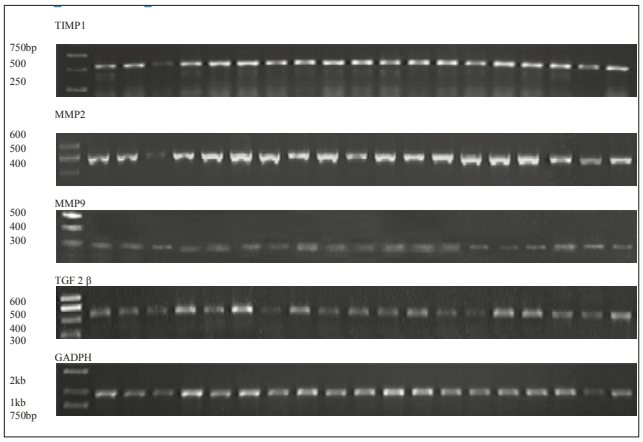

Figure 1. TIMP-1, MMP-2, MMP-9, TGF-β2 and GADPH expression in endometrium (patients 1–19)

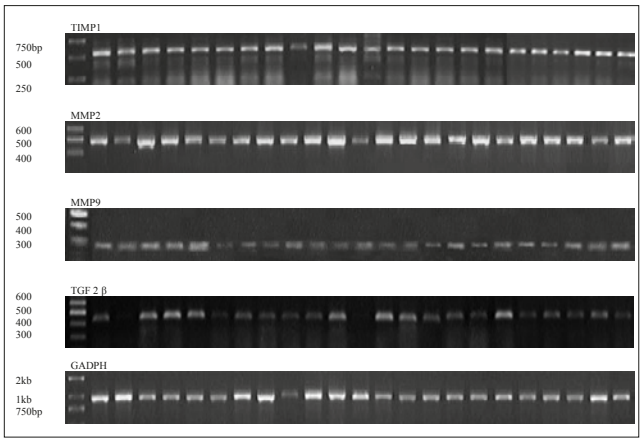

Figure 2. TIMP-1, MMP-2, MMP-9, TGF-β2 i GADPH in endometrium (patients 20–42)

Table 2. Means and standard deviations of the expression of studiem genes in women with and without endometriosis

Group	Studied gene	Product of studied gene/GAPDH	Standard deviation	P
With endometriosis	MMP2	1,67	0,25	NS
With endometriosis	MMP9	0,30	0,12	NS
With endometriosis	TIMP1	1,62	0,26	NS
With endometriosis	TGF-β2	0,62	0,15	NS
Without endometriosis	MMP2	1,56	0,26	NS
Without endometriosis	MMP9	0,25	0,07	NS
Without endometriosis	TIMP1	1,54	0,33	NS
Without endometriosis	TGF-β2	0,55	0,13	NS

P value corresponds to the expression of each gene in women with and without endometriosis.

In the group of women without endometriosis (n=24) only a correlation between MMP-2 and IMP-1 was found ($p<0.001$).

No statistical difference was found in serum progesterone levels between the groups of women with and without endometriosis. There were no correlations between serum levels of progesterone and MMP-2, MMP-9, TIMP-1 or TGF-β2 endometrial gene expressions.

DISCUSSION

Clinical trials *in vivo* regarding early endometriotic foci development, are impossible to perform for obvious ethical reasons. Therefore, knowledge of endometriosis development arises from experimental works, animal studies, as well as the observations of women with minimal or mild endometriosis. The results of the studies analyzing relationship between MMPs expression and endometriosis are difficult to compare since different techniques are utilized, different selection of MMPs are used, and furthermore, different study and control groups are enrolled. The results of such studies are still conflicting. Although the expression of a number of MMPs have been described, information about the function of these enzymes in the initial steps of endometriosis development is lacking.

Matrix metalloproteinases activity in the eutopic endometrium has been a topic of publications within the last few years, although there have not been many clinical trials undertaken. Among them these concerning women with endometriosis are scarce [10, 11, 12, 13]. The authors of the current study did not find any data on MMPs activity in eutopic endometrium of women with minimal or mild endometriosis. The obtained results did not reveal any statistical difference in the endometrial expression of MMP-2, MMP-9,TIMP-1, and TGF-β2 or serum progesterone level between women with endometriosis and without visible signs of this illness. Despite the lack of statistical differences, the authors observed interesting trends in the results. Both examined metalloproteinases expressed tendency to higher gene expression in eutopic endometrium of women with endometriosis. However, both TIMP-1 expression and TGF-β2 expressions had the same tendency m – higher values in endometriosis patients. It is probable that larger groups will allow the acquisition of statistical differences. But the presently observed tendencies seem to show a sort of readiness of the eutopic endometrium in women with endometriosis for implantation.

The afore-mentioned works of Chung et al. [7] revealed higher activity of MMPs than their inhibitors. The authors suggested that eutopic endometrium of women with endometriosis possess higher proteolytic activity, thereby enabling implantation in the peritoneal cavity. This study was performed on women with advanced endometriosis (III° and IV°; rAFS classification), and the authors suggest that the expression of MMP-2 and MT1-MMP changed after the initial stage of endometriosis, so that after the development of ovarian endometriotic cysts, enzyme activity was no longer significant. The authors of the presented study do not consider that such a conclusion may be drawn on the basis of that study. The final suggestion of Chung et al. was that eutopic endometrium in women with endometriosis is more prone to peritoneal implantation than the endometrium from women without endometriosis. Furthermore, they speculated that evaluation of the MT1-MMP and MMP-2 system may be used as a prognostic marker of endometrial invasion.

However, the main question remains: what is the primary phenomenon? Is endometriosis caused by different properties of eutopic endometrium, or does the growth of endometriotic implants change characteristic of the eutopic endometrium? In previous animal experiments, the authors of the current study showed that apoptosis could induce changes in the properties of the eutopic endometrium [13]. However, these

animal studies also revealed that endometriosis development in the abdominal cavity provoked further changes in the integrin pattern [14, 15]. Whether a similar situation exists with regard to metalloproteinases expression and their activity in eutopic endometrium remains a mystery.

The observed tendency for higher gene expression of TGF-β2 in eutopic endometrium in women with endometriosis could possibly provoke the above-mentioned changes. Chegini [16] has shown an increase of the metalloproteinases tissue inhibitors and a decrease of metalloproteinases expression after TGF-β2 application. Pizzo et al. [17] have shown truly higher levels of TGF-β2 in the peritoneal fluid of women with endometriosis, compared to endometriosis-free patients ($p<0.001$). This cytokine is also responsible for MMPs expression, being a mediator of progesterone action [18]. In the meantime, TGF-β2 was shown to provoke an increase in TIMP-1 expression in the stromal cells of human endometrium [19]. With this in mind, the lack of correlation between the examined MMPs and TIMP-1 expressions seems surprising.

Analysis of the correlations between MMP-2, MMP-9, TIMP-1 and TGF-β2 expression also revealed very interesting results. All these data may emphasize the role of the meticulous balance between extracellular matrix reabsorbtion and stabilization for homeostasis.

The obtained data does not permit the use of MMPs and their inhibitors expression in eutopic endometrium in the diagnostic process of peritoneal endometriosis. Nevertheless, this study encourages the authors to continue investigations in an attempt to further explain the pathogenesis of endometriosis.

CONCLUSIONS

Progesterone directly or indirectly stimulates MMP-9 expression in both the glandular and stromal cells [20]. The results of experimental studies regarding the progesterone and TGF–β [18] suggest their complex action on the expression of MMPs. Increased activity of MMPs was shown in endometriotic lesions which subsided during treatment. The blocking of the TGF–β action removes the endometrial metalloproteinases supression caused by progesterone. Defects in the TGF–β make the action of progesterone upon the endometriotic lesions more difficult. Therefore, very crucial points remain to be explained.

It is worth mentioning that estimation of MMPs mRNA does not reveal what the true activity of the enzymes will be. This is only the first step in the authors' considerations. At the moment they searching for aberrant activity of MMPs through active and inactive forms, a problem presented by Laird et al. [21], who suggested that the bands obtained by zymography with molecular masses of 73 and 67 kDa corresponded to inactive and active forms of MMP-2, respectively, and that at 98 and 90 kDa could correspond to inactive and active forms of MMP-9. Furthermore, concentrations of MMP-9 in individual uterine flushings detected by ELISA showed an excellent correlation with the 98K/90K activity bands seen by zymography.

Recently, Bruner et al. [18, 22] and Nap et al. [23] demonstrated in murine and chicken chorioallantoic membrane models the prevention of early endometriotic lesions formation when MMPs activity was blocked. These results allow the assumption that imbalance between MMPs and their inhibitors is the immanent feature of the eutopic endometrium, thus provoking ectopic implantation in some women. Furthermore, changing MMPs activity locally may be effective in endometriosis prophylaxis, or even treatment.

Studies by the authors on endometriosis are still in progress, and it hoped that the acquisition of much more interesting data with statistical power concerning the role of the MMPs and their inhibitors will result through bigger study groups, and newly-programmed animal studies.

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