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Matrix metalloproteinases-2, -7 and tissue metalloproteinase inhibitor-1 expression in human endometrium

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Abstract

Introduction. Endometrium undergoes regular, cyclic tissue remodeling mostly associated to the endocrine system status. It is well-known fact that steroid hormones are strongly responsible for changes in endometrium. The precise mechanism of their action is still under investigation. The aim of the study was to evaluate the expression of metalloproteinases 2 and 7 (MMP-2, -7) and tissue inhibitor of metalloproteinase 1 (TIMP-1) in human endometrium in relation to serum concentrations of estradiol and progesterone during different phases of menstrual cycle.

Material and methods. The study material consisted of 52 biopsy samples; 12 obtained in the proliferative phase, 11 in the secretory phase and 29 during menstruation. Expression of MMP-2, MMP-7 and TIMP-1 was assessed by immunohistochemistry. Serum concentrations of estradiol and progesterone at time of biopsy were evaluated by immunochemistry assay. Results of the study were statistically assessed by linear regression model.

Results. Increased serum concentration of estradiol was associated with increased MMP-2 expression in proliferative phase but decreased in secretory phase and during menstruation. No significant relationship was found between progesterone concentration and MMP-2 expression. Moreover, no difference in the expression of MMP-7 and TIMP-1 in the endometrium in relation to hormone levels and menstrual cycle phases were observed.

Conclusions. The results of the study indicate that estradiol influence MMP-2 expression in the endometrium depends on the phase of menstrual cycle. Such relationships were not found for MMP-7 and TIMP-1 and further tests clarifying association between estradiol and MMPs are needed. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 3, 133–140)*

Key words: metalloproteinase; MMP; tissue inhibitor of metalloproteinase; TIMP; endometrium; estradiol; progesterone

Introduction

Endometrium is an inner layer of the uterus which consists of the simple columnar epithelium and connective tissue stroma. A stroma develops and maintains its function under the influence of endocrine system, mostly ovarian hormones. In endometrium of women in reproductive age the functional layer and

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the basal layer may be distinguished morphologically. Monthly during physiological cycle the functional layer undergoes proliferative and secretory changes preparing it for blastocyst implantation. However, it routinely exfoliates during menstruation; when fertilization occurs this process does not take place. The basal layer remains intact during menstrual bleeding enabling regeneration of endometrium. In the proliferative phase of the menstrual cycle, all the components of the functional layer undergo proliferation, leading to the formation and expansion of uterine glands. After ovulation corpus luteum appears, releasing progesterone which induces secretory changes. Glandular cores are expanding, becoming twisted and their epithelial cells release secretion containing significant amounts of glycogen, an important nutrient needed for embryo development. Steroid hormones like estradiol and progesterone have a strong influence on cell proliferation and their secretory function, whereas they are also responsible for the regulation of matrix metalloproteinases (MMPs) activity. The regulatory role of MMPs in the endometrium was investigated in both immunohistochemical and molecular studies [1–3].

MMPs are a family of proteolytic enzymes containing zinc ion in their active center. They are expressed in various tissues located in the extracellular matrix (EMC) as inactive proenzymes or remain attached to cell membranes [4]. Their primary role is degradation of EMC, facilitating cell migration during tissue remodeling. Moreover they affect other cell functions, *e.g.* induce release of growth factors controlling tissue restoration processes, and angiogenesis [5–9]. Their role in the menstrual cycle of endometrial changes in women of reproductive age as well their involvement in embryo implantation has been studied [10, 11]. Their activity influences the regeneration and organization of both endometrial components. While expression of many MMPs have been described in pathological states [12], significant differences in their expression and localization in the various phases of the physiological menstrual cycle are still not known. For instance, MMPs expression is present primarily in the stromal and vascular cells, but *in-situ* hybridization demonstrated that MMP-2 mRNA is also expressed in epithelial cells but MMP-7 mRNA and MMP-26 mRNA only in the epithelial glandular cells [13–15].

Important regulators of MMPs activity are tissue inhibitors of metalloproteinases (TIMP-1 to TIMP-4) [16]. TIMP-1 is the main inhibitor of MMP-2 and MMP-9 [17, 18]. Although their significance for activation of proenzymes has been confirmed, studies on the role of TIMPs in the regulation of tissue activity and function are ongoing [19]. Moreover, immunohistochemical analysis showed that TIMP-1 expression

within the endometrium correlates with MMP-2 and MMP-7 expression [20].

Many studies describe the role of MMPs in the development of endometrial neoplasms [21], whereas, only a few focus on their expression in the normal endometrium, mostly in outer layer. It seems that finding of factors that influence proliferation and activity of epithelial cells may be useful for diagnostics and treatment of disorders caused by MMPs/TIMPs imbalance, *e.g.* abnormal uterine bleeding [20].

Most studies verify the effect of steroid hormones on MMP activity in endometrium *in vitro* (tissue explants cultures). For a more realistic assessment, in this investigation we evaluated MMP expression directly in the endometrium from particular days of menstrual cycle in regard to serum concentrations of estradiol and progesterone.

Material and methods

Patients. Fifty two premenopausal, healthy women aged 25–40 with 25–30 days long menstrual cycles were qualified for the study. None of the participants was taking hormone treatment or hormonal contraception prior and during research. All participants signed informed consent prior the inclusion to the trial. The research was approved by the Bioethical Committee of the Medical University of Warsaw (KB/243/2012). Clinical data are presented in Table 1.

Material sampling. Endometrial sampling was made in out-patient clinic with Pipelle de Cornier® (Laboratoire CCD, France). Indications for the biopsy were: "endometrial scratching" in 10 women with male factor infertility, cervical polyp in 13 women and 29 women voluntarily agreed for biopsy during menstruation. It was performed in 12 women in the proliferative phase of the cycle (between day 8 and 13 of the cycle, mean age 34.5 years), in 11 in the secretory phase (day 18–23 of the cycle, mean age 36 years) and in 29 during bleeding (mean age 31.3 years). The material was immediately placed in 10% formalin and sent to histopathological and immunohistochemical examination. Histopathological examination performed on hematoxylin and eosin stained paraffin sections did not show any abnormalities in microscopic image and was consistent with the phase of cycle in all cases.

Additionally, in all women, 5 ml of venous blood sample was obtained on the day of biopsy; serum concentrations of estradiol and progesterone were determined by the electrochemiluminescence immunoassay (ECLIA; Elecsys, Cobas 6000, Roche, Basel, Switzerland).

Immunohistochemistry. Immunohistochemical (IHC) reactions were carried out on 4 μm deparaffinized sections. All procedures were conducted according to running IHC protocols of Department of Pathology, Medical University

Table 1. Clinical data of 52 patients included in the study

Covariates	Mean ± SD	Range (min–max)	Median
Age [years]	33.02 ± 5.44	23–40	34
BMI [kg/m²]	24.55 ± 5.54	15.44–50.78	22.98
Length of bleeding [days]	18.12 ± 26.57	0–102	4.50
Estradiol conc. [pg/mL]	102.2 ± 84.03	22.37–450.7	79.56
Progesterone conc. [pg/mL]	4.88 ± 5.58	0.24–22.76	1.77
Estradiol/progesterone ratio	55.67 ± 74.18	5.7–367.1	23.41
Pregnancies [no.]	0.67 ± 0.76	0–2	0.50
Miscarriages [no.]	0.07 ± 0.43	0–3	0

SD — standard deviation

of Warsaw, (Dako AutostainerLink 48, Dako Omnis and Dako PT Link, Dako, Glostrup, Denmark). Direct mouse monoclonal antibodies against MMP-2 (clone 17B11, dilution 1:50; Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK), MMP-7 (clone ID-2, dilution 1:75; Millipore, Billerica, MA, USA), and TIMP-1 (clone 6F6a, dilution 1:50; Novocastra) were used. For the detection of monoclonal antibodies, peroxidase-conjugated anti-mouse ImPress ™ reagent kit (Vector Laboratories, Burlingame, CA, USA) was used. 3,3-diaminobenzidine was used as chromogen. As internal control reactions with non-essential mouse IgG1 (Sigma-Aldrich, St. Louis, MO, USA) instead of primary antibodies were performed.

Morphometric assessment. Expression of MMP-2, MMP-7 and TIMP-1 was analyzed with a microscope (Nikon, Labophot, Tokyo, Japan) equipped with a motorized stage and Image Pro Plus software (Medra Cybernetics Inc., Rockville, MD, USA). 10 random fields of each sample were photographed at × 10 magnification. Each image was thresholded, and the areas covered by MMP-2, MMP-7 and TIMP-1 expression, as well as the average staining intensity, were quantified. Fields with histological artifacts or large areas of hemorrhage and necrosis were excluded. Total immunoreactivity was approximated as the product of an area of a given field times expression intensity. As our goal was to analyze the expression in both the cells and the extracellular matrix (ECM was considered as an approximation of the total result, and displayed in arbitrary units (AU). This dimensionless quantity commonly serves in biological science to compare multiple measurements performed in similar environment and equipment. This modality of assessment gives opportunity to compare expression of proteins both inside of the cells and in the surrounding ECM. It is frequently used both clinically and in laboratory experiments [22].

Statistical analysis. In the analysis three response variables were considered: expression of MMP-2, MMP-7 and TIMP-1. A multiple linear regression using the ordinary least squares (OLS) estimator was used. This approach enables analysis of the isolated impact of the individual factors. Functional forms with non-transformed and logarithmically transformed response variables and hormone levels were considered. Additionally, for hormone levels polynomials and interactions with phase of the cycle were considered. The choice of the final functional form was based on a general-to-detail approach using the F test. For the final model diagnostics was performed to confirm its correctness (Ramsey test, Jarque-Bera test, Breusch-Pagan homoscedasticity test). The variance inflation factor (VIF) measure of the co-linearity of the variables explained in the model was also performed for either non-transformed or logarithmic values. Isolation of the impact was crucial benefit of this study, making presented approach better comparing to using simple univariate analyzes such as Kruskall-Wallis or Spearman tests. All calculations were done using the STATA 14 software (StataCorp LLC, College Station, Texas, USA). Unless otherwise indicated, the tests were carried out at a significance level of 5%.

Results

The IHC expressions of MMP-2, MMP-7 and TIMP-1 were analyzed in cytoplasm of epithelial and stromal cells in proliferative, secretory and menstruation phases (Fig. 1). In proliferative phase strong expression of MMP-2 was found in epithelial and stromal cells (Fig. 1A), whereas moderate expression of TIMP-1 was observed only in epithelial cells (Fig. 1G). In secretory phase moderate expression of MMP-2 and weak expression of MMP-7 and TIMP-1 was found in epithelial cells (Fig. 1B, E, H). During menstruation stromal portion of endometrium was found to be relaxed as well strong expression of all analyzed markers was observed in cytoplasm of stromal cells (Fig. 1C, F, I).

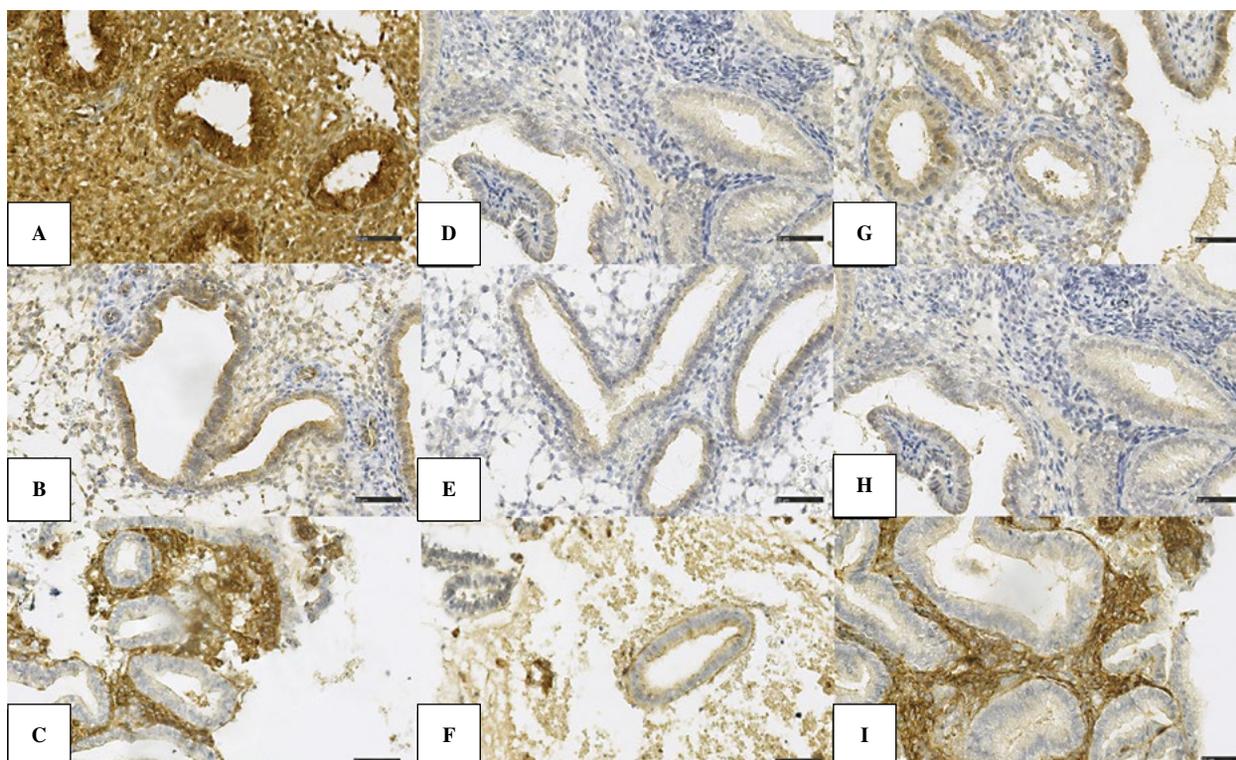The results of expression of MMP-2, MMP-7 and TIMP-1 in endometrium are presented in Table 2.

Figure 1. Immunohistochemical expression of MMP-2 (**A–C**), MMP-7 (**D–F**) and TIMP-1 (**G–I**) in the human endometrium during different phases of menstrual cycle. Proliferative phase served as a reference (**A, D, G**), so that increased expression is seen in the glandular epithelium in secretory phase (**B, E, I**), and increased expression in stromal cells during menstruation (**C, F, I**). The immunohistochemical staining was performed as described in Methods. Total magnification 100×, space bars 10 μm.

Table 2. Expression of metalloproteinases (MMP) 2 and 7 and tissue inhibitor of metalloproteinases 1 (TIMP-1) during successive phases of menstrual cycle assessed by immunohistochemistry in arbitrary units [AU]

	MMP-2	MMP-7	TIMP-1
Secretory phase	141.38 ± 11.83	131.50 ± 11.53	139.38 ± 11.99
Proliferative phase	131.81 ± 10.06	133.81 ± 14.89	137.81 ± 5.51
Menstruation	141.86 ± 12.89	136.39 ± 11.49	143.47 ± 9.82
Menstrual cycle mean expression [range]	139.4 ± 12.58 [54.64]	134.8 ± 12.26 [61.72]	141.3 ± 9.6 [49.97]

Values represent mean ± SD

There was a significant dependence between serum estradiol and MMP-2 expression in the endometrium. It was different depending on the phase of the menstrual cycle. In the proliferative phase of the cycle, with 1% increase in serum estradiol levels, expression of MMP-2 in the endometrium also increased (on average by 0.11 AU). During the secretory phase of the cycle and during the menstruation, an increase in estradiol concentration of 1% was associated with a decrease in expression of MMP-2 (by 0.08 AU). Immunohistochemical expression of MMP-2 in different phases of the cycle is presented on Figures 1A–C.

Linear regression results presented in Table 3 indicate that MMP-2 expression is lower (by 64.6 AU) in the proliferative phase of the cycle compared to the secretory phase (p < 0.01; OLS results — t test). During the menstrual bleeding expression of MMP-2 decreases with each day of bleeding (by an average of 0.147 AU, p < 0.05; OLS results — T test). There were no significant differences in progesterone-dependent MMP-2 expression.

The expression of MMP-7 and TIMP-1 in the endometrium remained at a certain level and was not dependent on hormone levels or menstrual cycle

Table 3. Factors that significantly affect MMP-2 expression in endometrium (linear regression model)

Covariates	Existence MMP-2 Coef. (SE)
Menstrual cycle:	
Proliferative phase	–64.554** (22.735)
Length of bleeding	–0.149* (0.061)
Hormones:	
Log (Estradiol)	–8.083*** (2.559)
Proliferative Phase × Log (Estradiol)	11.758* (4.834)
Miscarriage:	
3 times miscarriages	25.175* (10.645)
Constant	179.009*** (11.525)

SE — standard error

phases. Their immunohistochemical expressions are presented on Figures 1D–F and G–I, respectively.

Discussion

Expression of MMPs in human endometrium is uneven during subsequent phases of menstrual cycle and most MMPs have much higher expression during menstruation [23]. Up to date there are very few original reports on MMPs expression in physiological endometrium, as most studies concentrated on endometrial cancer cases. Defining the role of MMPs in the course of endometrial transformation, proliferation, secretional changes, exfoliation and re-growth is essential to understand how they work in pathological conditions.

Knowing the interplay between MMPs and sex hormones concentration in the tissues may enhance the treatment of many disorders from abnormal uterine bleeding (AUB) spectrum.

Correlation between MMP-2 expression and steroid hormones concentration

At physiological concentrations estradiol stimulates MMP-2 activity by increasing production and release of MMP-2 protein in a receptor-mediated process [24]. 17β-estradiol has been shown to stimulate the release of MMP-2 from smooth muscle of coronary arteries as well as from muscle cells of umbilical artery wall [25]. Similarly Huang *et al.* showed a positive correlation between serum estradiol concentration and serum MMP-2 concentration in the first stage of the menstrual cycle [26].

Our results indicate that the expression of MMP-2 in the endometrium was seen throughout the cycle both in stroma and epithelial cells and was dependent on serum estradiol levels. Mutual correlations depend on the phase of the menstrual cycle. The immunohistochemical studies have shown that in the proliferative phase of the cycle, as the estradiol level increased, MMP-2 immunoreactivity in the endometrium also increased. It was reversed in the secretory phase since estradiol increase was accompanied by a decrease in MMP-2 expression. The role of MMP-2 in the proliferative phase of the menstrual cycle seems to be stimulation of endometrial cell proliferation and angiogenesis [27, 28]. In the secretory phase the increase in the synthesis and release of estradiol and progesterone is almost parallel [29]. This may explain the negative relationship between estradiol levels in the blood and expression of MMP-2 in the endometrium. In physiological conditions progesterone prevents endometrial breakdown by inhibiting MMPs *via* its nuclear receptor [30]. However, it was showed *in vitro* that adding RU486, progesterone receptor inhibitor, to the stromal cell culture increased MMP-2 production which was dependent on *de novo* protein synthesis since it was suppressed by cycloheximide [31]. In other studies, also conducted *in vitro,* Zhang *et al.* have demonstrated that progesterone inhibited MMP-2 activation [32]. In another study conducted on ovariectomized monkeys, Brenner *et al.* demonstrated an increase in MMP-2 expression both by IHC and PCR after removal of progesterone-releasing implant; however, in subsequent days the MMP-2 expression decreased despite lack of progesterone [33].

Increased expression of MMP-2 in the secretory phase may be associated with increased synthesis of collagens type IV, V, fibronectin, and laminin by stromal cells [34]. Balance between various components of the extracellular matrix during endometrial changes is regulated by the activity of MMPs and other enzymes. The reduction of steroid hormone levels strongly induces MMP-2 activity leading to the degradation of basal membranes and stromal breakdown resulting in the onset of menstruation [31].

Correlation between MMP-7 expression and steroid hormones concentration

The expression of MMP-7 was found mainly in the epithelial cells. In contrast to MMP-2, MMP-7 expression within the endometrium remained at a certain level and did not differ between the proliferative and the secretory phase, and was irrespective of estradiol and progesterone concentrations. MMP-7 expression during proliferative and secretory phases suggests that MMP-7 participates not only in the extracellular matrix breakdown but also in the remodeling processes ongoing in non-menstrual endometrium,

such as epithelial cell migration, proliferation and apoptosis, which are needed to regenerate the endometrium after menstruation. Berton *et al.* showed that MMP-7 targeting the plasma membrane of epithelial cell focally promotes its activity, a mechanism which could be important to allow MMP-7 action on substrates associated to the epithelial cell membrane, such as E-cadherin, β_4-integrin, TNF-α, Fas, Fas ligand, heparin-binding EGF, IGF binding proteins and plasminogen [35]. There are limited recent studies concerning correlation between MMP-7 expression and hormone levels, nevertheless, some classical experiments by Osteen's and Rudolph-Owen's groups explain these dependences well. Osteen *et al.* demonstrated that epithelial cells treated with estradiol (proliferative phase conditions) produced only MMP-7 [36]. The lack of increased expression of MMP-7 along with an increase in estradiol levels possibly indicate the inhibitory effect of other factors, probably such as progesterone and local inhibiting factors that are released directly into the stroma [35]. Rudolph-Owen *et al.* demonstrated that endometrial expression of MMPs, including MMP-7 in endometrial proliferative phase, is similar in regular proliferative phase as well in ovariectomized animal model. In their study increased expression of MMP-7 occurred only until 2 days after discontinuation of progesterone and then decreased and persisted low for a further 9 days despite the absence of progesterone [37].

Physiologically, the increase in MMP-7 expression was found only in the first days of menstruation, indicating the importance of this enzyme for the normal process of endometrial exfoliation. Regeneration of the endometrium usually begins at the second day of the cycle, whereas bleeding disappears when the endometrium reepithelization process is complete [38, 39].

Correlation between TIMP-1 expression and concentrations of steroid hormones

The results of our study indicate that TIMP-1 may be important for maintaining endometrial stability. TIMP-1 expression was present in the endometrium throughout the whole cycle at a constant level and was not dependent on estradiol or progesterone concentrations. The lack of this dependence was shown in *in vitro* studies. Lockwood *et al.* showed by Northern-blot analysis that supplementation of cell culture with both steroid hormones or progesterone separately did not affect TIMP-1 mRNA expression [40]. There is an evidence for changes of TIMP-1 mRNA expression depending on the phase of menstrual cycle. Maatta *et al.* using *in-situ* hybridization and Northern-blot techniques showed decreased expression of TIMP-1

mRNA in the proliferative phase, while increased expression was found in the late secretory phase [41]. Our results are similar to those of Rogers *at al.* and Lockwood *et al.*, who in *in-situ* hybridization studies, showed lack of temporal regulation of TIMP-1 mRNA expression during menstrual cycle [42].

In our study TIMP-1 was expressed equally throughout the cycle. The slight increase at menstruation was not statistically significant. TIMP-1 was immunolocalized in the epithelial, stromal and vascular compartments of the endometrium. Maatta *et al.* using *in-situ* hybridization and Northern-blot technique showed decreased expression of TIMP-1 mRNA in the proliferative phase, while increased expression was found in the late secretory phase [41]. Our results are similar to Lockwood *et al.*, who in *in-situ* hybridization studies, showed lack of temporal regulation of TIMP-1 mRNA expression during menstrual cycle [40, 42].

The TIMP-1 expression in the endometrium was not dependent neither on estradiol nor progesterone concentration. The lack of hormonal dependence was shown in other *in vitro* studies [40, 43]. The results of our study indicate that TIMP-1 may be important for maintaining endometrial stability and prevent tissue breakdown during the menstrual cycle.

Conclusions

The mechanism by which estradiol and progesterone regulate expression of MMPs and TIMPs remains unclear. The results of our study confirm the involvement of estrogens in the regulation of MMP-2 expression in the endometrium. However, complex dependence on the hormone concentration as well on the phase of menstrual cycle strongly suggests interplay with other factors. Such relations were not found for MMP-7 and TIMP-1 and further studies should be focused on their evaluation.

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