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Expression of matrix metalloproteinase enzymes in endometrium of women with abnormal uterine bleeding

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Abstract

OBJECTIVES: Abnormal uterine bleeding (AUB) is caused by derangement of physiological processes of tissue growth, shedding and regeneration. It is known that interplay between metalloproteinases (MMP's) and tissue inhibitors of metalloproteinases (TIMP's) may play a crucial role in its occurrence.

AIM: To define if expression of proMMP-2, MMP-2 and TIMP-1 in endometrium of women with AUB is dependent on steroid sex hormone concentration and histopathological picture.

MATERIALS AND METHODS: Endometrial scraps were taken from 21 women with AUB and 19 controls. Samples were evaluated in light microscopy by a certified pathologist. Activity of proMMP-2 and MMP-2 proteins levels were evaluated by gelatin zymography and TIMP-1 by reversed zymography. The results has been correlated with serum estradiol and progesterone concentrations in linear regression model.

RESULTS: Expression: of proMMP-2 in endometrium of women with AUB is correlated with estradiol concentration and inversely correlated with progesterone levels. It was significantly higher in women with dysfunctional endometrium ($p<0.001$). Expression of MMP-2 was highest in women with endometrial polyps and longer bleeding ($p<0.01$), while expression of TIMP-1 was independent from hormone concentration.

CONCLUSION: Lack of correlation between proMMP-2 and MMP-2 levels suggest different pathway of their activation in AUB. ProMMP-2 is up regulated by estradiol and down regulated by progesterone while MMP-2 levels increase with the length of bleeding.

ORIGINAL ARTICLE

INTRODUCTION

Abnormal uterine bleeding (AUB) is one of most significant disorders deteriorating the quality of life of young women. It is estimated that AUB affects 11–13% of all female (Marret *et al.* 2010). Most frequent reasons are uterine myomas, endometrial polyps, hormone imbalance, endometrial hyperplasia and endometritis, but in approximately 40–50% of women the substantial cause remain unknown (Kotdawala *et al.* 2013). They differ from menstrual bleeding not only in regularity but also in length and abundance. All cases of AUB are resemblance of derangements of physiological processes occurring in menstruating female. Despite the variety of possible causes of an impairment of hemostasis or vascular damage, the increased bleeding is often mediated through the matrix metalloproteinases (MMPs), group of more than 20 proteolytic enzymes which regulate degradation of extracellular matrix in the endometrium (Lockwood 2011; Apte & Parks 2015).

Ability of MMPs to decompose extracellular matrix at natural pH makes them essential for endometrial shedding and regeneration during normal menstrual cycle. Numerous MMPs are found in the uterine mucous membrane during regular cycle (Goffin *et al.* 2003). Menstrual bleeding and following regeneration of stroma and re-epithelialization of glandular epithelium are, to a large extent, dependent on expression of individual MMPs, which are regulated by steroid hormones. In physiological conditions decrease of hormones concentration (mainly progesterone) leads to up regulation of MMP-1, MMP-3 and MMP-9 expression, which consequently causes tissue degradation and initiates menstruation. The end of tissue breakdown and bleeding is initiated by the increase of expression of tissue inhibitors of metalloproteinase's (TIMPs), mainly TIMP-1 and TIMP-2 (Dong *et al.* 2002). Specific links between steroid hormones and activity of MMPs and TIMPs are very complex and still nurture researchers worldwide. Imbalance of physiological equilibrium between processes of epithelial exfoliation and stroma regeneration may lead to pathological changes in endometrium structure like hyperplasia or endometrial cancer (Bogusiewicz *et al.* 2007; Hickey *et al.* 2008). However, little is known about the role of MMPs expression in AUB. This is why we decided not only to evaluate the activity of MMP-2 and TIMP-1 in endometrium of women with AUB, but to correlate it with histopathological diagnosis and estradiol and progesterone serum concentration and compare the results with normally menstruating women. MMP-2 and TIMP-1 was selected because they are the only enzymes which expression is detectable during whole menstrual cycle and reach maximum levels during the bleeding period (Brenner & Slayden 2012; Rodgers *et al.* 1994). Additionally MMP-2 is the only MMP present in the endometrium during the secretive phase, which strongly suggest that it's expression is dependent on steroid hormones. Research on correlation between concentration of steroid hormones and MMPs activity in women with AUB can not only clear the pathomechanism leading to prolonged and abundant bleeding, but will help to adjust length, doses and timing of the hormone based therapies.

MATERIAL AND METHODS.

Baseline characteristics

The samples were taken from 23 women with normal menstrual periods and 29 women with AUB aged 25–40 years. All women signed informed consent before the inclusion to the trial. The research was approved by Bioethical Committee of Medical University of Warsaw (KB/243/2012). AUB was defined as acyclic vaginal bleeding of various degree. None of the participants was taking hormone treatment nor oral contraceptives prior the research. Descriptive statistics of participants are presented in Table 1.

Endometrial sampling in healthy subjects was made with Pipelle device during proliferative phase (8–13 day of cycle) or in secretive phase (18–23 day) while in AUB patients the samples were obtained during their first

Tab. 1. Descriptive statistics of all patients included in the research.

Covariates	No.	Mean	SD	Minimum	Maximum	Range	Q1	Median	Q3
Age (years)	40	33.10	5.28	23	40	17	29.5	34	38.50
BMI (kg/m2)	40	24.43	5.79	15.44	50.78	35.34	21.3	22.32	25.76
Length of bleeding (days)	40	18.18	26.2	0	90	90	0	3.500	31
Estradiol (pg/ml)	40	95.42	67.7	22.37	328.1	305.8	43.8	84.41	131.4
Progesterone (ng/ml)	40	5.80	6.00	0.90	23	22.10	1.50	1.810	10.31
E/P	40	36.55	46.80	5.70	212.5	206.8	10.8	18.47	37.55
Deliveries	40	0.65	0.77	0	2	2	0	0	1
Miscarriages	40	0.075	0.47	0	3	3	0	0	0

SD – Standard Deviation, Q1,Q3 – Quartile 1 & 3

consultation. Part of material was immediately frozen in liquid nitrogen and the rest was put in 4% formaldehyde for histopathological evaluation.

In 12 cases we failed to obtain diagnostic material, leaving 21 patients with AUB and 19 controls included in the study. In all patients we assessed the estradiol and progesterone levels on the day of endometrial sampling. Hormone levels has been assessed by immunochemiluminometric assay (ELISA, Elecsys, Cobase) while the expression of proMMP-2, MMP-2 and TIMP-1 was assessed by zymography (Sliwowska & Kopczynski 2007; Snoek-van Beurden & Von den Hoff 2005).

Zymography

Proteins preparation. Endometrial samples were homogenized in 500 µl of homogenization buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 5 mM $CaCl_2$; 0.05% Brij-35; 0.02% NaN_3) with 1% Triton X-100 in TissueLyser LT (Qiagen) for 5 min. Samples were then centrifuged for 5 min (12 000 rpm, 4 °C) and the supernatant was obtained. Protein concentration was determined in all samples in SmartSpec™ Plus spectrophotometr (Bio-Rad) by Bradford method.

Gelatin zymography. Gelatin zymography was used to detect the activity of MMP-2 in tissue extracts from endometrium. Each sample were analyzed in duplicate. Proteins separation was performed on 7.5% polyacrylamide gels containing 10% sodium dodecyl sulfate (SDS) and 1 mg/ml of porcine gelatin (Sigma-Aldrich), as substrate for MMP-2. Samples mixed (1:1) with sample buffer (with 4% SDS) were loaded onto a gel. Molecular weight marker (Precision Plus Protein™ Dual Xtra standards, Bio-Rad), and recombinant human MMP-2 Western Blotting standard (R&D Systems) were also loaded onto each gel. After electrophoresis gels were washed in distillated water, and then in 2.5% solution of Triton X-100 two times for 20 min, to remove SDS. Gels were than incubated in activation buffer (50 mM Tris-HCl pH 7.6; 200 mM NaCl; 5 mM $CaCl_2$; 0.02% NaN_3; 1% Triton X-100), at 37 °C for 18 h. Gels were then stained in 0.1% Coomassie Brilliant Blue R250 in 40% methanol and 10% acetic acid solution for 1 h and destained in 40% methanol and 10% acetic acid to obtained clear bands against blue background. The zymograms were scanned, and analysed by use of Image J software. Clear bands in gels were quantified densitometrically. Proteolytic activity of MMP-2 was determined in arbitrary units (AU), in comparison to MMP-2 standard.

Reverse zymography. Reverse zymography was used to detected the activity of TIMP-1. Electrophoresis was performed on 12% polyacrylamide gels containing 10% sodium dodecyl sulfate (SDS), 1 mg/ml of porcine gelatin (Sigma-Aldrich) and 8% of collagenase (Collagenase Type I, Clostridiopeptidase A from Clostridium histolyticum, 125 U/mg, Sigma-Aldrich) solutions (1 mg/ml) in TESCA buffer (50 mM TES, 0.36 mM $CaCl_2$, pH 7.4). Collagenase degraded gelatin in gel where inhibitors of MMPs were absent. Next stages of reverse zymography were performed following the same method as described for gelatin zymography. Recombinant human TIMP-1 Western Blotting standard (R&D Systems) and Precision Plus Protein™ Dual Xtra standards (Bio-Rad) were used. The inhibitory activity of TIMP-1 appeared as blue bands against clear background. Gels were scanned and analyzed as described for gelatin zymography.

Histopathological examination

After fixation in formalin, fragment of the endometrium were transferred into plastic cassettes and embedded in paraffin. Once cooled, 4-µm sections were shaved and applied to a slide. Slides were then deparaffinized in xylene and alcohol, stained with hematoxylin and eosin, and then dehydrated and sealed. All slides were viewed and evaluated by a certified pathologist with respect to the phase of the menstrual cycle and the presence of pathology.

Statistic method

All variables such as proMMP-2, MMP-2 and TIMP-1 expression were obtained in quantity large enough to conduce linear regression with OLS estimator. This allowed us to determine the effect of selected factors, such as hormone concentration, estradiol to progesterone ratio, phase of menstrual cycle, age, body mass index (BMI) and parity on the variables.

During the functional form choice it was considered that either depended variables or hormone levels were expressed in original values or logarithms. Additionally, for hormone levels polynomial functions were analysed (i.e. squares of hormone levels). As a possible extension assumption that hormone levels affect dependent variables differently in the proliferative and secretive phase of the cycle were considered as well (interactions). The choice of the final functional form was based on a general-to-specific approach using the F test. For the final model appropriate diagnostics were performed (the Ramsey test of correct specification in linear regression, the Jarque-Bera test of error terms normality, the Breusch-Pagan test of error terms homoscedasticity and VIF measures in order to assess collinearity). All calculations were performed in STATA 14 (StataCorp LLC. Texas, USA.). Unless indicated otherwise, 5% was considered as a level of significance.

RESULTS

In the healthy subjects the histopathological appearance of the endometrium was concordant with the phase of menstrual cycle, 8 patients were in proliferative and 11 in secretive phase (mean age 33.5 and 36.0 years respectively). In AUB group (mean age 33.5 y) the histopathological results were as follows: proliferative endometrium 4 patients, secretive endometrium 4 patients, endometrial polyp 3 patients, simple endome-

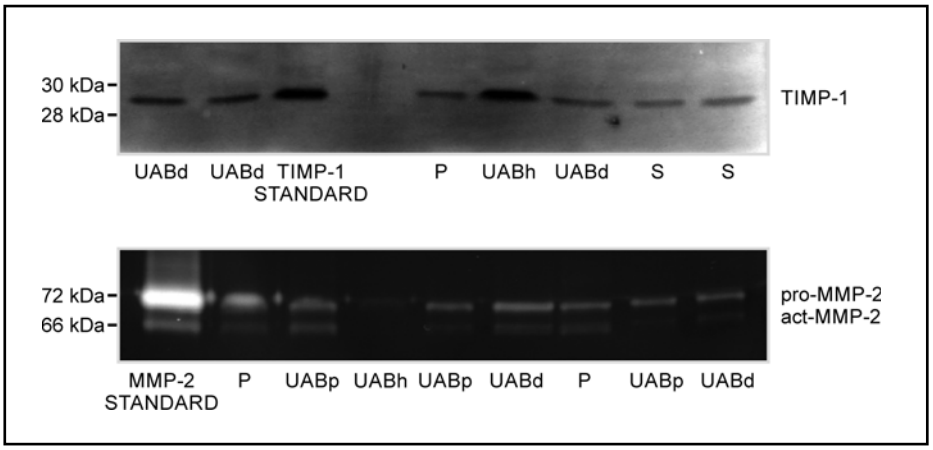

Fig. 1. Representative gelatin and reverse gelatin zymograms of human endometrium homogenates. Samples were obtained from women in proliferative (P) and secretive (S) phase of menstrual cycle and those with abnormal uterine bleeding diagnosed with: endometrial polyps (UABp), endometrial hyperplasia (UABh) or endometrial dysfunction (UABd). Gelatinolytic activities of active and pro-MMP-2 were observed at 66 and 72 kDa. Inhibiting of gelatinolytic activity by TIMP-1 was observed at about 28-30 kDa.

Tab. 2. Expression of proMM2-2, MMP-2 and TIMP-1 in the population.

Covariates	No.	Mean	SD	Minimum	Maximum	Range	Q1	Median	Q3
proMMP-2	40	**0.256**	0.0903	0.0890	0.454	0.365	0.192	0.253	0.300
MMP2	40	**0.160**	0.125	0	0.596	0.596	0.0736	0.129	0.218
TIMP-1	40	**2.133**	1.726	0.185	8.140	7.954	1.006	1.483	2.996

SD – Standard Deviation, Q1,Q3 – Quartile 1 & 3

Tab. 3. Linear regression. The table presents only factors which affects the dependent variables (with significance of min. 10%, results with very strong correlation of <0.001 marked in bold).

Covariates	Exposure	
	ProMMP2	MMP2
	Coefficient (Standard Error)	Coefficient (Standard Error)
Menstrual cycle		
Secretory Phase	**0.618*** (0.222)**	
Length of bleeding		0.002** (0.001)
Hormones		
Log(Estradiol)	**0.061*** (0.021)**	
Secretory Phase. x Log(Estradiol)	**–0.183*** (0.061)**	
Log(Progesterone)	–0.043** (0.017)	
Secretory Phase. x Log(Progesterone)	**0.153*** (0.046)**	
Diagnosis		
Polyp		**0.176*** (0.056)**
Hormonal dysfunction	**0.171*** (0.041)**	–0.103** (0.049)
Endometrial hyperplasia		–0.103** (0.049)
Miscarriage		
3 times miscarriages		0.196* (0.108)
Constant	–0.004 (0.086)	**0.129*** (0.021)**
Number of observations	40	40
R^2 – goodness of fit measure	0.50	0.39

Significance – *p<0.10, **p<0.05,***p<0.01

trial hyperplasia 7 patients, hormonally dysfunctional endometrium 3 patients. All descriptive statistics are presented in Table 1 while results of proMMP-2, MMP-2 and TIMP-1 expression are presented in Table 2.

The presence of two forms of MMP-2 – 66 kDa active and 72 kDa pro-MMP-2 were detected by gelatin zymography (Figure 1A). While reverse zymography allowed to detect the presence of TIMP-1 with a molecular weight of about 28–30 kDa (Figure 1B).

Linear regression analysis revealed that proMMP-2 expression is significantly increased during secretive phase (by 0.618 AU, p<0.001) comparing to proliferative phase and abnormal bleeding. Surprisingly correlation between proMMP-2 expression and estradiol and progesterone concentrations was different depending on the phase of cycle. During abnormal bleeding with 1% increase of estradiol concentration the expression of proMMP-2 raised by 0.0006 AU while during secretive phase dropped by 0.0018 AU. When correlated with 1% increase of progesterone during abnormal bleeding proMMP-2 expression decreased by 0.0004 AU and in secretive phase increased by 0.0015 AU. Women with hormonally dysfunctional endometrium had significantly increased proMMP-2 expression when compared with other AUB (0.171 AU, p<0.001).

MMP-2 expression was stable during the cycle, with insignificant tendency to raise during secretive phase, while during abnormal bleeding it's expression was increasing 0.002 AU daily. MMP-2 expression was not correlated with estradiol nor progesterone concentrations, but in patients with endometrial polyp it's activity was increased by 0.176 AU (p<0.01) and decreased by 0.103 AU (p<0.05) in patients with hormonally dys-

functional endometrium and hyperplasia. Interestingly the expression of pro-enzyme and it active form was not correlated (Pearson correlation index 0.0607, p=0.71).

Expression of TIMP-1 was similar during both phases of cycle and abnormal bleeding and did not differ in patients with different histopathological diagnoses. It did not correlate with hormone levels neither. All results of linear regression analyzes are presented in Table 3.

DISCUSSION

Role of proMMP-2 and MMP-2 in normal menstrual cycle and AUB

Main mechanism of MMP-2 activity is degradation of type IV collagen, which can be found in most cellular membranes. MMP-2 has confirmed role in tissue rearrangement both in physiological and pathological conditions (Unemori *et al.* 1990). It's expression was found in stromal cells during tissue development and regeneration (Morgunova *et al.* 1999). Previous research confirms complicated mechanism of MMP-2 secretion and activation. It is synthesized as pre-proenzyme and it activity is regulated on various levels, from gene transcription through intracellular pro-enzyme activators which cut out the signal peptide and release MMP to extracellular matrix to specific tissue inhibitors like membrane-type 1MMP (MT1-MMP), TIMP-1, TIMP-2 and serine proteases (Bian & Sun 1997; Carmeliet *et al.* 1997; Mazzieri *et al.* 1997; Lafleur *et al.* 2001; Nagase *et al.* 2006).

The analysis indicate that steroid hormone concentrations have an effect on proMMP-2 expression but did not directly affect MMP-2 expression. There was greater expression of MMP-2 during abnormal bleeding when compared to expression in the proliferative and secretory phase and no correlation with hormone levels. Lack of correlation between proMMP-2 and MMP-2 expression suggest another activation mechanism.

The above observations together with the increase in MMP-2 expression along with the length of bleeding indicate the role of local, possibly paracrine factors. The stimulating factor may be type 1 membrane MMP (MT1-MMP), released from neutrophils, which accumulation was found in the endometrium during bleeding. The role of local factors, cytokines, IL-1 alpha, TNF-alpha, prostaglandins are described in previously published research (Rudolph-Owen *et al.* 1998; Henriet *et al.* 2002). The role of local factors and the independence of MMP-2 expression from hormone levels during AUB also explains its increased expression in women with endometrial polyps compared to women with hormone disorders (endometrial dysfunction). Endometrial polyps histopathology occurred more often in patients reporting abnormal bleeding (73%) when compared with patients with no symptoms (61%) (Radowicka *et al.* 2016). Increased expression of MMP-2 in women of childbearing age with AUB was also reported by other authors (Erdemoglu *et al.* 2008). Its correlation with aberrant angiogenesis leads to suggestion that MMP-2 has role in the development of endometrial polyps and polyp-associated AUB (Tokyol *et al.* 2009).

In contrast, the expression of proMMP-2 was higher in women with hormonal dysfunction of endometrium and significantly related to hormone levels. Higher concentration of estradiol were associated with greater activity of proMMP-2, while higher levels of progesterone lead to turning it off. The fact that progesterone is an inhibitor of proMMP-2 activation concurs with previous publications (Zhang *et al.* 2000).

Role of TIMP-1 in normal menstrual cycle and AUB

TIMP-1 is a glycoprotein with molecular weight of 30 kDa. In physiological state it is main known inhibitor of MMPs activity (Nagase & Woessner 1999), but also regulate activity of plasmin, trombine and urokinase (Chesler *et al.* 1995).

Unlike MMPs, the role of TIMP-1 in the endometrium during abnormal bleeding is less well known. In immunohistochemistry, menstrual endometrium has been shown strong heterogeneous expression for TIMP-1 during menstruation, which varied greatly from region to region showing lower level in necrotic tissue compared to non-necrotic tissue (Freitas *et al.* 1999). Observations indicate its role in inhibiting proteinase activity and relevance to end of bleeding and start of endometrial regeneration (Freitas *et al.* 1999).

In the case of abnormal bleeding it acts differently. As shown by the results of the studies conducted in AUB patients, TIMP-1 expression in the endometrium was not increased. Expression was not affected by estradiol concentration. The results of our study are confirmed by the results of in vitro studies showing no correlation of TIMP-1 protein expression from estradiol concentration (Brenner & Slayden 2012). No correlation with progesterone concentrations was found neither. Other available studies of the expression dependence on progesterone concentrations mainly describe TIMP-1 mRNAs and similarly to our results indicate lack of significant dependence (Curry *et al.* 2001; Rodgers *et al.* 1994; Goffin *et al.* 2003). On the other side in the endometrium of women using depot medroxyprogesterone, a significantly less TIMP-1 was observed in immunostaining compared to perimenstrual endometria from normal cycling women and no correlation of its concentration with bleeding length was found (Vincent *et al.* 2002).

Similarly in our group in women with AUB, irrespective of histopathological diagnosis, there was no correlation between TIMP-1 expression and bleeding length. It has been previously shown that under physiological conditions the increase in TIMP-1 expression is induced by a dramatic decrease in the concentration of steroid hormones that induce menstrual bleeding (Hampton *et al.* 1995). AUB is not usually associated

with such a rapid and simultaneous decrease in estradiol and progesterone concentrations as occurs just prior to the onset of regular menstrual bleeding. This may explain the lack of difference in TIMP-1 activity during abnormal bleeding compared to normal cycles. We postulate that the persistence of TIMP-1 activity at constant levels with increasing MMP-2 activity may determine the duration of abnormal bleeding. Those results indicate the importance of MMP to TIMP ratio balance in maintaining endometrial integrity and in pathomechanism of abnormal bleeding.

The results of this study suggest an explanation for the efficacy of treatment of abnormal bleeding resulting from existing hormonal disorders, including progestogen-containing formulations that decrease MMP-2 activity during bleeding. Their abrupt withdrawal stimulates TIMP-1 activity and restores endometrial remodeling on the normal pathway.

CONCLUSION

ProMMP-2 and MMP-2 activity in endometrium of women with abnormal bleeding are not correlated. ProMMP-2 expression during bleeding is affected by steroid hormones. It is raising along with estradiol and progesterone concentrations and it's levels are significantly higher in patients with diagnosis of hormonal endometrial dysfunction.

MMP-2 expression in endometrium in women with abnormal bleeding is not dependent on estradiol and progesterone concentrations. Its concentration is raising with the length of bleeding which suggest important role of paracrine factors in its activation.

TIMP-1 expression in the endometrium in case of abnormal bleeding is not affected by hormone levels, bleeding length or histopathological cause.

The inhibitory effect of progesterone on the expression of proMMP-2 and increase in MMP-2 activity along with the length of bleeding may indicate the efficacy of progestagens treatment for abnormal bleeding caused by hormonal disturbances only in the case of short-term bleeding. This conclusion however needs to be confirmed by further clinical studies.

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