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Expression of membrane-type 5 matrix metalloproteinase in human endometrium and endometriosis

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Abstract

Background. The metalloproteinases (MMPs) are a family of proteolytic enzymes involved in tissue remodeling and cell migration. Endometrial tissue remodeling proceeds during the menstrual cycle and requires a temporary and spatially balanced expression of several different MMPs. Various members of the MMPs also seem to play an important role in the invasion process of endometriosis; however, so far only a limited number of studies have focused on membrane-associated MMPs.

Methods. The present study investigated the expression of membrane-type 5 metalloproteinase (MT5-MMP) in the human endometrium and endometriotic lesions by microarray hybridization, real-time polymerase chain reaction (PCR) and immunofluorescence.

Results. Both the gene chip expression analyses as well as PCR indicated expression of MT5-MMP in normal human endometrium and strongly elevated transcript levels in most peritoneal endometriosis lesions analyzed. Moreover we detected enhanced MT5-MMP expression in the eutopic endometrium from patients suffering from endometriosis, further supporting a role of MT5-MMP in the formation of endometriosis. Immunohistochemical analysis was used to determine the intracellular localization and tissue distribution of MT5-MMP. While the MT5-MMP antigen expression could be clearly attributed to the membrane of epithelial cells, a highly complex differential immunohistochemical staining of MT5-MMP in the various compartments of endometrial tissue was observed. The strongest staining was seen in luminal epithelial cells, whereas endometrial glands frequently showed partial expression of MT5-MMP.

Conclusion. Our microarray analysis and real-time PCR of MT5-MMP transcripts may point to an elevated tissue remodeling and cell migration in endometrium from endometriosis patients as implied by the function of related MMPs.

Keywords: *Endometrium, metalloproteinases, membrane-type 5 metalloproteinase, microarray*

Introduction

The metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes including collagenases, gelatinases, stromelysins and membrane-type proteinases. Membrane-type 5 metalloproteinase (MT5-MMP) contains a transmembrane domain that localizes the protein to the cell membrane [1]. The MMPs and their associated endogenous inhibitors – tissue inhibitors of metalloproteinases, serum panproteinase inhibitors – are implicated in normal and pathologic tissue remodeling processes through regulation of the composition and turnover of the extracellular matrix [2]. In human endometrium, the MMP system plays an important role in menstrual breakdown, in re-epithelialization of the luminal endometrial surface at the beginning of the new cycle, elongation of glandular tubes, implantation and placentation [3,4]. Alteration of MMP expression has been demonstrated in patients with endometrial bleeding disorders and endometriosis, and in patients with endometrial carcinogenesis [5–8]. Progesterone and – in response to ovarian steroids – a variety of growth factors and cytokines influence the expression of MMPs. Cyclic changes in various MMPs have therefore been described in several reports.

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The present study investigated the expression of MT5-MMP in human endometrium and demonstrated an increased mRNA expression in peritoneal endometriosis tissue as well as in eutopic endometrium from patients suffering from endometriosis. In addition, differential immunohistochemical staining of MT5-MMP in the various compartments of endometrial tissue was observed.

Methods

Materials

Endometrial biopsies were taken from patients undergoing hysterectomy, dilation and curettage, or other procedures for benign gynecological diseases. The study was approved by the local ethics committee. Polyclonal antibodies directed against MT5-MMP were obtained from Abcam Ltd, Cambridge (UK). Secondary Cy3-conjugated goat anti-rabbit antibody was purchased from Dianova (Hamburg, Germany).

Microarray analysis

Microarray analyses were carried out on peritoneal endometriosis samples ($n=3$) as well as eutopic endometrium ($n=3$) and peritoneal samples ($n=3$) from patients without endometriosis. The clinical diagnoses of the endometriosis patients were based on the revised criteria of the American Fertility Society – rAFS grade I ($n=2$) and rAFS grade III ($n=1$); diagnoses were uterus myomatosis ($n=2$) and cervical intraepithelial neoplasia grade III ($n=1$) for the endometrium group; and uterus myomatosis ($n=2$) and sterility ($n=1$) for the peritoneal samples. RNA preparation and microarray analysis using the Affymetrix Human Genome U133A GeneChip platform (Affymetrix, Inc., Santa Clara, CA, USA), containing 22,283 probes, was performed as described elsewhere [9]. Hybridization intensity data were automatically acquired and processed using the Affymetrix Microarray Suite 5.0 program. The expression level of each gene was determined by calculating the average differences in intensity (perfect match – mismatch) between its probe pairs. Scans were rejected if the scaling factor exceeded 2 or a 'chip surface scan' revealed scratches, specks or gradients affecting the overall data quality (Refiner; GeneData, Inc., Basle, Switzerland).

The expression data were subsequently analyzed using the Cluster and Treeview software package [10]. Prior to cluster analysis, gene chip expression values were adjusted by log transformation and median centering of the gene chips. Hierarchical gene clustering was carried out using the Pearson correlation as distance metric and average linkage clustering.

Real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) analysis was carried out on samples from peritoneal endometriosis ($n=12$), as well as eutopic endometrium samples from patients without endometriosis ($n=8$), with peritoneal endometriosis ($n=5$) and with adenomyosis uteri ($n=2$). RNA extraction and real-time PCR analyses were performed as described previously [11] using the ABI 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA, USA). A pre-developed assay on demand (PDA) was applied to detect MT5-MMP mRNA (assay ID: Hs00198580_m1; PE-Applied Biosystems). cDNAs for all PCRs were generated by random primed reverse transcription (RT) (ProSTAR cDNA synthesis kit; Stratagene, La Jolla, CA, USA). PCR reactions were carried out in accordance with the manufacturer's protocols (PE-Applied Biosystems). VIC-fluorophore-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan probes served as internal quantification markers in multiplex PCR reactions. Each quantification was reproduced three times and normalized by GAPDH using the $\Delta\Delta c_t$ method.

Immunofluorescence

The samples used for immunofluorescence included five eutopic and ectopic endometrium tissues from patients with endometriosis (rAFS grade III/IV, $n=3$; rAFS grade I, $n=1$; and adenomyosis uteri, $n=1$) as well as 14 endometrium samples from patients without endometriosis. The clinical diagnoses of these latter patients were intraepithelial cervical dysplasia ($n=3$), uterine fibroids ($n=6$) and bleeding disorders ($n=5$). Immunofluorescence was carried out on samples quick-frozen in liquid nitrogen. Tissue sections of 8 μm thickness were cut and mounted directly on Superfrost Plus slides. Tissue sections were fixed by incubation in absolute methanol for 5 min (-20°C), air-dried and subsequently incubated with the primary antibody (1:100 dilution in $1\times$phosphate-buffered saline (PBS) containing 10% fetal calf serum) at 4°C overnight. After three rounds of washing, the secondary antibody was added at a dilution of 1:100 and incubated for 1 h at room temperature. Unbound Cy3-labeled antibody was removed by three rounds of washing with $1\times$PBS containing 0.1% Tween 20. Fluorescence was detected using a Zeiss Axiovert microscope.

Statistical analyses

All reported p values are two-sided, with p values of less than 0.05 considered to indicate a significant result. The Mann–Whitney test was used to compare

expression values between different tissues. All analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Increased expression of membrane-type 5 matrix metalloproteinase in endometriosis as detected by microarray analysis and quantitative polymerase chain reaction

In human endometrium, the MMP system plays an important role in menstrual breakdown as well as in re-epithelialization of the luminal endometrial surface at the beginning of the new cycle. Alteration of MMP expression has been demonstrated in patients with endometrial bleeding disorders, endometrial carcinogenesis, and in endometriosis. To gain further insight into the transcriptional changes that take place before and after the development of endometriosis, we carried out microarray analysis of endometrial tissue, normal peritoneum and peritoneal endometriosis lesions (see Methods section). Using cluster analysis, we identified several candidate marker genes indicated to be specifically upregulated in endometriosis tissues (Table I). The principal plausibility of this approach was validated by the identification of MMP9 from these microarray analyses, which has been previously described to be elevated in expression in endometriosis [12]. Among the identified genes we detected the MT5-MMP metalloproteinase, which showed an 8.4-fold higher expression in endometriotic lesions in comparison with endometrium but only a slight elevation (1.4-fold) in peritoneum biopsies (Table I). Since the microarray profiling encompassed only a limited number of samples, we next assembled a larger tissue-sample group and carried out expression analysis of MT5-MMP using quantitative real-time PCR. Table II gives the results of these analyses. We observed higher mRNA expression of MT5-MMP in peritoneal endometriosis tissue ($n = 12$) compared with eutopic endometrium ($n = 15$; $p = 0.001$, Mann–Whitney test using Δc_t values of MT-MM5 compared with GAPDH). Interestingly, as shown in Figure 1, we also detected higher MT5-MMP expression in the endometrium from patients suffering from endometriosis ($n = 7$) vs. patients without endometriosis ($n = 8$; $p = 0.04$, Mann–Whitney test), supporting a role of MT5-MMP in the process of endometrial cell detachment and invasion of pelvic organs. Two endometrium samples from patients with adenomyosis uteri were included in these analyses. When these patients were excluded from the comparisons still higher MT5-MMP expression in endometriosis tissue ($n = 12$) compared with eutopic endometrium was observed ($n = 13$; $p = 0.001$). However, the trend of higher expression in endometrium from the five patients with peritoneal endometriosis vs. patients without endometriosis ($n = 8$) was no longer significant ($p = 0.1$).

Table I. Candidate marker genes differentially expressed in endometriosis tissue as identified by microarray analysis.

		Endometriosis vs. endometrium		Peritoneum vs. endometrium	
Gene	Affymetrix ID	Fold difference	SD	Fold difference	SD
Genes upregulated in endometriosis (> 5-fold difference)					
SMR3B	207441_at	51.6	1.9	2.3	1.2
NELL1	206089_at	23.8	1.7	1.7	0.7
IGSF4B	221921_s_at	23.3	2.1	4.2	0.8
TREM2	219725_at	18.6	3.3	0.5	1.3
GALNT14	219271_at	16.3	1.3	1.1	0.3
APLP1	209462_at	9.7	1.6	0.7	0.4
MT-MMP5	213171_s_at	8.4	1.2	1.4	0.5
PKIA	204612_at	7.4	1.5	1.4	0.3
GSTA1	203924_at	7.0	1.9	1.7	0.5
ZNF287	216710_x_at	6.8	1.2	0.9	0.2
IGSF4B	211677_x_at	5.6	1.3	1.7	0.5
CHRM3	214596_at	5.3	1.7	1.8	0.5
RARRES1	206391_at	5.3	1.4	1.9	0.3
C20orf7	219524_s_at	5.2	2.0	1.7	0.6
*MMP9**	203936_s_at	3.7*	1.4	1.2	0.3
Genes downregulated in endometriosis vs. endometrium (> 5-fold difference)					
AIPL1	219977_at	0.2	1.2	0.9	0.2
RPL12L2	216354_at	0.1	1.1	0.8	0.2

SD, standard deviation; *MMP9, which was reported previously to be differentially expressed, is included in the table despite not meeting the criterion of a 5-fold difference in expression.

Immunofluorescence analysis identifies epithelial cells as the source of membrane-type 5 matrix metalloproteinase expression

Endometrial specimens revealed immunostaining of cell membranes for MT5-MMP but varied widely in intensity and completeness of endometrial gland positivity. In general, MT5-MMP antigen expression was restricted to epithelial cells. The strongest staining was seen in luminal epithelial cells (Figure 2A). Endometrial glands frequently showed partial expression. MT5-MMP-negative glands and completely positive glands (Figure 2B) were seen in the same endometrial specimen, as well as glands with both strongly and weakly expressing epithelial cells (Figure 2C, arrows). Luminal epithelial cells and about half of the glandular MT5-MMP-positive epithelial cells showed circular immunostaining of the cell membrane, whereas in the other half of the glandular MT5-MMP-positive epithelial cells, expression was localized at the apical cell membrane (Figure 2D). Only in one case a weak immunostaining of endometrial stromal cells (less than 5% of the total number of stromal cells) and endometrial

Table II. Analysis of membrane-type 5 metalloproteinase (MT5-MMP) mRNA expression using real-time polymerase chain reaction.

Δc_t (MT-MMP5 – GAPDH)	c_t Value, MT-MMP5	Clinical diagnosis	Phase
Peritoneal endometriosis			
1.7	30.8	Endometriosis rAFS I	proliferative
6.7	28.1	Endometriosis rAFS I	proliferative
3.1	31.6	Endometriosis rAFS I	proliferative
6.6	28.0	Endometriosis rAFS I	oral contraceptive
6.8	30.0	Endometriosis rAFS I	oral contraceptive
5.5	30.0	Endometriosis rAFS I + ovarian cyst	proliferative
3.7	27.8	Endometriosis rAFS I + ovarian cyst	oral contraceptive
6.7	26.0	Endometriosis rAFS III/IV	secretory
4.6	27.0	Endometriosis rAFS III/IV	oral contraceptive
5.6	29.0	Endometriosis rAFS III/IV	NA
7.8	29.9	Endometriosis rAFS III/IV	NA
6.4	27.8	Endometriosis rAFS III/IV	oral contraceptive
Endometrium from patients without endometriosis			
10.2	26.7	Infertility	proliferative
8.7	30.1	Infertility	proliferative
10.8	32.0	Uterine fibroids	NA
10.7	29.2	Uterine fibroids	secretory
9.6	28.7	Intraepithelial cervical dysplasia	proliferative
11.7	29.4	Intraepithelial cervical dysplasia	proliferative
10.8	29.4	Intraepithelial cervical dysplasia	proliferative
10.2	27.4	Chronic pelvic pain	secretory
Endometrium from endometriosis patients			
7.6	28.4	Endometriosis rAFS I	proliferative
8.9	27.4	Endometriosis rAFS III/IV	proliferative
12.2	31.8	Endometriosis rAFS III/IV	proliferative
4.9	24.8	Endometriosis rAFS III/IV	secretory
4.6	28.3	Endometriosis rAFS III/IV	proliferative
9.3	26.9	Adenomyosis uteri	oral contraceptive
5.9	24.0	Adenomyosis uteri	secretory

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rAFS, revised criteria of the American Fertility Society; NA = not available.

vessels was found. The proportion of stained glands in 18 of the 19 analyzed endometrium samples ranged from 0 to 50%. Only in one case staining of almost all glands was observed. No significant correlation was detected between the proportion of stained glands, as well as the type of staining pattern of the endometrium described above and shown in Figure 2, and the diagnosis of the patient, nor was any menstrual cycle-dependency detected (Table III).

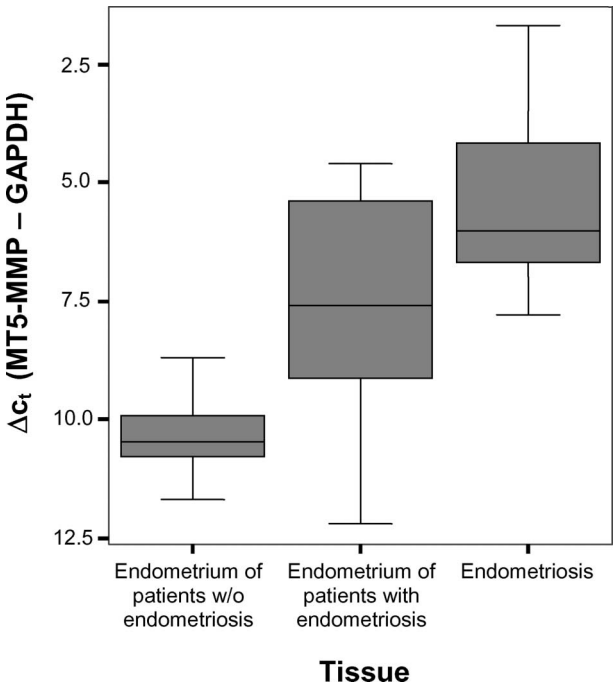

Figure 1. Membrane-type 5 metalloproteinase (MT5-MMP) mRNA expression in eutopic and ectopic endometrium as determined by quantitative real-time polymerase chain reaction (PCR); MT5-MMP mRNA expression in the endometrium from patients without endometriosis ($n = 8$) vs. endometrium from patients suffering from endometriosis ($n = 7$) and endometriosis lesions ($n = 12$). Box plots of the Δc_t values from quantitative real-time PCR analyses (normalized to glyceraldehyde-3-phosphate dehydrogenase, GAPDH) as given in Table II are shown.

Elevated membrane-type 5 matrix metalloproteinase protein expression in peritoneal endometriosis tissue

The enhanced transcription of *MT5-MMP* gene observed in microarray and PCR analysis of endometriosis tissues was also detected on the protein level, indicating translational processing of the corresponding mRNA. As shown in Figure 3, a very intense staining of glandular cells was prominent in each peritoneal endometriosis lesion analyzed. Although upregulation of MT5-MMP in ectopic endometrium was evident when comparing sections of eutopic and ectopic endometrium, we failed to accurately quantify differences in protein levels. This was largely due to the complex staining pattern with partial stained tubes and spotted accumulation of the antigen which did not allow a robust quantification.

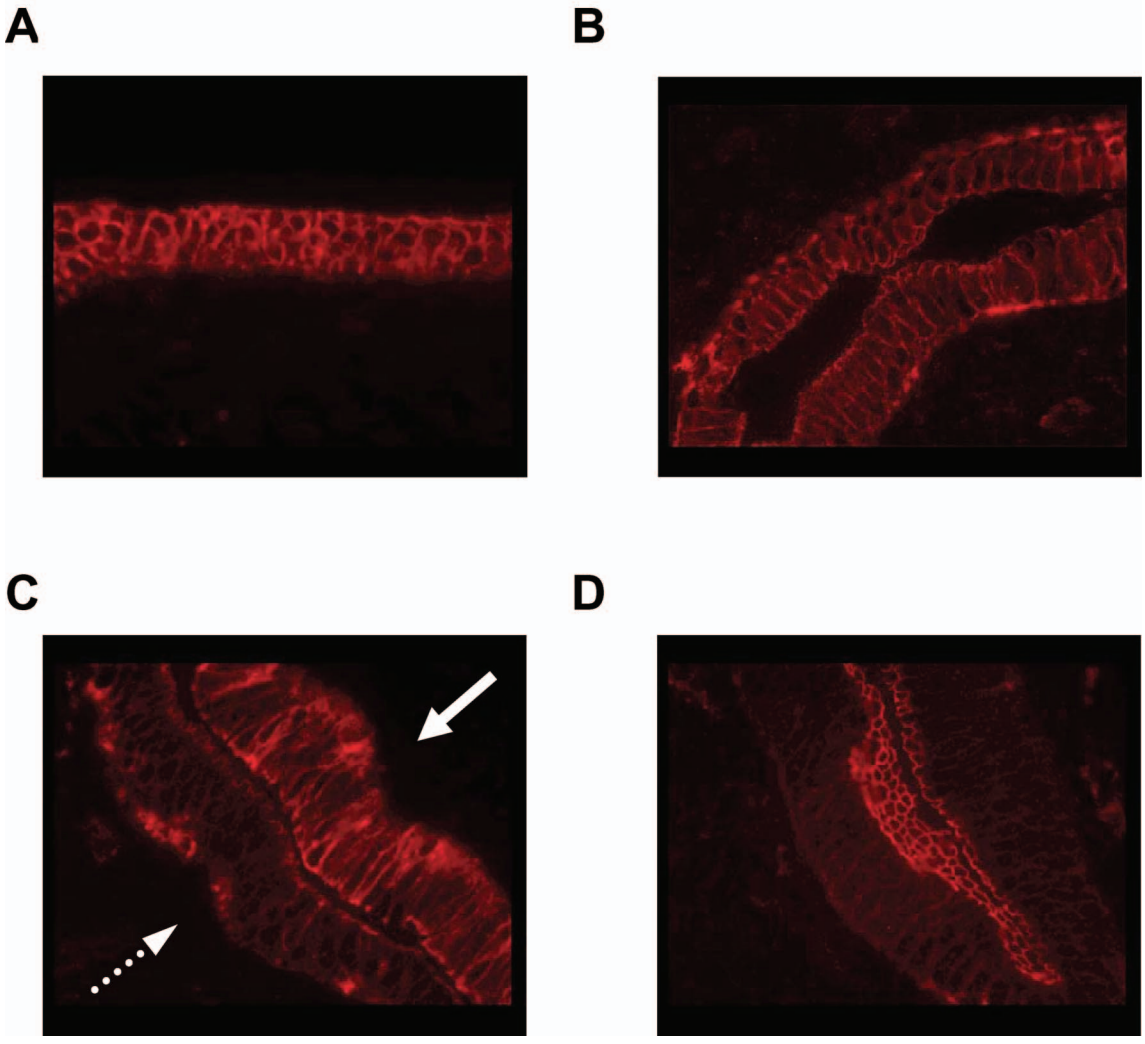

Figure 2. Expression of membrane-type 5 metalloproteinase (MT5-MMP) in human endometrium. Luminal endometrial epithelial cells (A) and endometrial glands (B) showing strong immunostaining of the cell membrane; endometrial gland with epithelial cells revealing strong expression of MT5-MMP on one side of the gland (solid arrow) and weak expression on the opposite side (dotted arrow) (C); and endometrial glandular epithelial cells showing strong staining of the apical cell membrane (D).

Table III. Analysis of membrane-type 5 metalloproteinase antigen expression in glandular epithelial cells using immunofluorescence.

Histology	Positive/total specimens*
Total	15/19
Proliferative phase	5/6
Secretory phase	5/7
Atrophic endometrium	2/2
Hyperplasia	3/4

*The number of specimens with positive immunostaining out of the total number of samples analyzed.

Discussion

The MMPs, through their regulation of the extracellular matrix, play a decisive role in embryonic development, wound healing processes, angiogenesis, and also in tumor diseases. Their cycle-dependent expression reported in the literature is the basis for the hypothesis that MMPs play an important role in the dynamic changes in the endometrium associated with the menstrual cycle and pregnancy. Relatively little is known as yet about the expression and function of MT-MMPs in the human endometrium. Recently it was suggested that membrane-type 2, 3 and 4 MMPs correlate with the angiogenesis involved in cyclic endometrial reconstruction [4].

In the present study we analyzed MT5-MMP in the human endometrium. Expression of the MMP was demonstrated in approximately three-quarters (15 of 19) of the endometrial biopsies examined using immunofluorescence (Table III). However, in those endometrial biopsies that showed positive immunostaining only a limited number of endometrial cells showed MT5-MMP expression. The proportion of positive epithelial cells in the total cell population varied between 0 and 50% of the glandular epithelial cells in the various biopsy

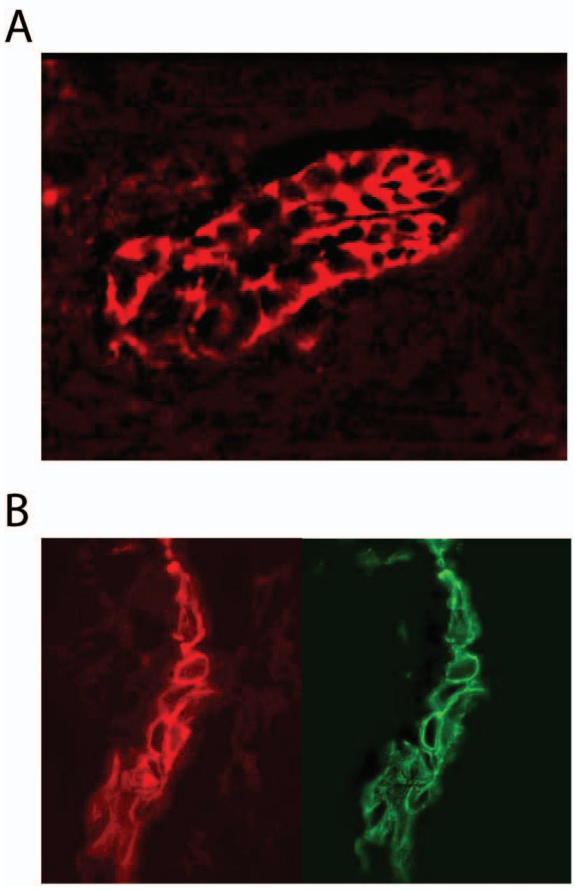

Figure 3. Expression of membrane-type 5 metalloproteinase (MT5-MMP) in peritoneal endometriosis tissue. Immunohistochemical analysis of MT5-MMP in two different endometriosis samples (A and B). Red fluorescence indicates the sites of MT5-MMP localization; green fluorescence marks the sites of cytokeratin (control).

samples. Despite the highly diverse MT5-MMP immunostaining patterns among the different samples, both ectopic and eutopic endometrium samples from endometriosis patients showed significantly increased amounts of MT5-MMP transcription as determined independently by RT-PCR (Figure 1).

Little is currently known regarding the function of MT5-MMP in the endometrium. By analogy with other MMPs and their postulated role in the endometrium, it might be speculated that MT5-MMP may be involved in the re-epithelialization of the luminal endometrial surface, elongation of the glandular tubes, and menstrual breakdown. Plaisier and colleagues [4] describe cycle-dependent expression of MT-MMP types 1 to 6. Yet the authors found a reduced staining index for MT5-MMP only in the early proliferative phase, while MT5-MMP expression in the epithelial cells in other phases showed no further cycle-dependent differences. In our study we observed no significant correlation of MT5-MMP expression with the phase of the menstrual cycle. However, since none of the samples in our group investigated was from the early proliferation phase, the two studies are not directly comparable. The reduced MT5-MMP expression reported by Plaisier's group in the early proliferative phase, as well as our own data on cycle independence of MT5-MMP appear to make it unlikely that MT5-MMP plays a decisive role in the re-epithelialization of the endometrium after menstruation. Equally, the homogeneous expression of MT5-MMP in other phases of the cycle also argues against it having a function in menstrual breakdown. No evidence was found for a correlation between the immunohistochemical staining pattern of MT5-MMP and endometriosis, endometrial hyperplasia or existing bleeding disturbances. The strong immunostaining of MT5-MMP in the luminal epithelial cells, its weaker expression in the deeper endometrial layers, and its location at the apical cell boundaries in the endometrial glands, suggests that MT5-MMP may have a function related to pericellular proteolysis, which is associated with tasks involved in delimiting the surface characteristics of the endometrium.

In general, whereas the soluble MMPs are presumably responsible for extracellular matrix degradation over broad areas of tissue, membrane-bound MMPs are believed to act locally in the focal degradation of the pericellular extracellular matrix. Accordingly, these enzymes are associated with specific functions in proliferation, migration and invasion. Recent studies of MT5-MMP expression in the brain point to a requirement of this MMP for the migration of neuronal cells and the formation of neurites [13]. In this context, the strong expression of MT5-MMP observed in all analyzed endometriotic lesion could indicate an improved implantation and spreading of scattered endometrial cells during retrograde menstruation. However, it is not yet clear if the increased expression is induced by attachment of endometrial cells to the peritoneal surface or if the cells from the endometrium of endometriosis patients already express higher levels of MT5-MMP. By analyzing several endometrial samples from endometriosis patients, we detected in a number of specimens strongly elevated MT5-MMP expression compared with endometrium from patients without endometriosis. This is in line with studies from other groups reporting altered transcription of various MMPs in eutopic endometrium from endometriosis patients [14–16]. Since refluxed endometrial tissue in patients with endometriosis seems to be more prone to establish endometriotic lesions, the level of proteolytic activity may play an important role in the development of endometriosis.

In conclusion, the enhanced expression of MT5-MMP in the endometrial epithelial cells from women with endometriosis thus may have a role in the etiology of endometriosis by enhanced capability of

invasion and breakdown of the extracellular matrix in host tissue.

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