Membrane-type 1 matrix metalloproteinase is induced in decidual stroma without direct invasion by trophoblasts

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Membrane-type 1 matrix metalloproteinase (MT1-MMP) in endometrium and decidua may greatly affect attachment of the embryo to the epithelium, invasion of the trophoblast into the stroma, and extracellular matrix remodelling in the endometrium and decidua. We investigated the expression of this enzyme in normally cycling endometrium and in decidua associated with normal and tubal pregnancies at both the gene and protein level. Localization of expression (but not the overall level of expression), differed between endometrium and decidua parietalis and tubal pregnancy decidua.

MT1-MMP mRNA was expressed mainly in epithelium and only faintly in stroma throughout the menstrual cycle, while in decidua parietalis and tubal pregnancy decidua, this mRNA was expressed predominantly in stromal cells. MT1-MMP protein was detected in the epithelium alone throughout the menstrual cycle, while in decidua parietalis and tubal pregnancy decidua, it was detected in stromal cells as well as the epithelium. Since decidua showed altered expression in the absence of trophoblastic contact, trophoblast invasion may not directly affect MT1-MMP gene expression.

Key words: decidua/endometrium/matrix metalloproteinases/MT1-MMP/stromal cells

Introduction

Embryonic implantation, wound healing, and tumour invasion are processes requiring modification of the microenvironment of the extracellular matrix (ECM) (Hulboy et al., 1997; Werb et al., 1999). Several types of proteinases including serine proteinases, cysteine proteinases, asparatic proteinases, and metalloproteinases participate in this degradative process. To date, 18 matrix metalloproteinases (MMPs), a multigene family of metal ion-requiring enzymes, have been characterized and classified into five broad categories based on their domain structure, including a group of four recently identified membrane-type metalloproteinases (MT-MMPs) (Seiki, 1999). MT-MMPs are generally made up of six domains including a transmembrane (TM) domain and short cytoplasmic tail at the C-terminus (Sato et al., 1994; Cao et al., 1995; Seiki, 1999). The TM domain provides membrane linkage and is required for activation of proMMP-2 at the cell surface (Sato et al., 1994; Cao et al., 1995; Tokuraku et al., 1995). MT-MMPs also have a furin motif at the cleavage site involved in generation of mature enzymes (Nagase, 1997; Polette and Birembaut, 1998; Seiki, 1999). Activation of pro MMP-2 is thought to be associated with ECM remodelling, especially basement membrane remodelling, that occurs in healing of injured tissues as well as during invasion by malignant cells and trophoblasts.

MT1-MMP was identified and isolated from lung adenocarcinoma cells (Sato et al., 1994). MT1-MMP has a molecular weight of 63 kDa and is made up of 583 amino acid residues. So far, the expression of MT1-MMP mRNA has been demonstrated in many types of malignant tissues and also in non-malignant tissues, e.g. proliferating mesangial cells (Hayashi et al., 1998), stretched cardiac fibroblasts (Tyagi et al., 1998), the cells of granulation tissue during wound healing (Madlener et al., 1998), and osteoclasts carrying out bone resorption (Pap et al., 1999). MT1-MMP mRNA has also been detected in placental tissues (Nawrocki et al., 1996; Bjorn et al., 1997) and fetal membranes (Fortunato et al., 1998). While cells of the decidua basalis are reported to express MT1-MMP mRNA and protein (Nawrocki et al., 1996), it has not been determined whether the changes in its expression in decidua require direct contact between trophoblastic and endometrial cells or whether a different mechanism is involved. We addressed this question using a quantitative reverse transcription–polymerase chain reaction (RT–PCR), in-situ hybridization and immunohistochemistry in endometrium and in decidua associated with normal and tubal pregnancies.
Materials and methods

**Specimens**

Human endometrial tissues were obtained from hysterectomy specimens from non-pregnant patients aged 43–48 years. The hysterectomies had been performed to treat leiomyoma or adenomyosis. Only histologically normal endometrium was included in the study. Endometrial samples were dated with respect to the menstrual cycle using histological criteria (Noyes et al., 1950); these were proliferative in 10 cases, early secretory (days 14–21) in four cases, and late secretory (days 22 or later) in four cases. With written consent, decidual samples were obtained from three women undergoing voluntary termination at 7–10 weeks gestation. In all cases, a fetal heartbeat was detected by transvaginal ultrasonography. With transabdominal ultrasonographic guidance, the samples were obtained from the decidua parietalis, which is located opposite from the placenta and has no contact with trophoblastic tissue. The absence of trophoblastic tissue was confirmed by immunostaining for cytokeratin. Decidual tissue from three tubal ectopic pregnancies was also studied. Again, immunostaining ruled out the presence of trophoblast. Samples to be studied by quantitative RT–PCR were frozen immediately in liquid nitrogen. When the volume of tissue samples was sufficient, a portion (n = 12; three each from proliferative endometrium, late secretory endometrium, normal decidua parietaris, and decidua from tubal pregnancy) was fixed in 4% paraformaldehyde for in-situ hybridization and immunohistochemistry. Use of the specimens was approved by the institutional review committees at Hiroshima University Hospital, Hiroshima Memorial Hospital, and Hiroshima Prefectural Hospital, Japan.

**Quantitative RT–PCR**

**RNA preparation and cDNA synthesis**

Frozen endometrial tissues were homogenized, and total RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). RNA concentration was measured spectrophotometrically in all samples. Complementary DNA (cDNA) was prepared by reverse transcription at 42°C for 60 min in 20 µl of reaction mixture containing 5 µg of total RNA, 0.5 µg of oligo (dT), and 200 IU of Moloney murine leukaemia virus (MMLV) reverse transcriptase (Life Technologies, Rockville, MD, USA) in 50 mmol/l KCl, 40 mmol/l Tris (pH 8.3), 4 mmol/l MgCl₂, 1 mmol/l dithiothreitol (DTT), and 1 mmol/l each of dATP, dGTP, dCTP, and dTTP. Transcription reactions were terminated by heating the samples at 70°C for 10 min.

**Primers**

Primers designed according to known nucleotide sequences were: MT1-MMP, 5' primer: 5'-ATG AAC ACT GCC TAC GAG AG-3' and 3' primer: 5'-GAG ACT TCA TCG CTG CCC AT-3' (nucleotides 1220–1540); and glyceraldehyde 3-phosphate dehydrogenase (G3PDH), 5' primer: 5'-TGA AGG TCG GAG TCA AGG GAT TTG GT-3' and 3' primer: 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (nucleotides 71–1053).

**PCR**

The G3PDH gene was used as an internal control, since it is ubiquitously expressed in most tissues. In initial experiments, the appropriate amount of template to obtain the exponential range of amplification for the G3PDH gene, was determined by sequential two-fold dilutions of solution containing 1 µl cDNA in sterile distilled water, resulting in dilutions ranging from 1:32 to 1:864. According to these initial results, the PCR reaction was performed in a total volume of 25 µl containing an aqueous 1:96 dilution of cDNA (equivalent to cDNA of 52.0 ng from RNA) in 2.5 µl of 10× PCR buffer (500 mmol/l KCl, 400 mmol/l Tris–HCl, pH 8.3 at 37°C, 25 mmol/l MgCl₂, and 100 µg/ml bovine serum albumin), 0.1 mmol/l each of dATP, dGTP, dCTP, and dTTP, 10 pmol each of 5' and 3' primers, and 1 IU of Taq polymerase (Perkin-Elmer Cetus; Fostor City, CA, USA). The amplification profile involved preincubation at 94°C for 5 min, then denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 1 min, for 24–32 cycles and, after the last cycle, all samples were incubated for an additional 10 min at 72°C.

**Quantification**

PCR products were electrophoresed in 0.5× Tris-borate electrophoresis buffer on a 1.2% agarose gel. Intensity of ethidium bromide luminescence was measured using a charge coupled device (CCD) image analyzer (Densitograph AE-6900-F; Atto, Tokyo, Japan).

**Subcloning and sequencing of MMP cDNA**

MT1-MMP and G3PDH cDNA fragments amplified by PCR were isolated from agarose gels, purified with a Gene Clean II (Funakoshi, Tokyo, Japan), and ligated into pBluescript SK(−) (Stratagene, La Jolla, CA, USA). Competent cells were transformed by introducing the ligated plasmid DNA with heat shock. The transformed bacteria were cultured overnight. By blue–white selection and a colony PCR method, transformed bacteria were selected and grown in liquid culture overnight. The ligated plasmid DNA was purified and its DNA sequences were validated with a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

**In-situ hybridization**

**Specimens**

Tissue samples were fixed immediately in 4% paraformaldehyde for 24 h. Sections (5 µm) were mounted on 3-aminopropyl-triethoxy silane-coated slides (Matsunami, Osaka, Japan).

**cRNA probe preparation**

cRNA probes for in-situ hybridization were prepared from MT1-MMP cDNA fragments generated by RT–PCR from human endometrial RNA and subcloned into Bluescript vectors (Stratagene). The plasmid containing MT1-MMP cDNA was linearized by BamH I and ClaI at a site suitable for transcription of cDNA. Linearized plasmids were used to construct probes using fluorescein-labelled uridine triphosphate by in vitro transcription with T3 and T7 RNA polymerase. The RNA probes were quantified by a spotting method by comparison with known amounts of fluorescein-labelled DNA.

**Pretreatment of specimens**

Tissue sections were deparaffinized with xylene, rehydrated in decreasing concentrations of ethanol, and rinsed in phosphate-buffered saline (PBS) containing 10% Tween-20 (PBST). The slides were fixed in 4% paraformaldehyde in PBS for 15 min and treated with proteinase K (1 µg/ml) at 37°C for 10 min, following by rinsing in PBS. The specimens were then incubated with chondroitin ABC lyase (0.02 IU/ml) in a buffer solution of 0.1 mol/l Tris–HCl (pH 8.0), 0.03 mol/l sodium acetate, and 0.05% bovine serum albumin at 37°C for 30 min. After rinsing in PBST, sections were rehydrated with xylene, rehydrated in decreas-
30 min with a mixture of 50% formamide and 2× SSC at 55°C, followed by incubation in a mixture of 10 mmol/l Tris–HCl (pH 8.0) and 500 mmol/l NaCl with 10 mg/ml RNase A for 30 min at 37°C. Sections were incubated with 0.5% of blocking reagent (Nucleic Acid Detection Kit; Boehringer Mannheim, Germany) in PBST for 1 h at room temperature and then were covered with alkaline phosphatase-conjugated anti-fluorescein antibody (dilution 1:2000) and 0.5% blocking reagent in PBST and allowed to react overnight at 4°C. After rinsing three times for 20 min with PBST including 2 mol/l EDTA; pH 8.0) at room temperature, sections were incubated in alkaline phosphatase buffer (0.1 mol/l NaCl, 0.05 mol/l MgCl2, 0.1 mol/l Tris–HCl, pH 9.5, 0.1% Tween-20, 2 mmol/l levamisole) for 5 min at room temperature. The colour reaction was developed with Nitroblue Tetrazolium-4-romo-3-chloro-indolyl phosphate (NBT-BCIP) in alkaline phosphatase reaction buffer in darkness overnight. Sections then were rinsed twice for 15 min with 1× TE (0.01 mol/l Tris–HCl; pH 8.0, 0.01 mol/l EDTA; pH 8.0) at room temperature, fixed in 4% paraformaldehyde in PBS for 30 min, and finally washed twice for 10 min in 1× TE. Sections were not counterstained.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated by routine methods. Sections then were heated to 90°C in a microwave oven in 10 mmol/l citrate buffer for 18 min, followed by cooling in the buffer for 20 min at room temperature. Sections then were incubated in methanol with 0.3% H2O2 for 10 min at room temperature to block endogenous peroxidase activity. Immunohistochemical staining was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). After blocking non-specific reactivity with diluted normal horse serum, sections were incubated with 10 μg/ml purified mouse anti-human MT1-MMP monocolonal antibody (Fuji Chemical, Toyama City, Japan) for 18 h at 4°C or prediluted purified mouse anti-cytokeratin monocolonal antibody (Nichirei, Tokyo, Japan) for 1 h at room temperature. Next, sections were exposed to biotinylated anti-mouse immunoglobulin G (IgG; Vector Laboratories) for 30 min at room temperature, followed by incubation with avidin–biotin complex (ABC) reagent (Vector Laboratories) for 30 min at room temperature. These reactions were carried out in a humid chamber. Reaction products were visualized using 3-amino-9-ethylcarbazole in the presence of 0.003% H2O2, and sections were counterstained with haematoxylin before clearing and mounting. Negative control sections were processed by substituting non-immune serum for the primary antibody.

Epithelial and trophoblastic cells were identified by cytokeratin immunostaining. Decidualized stromal cells in normal and tubal pregnancy decidua were identified as relatively large, round, cytokeratin-negative cells.

**Statistical analysis**

Data were analysed by Wilcoxon’s test (non-paired). P < 0.05 was considered to be statistically significant.

**Results**

**Quantification of MT1-MMP gene expression by RT–PCR**

To quantitatively analyse the level of MT1-MMP mRNA, RT-PCR was performed for 24–32 cycles. PCR products increased exponentially with each cycle until they reached a plateau according to the electrophoretic pattern (Figure 1). G3PDH-specific PCR products increased similarly in all samples (Figure 1). The intensity of ethidium bromide luminescence for MT1-MMP- and G3PDH-specific PCR products was measured using a CCD image analyser and plotted semilogarithmically (Figure 2), with the vertical axis indicating densitometric units corresponding to staining intensity of PCR products and the horizontal axis indicating number of PCR cycles (Figure 2). Linearity of the amplification patterns of MT1-MMP and G3PDH gene PCR products was observed from 24 to 26 cycles. The PCR product was quantified at 26 cycles in the exponential range of amplification. Expression levels of MT1-MMP in secretory endometrium, decidua parietalis, and tubal pregnancy decidua did not differ significantly from those in proliferative endometrium (P > 0.05; Figure 3).

**In-situ hybridization**

Throughout the menstrual cycle, antisense probes disclosed that MT1-MMP mRNA was expressed mainly in the epithelium and was not observed in stromal cells (Figure 4A and C). Neither sense probes nor sections pretreated with RNAase yielded substantial background hybridization (Figure 4B and D). In the late secretory phase, the hybridization signal was similar in distribution and intensity to that in the proliferative phase. On the other hand, the hybridization signal was observed predominantly in stromal cells in both normal decidua parietalis and tubal pregnancy decidua (Figure 4E, F, H and I). Endothelial cells and vascular smooth muscle cells did not express MT1-MMP mRNA at any time during the menstrual cycle or early pregnancy. Neither sense probes nor sections pretreated with RNAase yielded substantial background hybridization (Figure 4G and J).

**Immunohistochemistry**

Only epithelial cells were immunoreactive for cytokeratin in normally cycling endometrium, decidua parietalis, and tubal pregnancy decidua (illustrated only for decidua parietalis, Figure 5G). Throughout the menstrual cycle, epithelial cells stained for MT1-MMP protein (Figure 5A and B), while stromal cells in normally cycling endometrium showed no staining. In decidua parietalis, both epithelial cells and stromal cells stained for MT1-MMP (Figure 5C and E). The same was true for tubal pregnancy decidua (Figure 5D and F).

**Figure 1.** Agarose gel electrophoresis of polymerase chain reaction (PCR) products from membrane-type 1 matrix metalloproteinase (MT1-MMP) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA. The figure shows the result of human proliferative endometrium. MT1-MMP and G3PDH were detected as 320 and 982 bp products respectively. Lanes 1–9 = PCR performed or 24–32 cycles at one cycle intervals. We also investigated MT1-MMP and G3PDH mRNA in other human samples, including early secretory endometrium, late secretory endometrium, decidua parietalis and tubal pregnancy decidua and obtained similar results to those in this study. MW = molecular weight makers of 100 bp ladder.
Figure 2. Semilogarithmic plot of the intensity of polymerase chain reaction (PCR) products versus the number of PCR cycles in different tissues. (A) Exponential linearity of amplification pattern of membrane-type 1 matrix metalloproteinase (MT1-MMP) PCR products was observed in 24–26 cycles. (B) A similar pattern was seen for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) PCR products.

Figure 3. Ratio of densitometric units of membrane-type 1 matrix metalloproteinase (MT1-MMP) mRNA to glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA in normally cycling endometrium, decidua parietalis and tubal pregnancy decidua. The relative expression level of MT1-MMP did not vary greatly between these tissues.

discussion

The two most important findings in this report are as follows. Firstly, expression of MT1-MMP mRNA did not show any quantitative changes in endometrium during the normal menstrual cycle, or in decidua parietalis or tubal pregnancy decidua. Secondly, localization of MT1-MMP mRNA and protein in these tissues differed dramatically between cycling endometrium and decidua in the absence of direct contact with trophoblastic cells.

The expression level of MT1-MMP mRNA in endometrium did not alter during the menstrual cycle, similar to previous conclusions (Rodgers et al., 1994) from a qualitative study with respect to MMP-2 mRNA. MT1-MMP mRNA was expressed mainly in the epithelium with only minimal stromal expression throughout the menstrual cycle. The mRNA hybridization signal, however, was detected clearly in stromal cells in decidua parietalis and in tubal pregnancy decidua. The mRNA hybridization signal seemed to be increased in stroma of early pregnancy decidua compared with that in epithelium of normally cycling endometrium. However, the number of positive cells counted was found to be similar between epithelium of normally cycling endometrium and stroma of early pregnancy decidua. Moreover, each cell showed the same strength mRNA hybridization signal in epithelium as that in stroma. Similar results were recognized in other parts of tissue samples. These findings seem to show that the RT–PCR result is compatible with that of in-situ hybridization.

With regard to the localization of MT1-MMP protein, MT1-MMP immunoreactivity was detected in the epithelial cells alone throughout the menstrual cycles. In decidua parietalis and tubal pregnancy decidua, MT1-MMP protein was detected in stromal as well as epithelial cells. In contrast to hybridization studies, immunohistochemistry detected MT1-MMP protein in both stromal and epithelial cells. The difference in localization between MT1-MMP mRNA and protein may reflect a low-level expression in epithelial cells of early pregnancy decidua which, as in the case of tumour cells, is undetectable by in-situ hybridization (Polette and Birembaut, 1998).

Throughout the process of implantation and early placentation, trophoblastic cells penetrate the basement membrane of epithelium and invade the stromal compartment of the uterus. Penetration ability is mediated by MMPs in a manner resembling events in malignant tumour invasion (Yagel et al., 1988; Burrows et al., 1996; Lala and Hamilton, 1996). Expression of MT1-MMP on cell surfaces may greatly enhance...
the localized pericellular degradation of extracellular matrix macromolecules during cell migration (Nagase, 1997). If so, proMMP-2 secreted by trophoblast could be activated by MT1-MMP in the epithelium. The activated MMP-2 would degrade the basement membrane of the epithelium. An embryo attached to the epithelium would continue to secrete proMMP-2 which would be activated by MT1-MMP in decidualizing cells. These processes would allow trophoblasts and the embryo to degrade the extracellular matrix surrounding decidualized cells and invade the stroma.

A few studies have examined the transcriptional regulation of MT1-MMPs. Growth factors and cytokines, e.g. interleukin-1β, epidermal growth factor, basic fibroblast growth factor, and transforming growth factor-β, negligibly affect mRNA levels of MT1-MMP, although transforming growth factor-α slightly increases MT1-MMP gene expression in HT-1080 cells (Lohi et al., 1996). In our study, expression of MT1-MMP mRNA and protein was localized in epithelial cells throughout the menstrual cycle, while in decidua the localization of MT1-MMP mRNA and protein shifted from epithelial cells to stromal cells in no direct contact with trophoblasts. MT1-MMP mRNA and protein are also reported to be expressed in stromal cells of decidua vera, with which trophoblasts are in direct contact (Nawrocki et al., 1996). Our results showed that the

Figure 4. In-situ hybridization of membrane-type 1 matrix metalloproteinase (MT1-MMP) mRNA in the endometrium and decidua. (A and B) Proliferative endometrium; (C and D) late secretory endometrium; (E, F and G) decidua parietalis; (H, I and J) tubal pregnancy decidua. (A, C, E, F, H, and I) Samples were hybridized with an MT1-MMP antisense mRNA probe. The hybridization signal was positive in the cytoplasm (arrow) but negative in the nucleus (arrowhead). (B, D, G and J) Samples were hybridized with an MT1-MMP sense mRNA probe. Scale bar = 25 µm.
MT1-MMP mRNA is expressed in certain tissues in the process of healing from injury or remodelling of the extracellular matrices ECM (Hayashi et al., 1998; Madlener et al., 1998; Pap et al., 1998; Tyagi et al., 1998). Interestingly, MT1-MMP mRNA exhibited a distribution similar to that of α-smooth muscle actin in proliferating mesangial cells and fibroblasts in granulation tissue (Hayashi et al., 1998; Madlener et al., 1998), and α-smooth muscle actin is also induced in endometrial stromal cells in the process of decidualization (Oliver et al., 1999). These changes in α-smooth muscle actin in endometrial stromal cells, mesangial cells, and granulation tissue cells appear to be closely associated with MT1-MMP gene expression.

The functions and substrates of MT1-MMP are complex, varied, and not completely known. However, MT1-MMP
appears to play a dual role in extracellular matrix remodelling through activation of proMMP-2 and proMMP-13, and on the other hand through direct cleavage of extracellular matrix components, e.g. collagen I, II, III, fibronectin, laminin-1, vitronectin, and dermanan sulphate proteoglycan (Nagase, 1997; Seiki, 1999). Activation of proMMP-2 by MT1-MMP is the result of a very complicated process. Many factors including the pericellular concentration of TIMP-2 (Strongin et al., 1995; Butler et al., 1998; Zucker et al., 1998), plasmin activity for autoproteolysis (Baramova et al., 1997; Mazzieri et al., 1997), or direct binding of the soluble catalytic domain of MT1-MMP to proMMP-2 (Sato et al., 1996) are directly or indirectly involved in this process. Our data showed that MT1-MMP was expressed in the endometrium throughout the menstrual cycle and early pregnancy, as has been shown for TIMP-2 and proMMP-2 (Rodgers et al., 1994; Nawrocki et al., 1996; Bjorn et al., 1997; Salamonsen et al., 1997). Simultaneous expression of MT1-MMP and MMP-2, however, does not in itself prove that co-expression results in activation of proMMP-2, as described above.

In summary, we studied the expression pattern of MT1-MMP in endometrium throughout the menstrual cycle and in early pregnancy by quantitative RT–PCR, in-situ hybridization, and immunohistochemistry. Our findings showed that even in decidua lacking trophoblastic contact, pregnancy shifted the localization of MT1-MMP mRNA and protein from epithelial cells to decidual stromal cells without significantly changing the overall level of MT1-MMP mRNA expression. These data indicate that MT1-MMP in the endometrium and decidua may be important for attachment of the embryo to the epithelium and invasion of the trophoblast into the stroma, as well as for ECM remodelling in the endometrium and decidua. In future studies we hope to verify the transcriptional regulation of MT1-MMP in the endometrium and decidua, and also to measure the activity of MMP-2 in vivo.

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