Identification and specific expression of matrix metalloproteinase-26 in rhesus monkey endometrium during early pregnancy

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Matrix metalloproteinases (MMPs) and their tissue inhibitors may play important roles in tissue remodelling processes of the uterus. This study identified MMP-26 (endometase/matriplysin-2) in the endometrium of pregnant rhesus monkeys (Macaca mulatta) and monitored the spatial and temporal expression of the transcript and protein in the uterus on days 12, 18 and 26 of pregnancy. The partial monkey MMP-26 gene sequence of 289 nucleotides was 98% identical to that of its human homologue and its protein fragment contained a PHCGVPDGDSD sequence in the prodomain identical to that in human MMP-26. RT–PCR analysis demonstrated that the average level of MMP-26 mRNA in the endometrium was high on day 12 of pregnancy, but significantly decreased on days 18 and 26 (P < 0.05). In-situ hybridization confirmed that MMP-26 mRNA is specifically localized in the endometrial compartments, with intense signals in the glandular epithelium on day 12 and in the walls of spiral arterioles adjacent to the implantation site on day 26. The hybridization signal for MMP-26 mRNA in the glandular epithelium decreased dramatically on day 18 and was undetectable on day 26. No MMP-26 mRNA transcripts were detected in the placental villi on days 18 and 26. Immunohistochemistry showed that the expression pattern of MMP-26 protein was similar to that of its mRNA. The restricted expression pattern of MMP-26 in the monkey uterus implies that this new MMP is involved in the highly regulated tissue remodelling processes of the glandular epithelium and spiral arteries during early pregnancy.

Key words: implantation/matrix metalloproteinase/placenta/primate/uterus

Introduction

Dramatic structural changes and extensive tissue remodelling take place in the endometrium during pregnancy, and experimental data are accumulating to suggest that matrix metalloproteinases (MMPs) are associated with embryonic implantation (Alexander et al., 1996; Menino et al., 1997; Hurst and Palmay, 1999; Bischof and Campana, 2000; Paria et al., 2000). Although at least 25 MMPs have been discovered to date, only a limited number have been demonstrated to be associated with the physiological processes of the female reproductive tract (Sternlicht and Werb, 2001). Among these matrix degrading proteases, the gelatinases, including gelatinase A (MMP-2) and gelatinase B (MMP-9), have been extensively investigated for their prominent roles in various reproductive processes (Turpeenniemi-Hujanen et al., 1995; Goto et al., 1999; Curry and Osteen, 2001; Riley et al., 2001). MMP-14 (MT1-MMP), an activator of proMMP-2, also participates in blastocyst implantation and in the development of placenta in the rhesus monkey (Wang et al., 2001).

It is both interesting and important to identify new MMPs involved in reproductive processes. The two newest members of the MMP family are MMP-26 and MMP-28 (de Coignac et al., 2000; Park et al., 2000; Lohi et al., 2001). MMP-26, also known as endometase or matriplysin-2, is the smallest of the MMP family with a molecular mass of 28 kDa (de Coignac et al., 2000; Park et al., 2000). It has a prodomain structure with a unique cysteine switch sequence, PHCGVPDGDSD, and a catalytic domain with the characteristic zinc-binding motif. The substrates of MMP-26 include rat-tail tendon type I gelatin, human plasma α1-proteinase inhibitor, type IV collagen, fibronectin, fibrinogen and vitronectin (Park et al., 2000; Uría and Lopez-Otin, 2000; Marchenko et al., 2001).

Interestingly, the MMP-26 gene was obtained from a human endometrial tumour cDNA library of more than three million expressed sequence tags (Park et al., 2000). Moreover, MMP-26 exhibits a restricted expression pattern in normal human tissues (de Coignac et al., 2000; Park et al., 2000; Marchenko et al., 2001); our previous study detected its mRNA only in the human uterus (Park et al., 2000), indicating that this new member may have a functional role specific to the uterus. However, little is known about the expression pattern of MMP-26 in the uterus. Therefore, in the current study we investigated the expression pattern of MMP-26 transcript and protein in the endometrial and placental compartments of the rhesus monkey uterus to further understand the role of MMP-26 in the primate uterus during early pregnancy.
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**Materials and methods**

*Animal and tissue preparation*

Twelve female rhesus monkeys (*Macaca mulatta*) with normal menstrual cycles and histories of pregnancy were obtained from the Center for Medical Primates, Institute of Medical Biology, Chinese Academy of Medical Sciences. Experimental protocols were approved by the ethical committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Pregnancy prediction and sample preparation were conducted as previously described (Wang et al., 2001). Uteri were obtained from rhesus monkeys under ketamine hydrochloride anaesthesia on days 12, 18 and 26 of pregnancy. The implantation site was then located, and the sample was divided into two parts. One part was embedded in OCT tissue freezing medium (Triangle Biochemical Sciences, Durham, NC, USA) for in-situ hybridization. Endometrium was isolated from the other parts of the sample by dissection with fine scissors (Rudolph-Owen et al., 1998), and the implantation site and placental villi were carefully excised. The endometrial samples were then quickly frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction.

*RT–PCR analysis of MMP-26 mRNA in macaque endometrium*

Total RNA was extracted from the endometrium of rhesus monkey with Trizol reagent (Gibco BRL Life Technologies Inc., Rockville, MD, USA) according to the manufacturer’s instructions. The cDNA was synthesized using Superscript II reverse transcriptase (Gibco) and oligo dT from 2 µg of total RNA. The reaction was carried out at 42°C for 50 min and 70°C for 10 min. In the PCR analysis, the cDNA was amplified by 25 cycles (denaturing at 95°C for 45 s, annealing at 55°C for 45 s and elongating at 72°C for 45 s) using MMP-26 primers (antisense primer 5'-GCCACGAGAACAAACGGCA-3' and sense primer 5'-TCCATCGGAATTGGGACACGAC-3') according to a previously described method (Marchenko et al., 2001). The 25 µl PCR system contained 2 µl of RT products, 200 µmol/l dNTPs, 2 mmol/l MgCl2, 1 IU Taq polymerase and 10 pmol of each MMP-26 primer. The anticipated size of the amplified fragment was 289 bp. A PCR system devoid of template cDNA (RNA replaced by water or reverse transcriptase omitted) was included as a negative control to exclude genomic DNA contamination. β-actin was amplified to confirm RNA integrity and efficacy of the reaction (primers were 5'-GGGCGCCCCAGCCACCA-3' and 5'-CTCCTTAATGTCAGCA-3') with an expected size of 548 bp.

Figure 1. RT–PCR analysis of MMP-26 mRNA transcript variation in the rhesus monkey endometrium during early pregnancy. (A) Representative RT–PCR products of MMP-26 and β-actin from the rhesus monkey endometrium on days 12 (D12), 18 (D18) and 26 (D26) respectively. M, low molecular weight nucleotide marker. (B) Graphical illustration of MMP-26 mRNA levels, as calculated by correcting for the levels of β-actin mRNA in each individual sample. Values are the mean ± SEM from three experiments. Bars with different letters on the top are significantly different (P < 0.05). (C) Alignment of the 289 nucleotide fragment of the rhesus monkey MMP-26 gene (GenBank acc. no. AY090610) amplified by PCR and the homologous region on human cDNA (GenBank acc. no. AF248646). The partial MMP-26 sequence is 98% identical to the corresponding fragment of human origin (from nucleotide 191 to 479). Four point mutations (shown in bold) were observed in the monkey gene fragment compared with its human homologue.
The PCR products were separated on a 1% (w/v) agarose gel containing ethidium bromide and then photographed. The bands were analysed using MetaView image analysing system (version 4.50; Universal Imaging Corp., USA). Furthermore, the amplified band was excised from the gel and purified using the CONCERT™ Rapid Gel Extraction system (Gibco). The purified fragment was inserted into pGEM-Z (Promega Corp., Madison, WI, USA). Some white colonies were identified by PCR using the above MMP-26 primers and the inserted nucleotide sequences of three colonies containing the RT–PCR products were determined (Sangon Corp., Shanghai, China). The partial MMP-26 fragments from four independent RT–PCRs were sequenced to ensure the accuracy of the gene sequence.

MMP-26 cDNA subcloning and cRNA probe labelling

The 742 bp MMP-26 cDNA fragment was cut from pCR 3.1 vector [constructed in our previous work (Park et al., 2000)] using EcoRI and EcoRV and was then subcloned into pGEM-3Z at EcoRI and BamHI restriction sites. To generate an antisense cRNA probe, the plasmid was linearized with EcoRI and transcribed in vitro with SP6 RNA polymerase (Promega); the sense probe was synthesized using BamHI and T7 RNA polymerase (Promega). The cRNA probes were labelled with digoxigenin (DIG) RNA labelling mix (Roche Molecular Biochemicals, Mannheim, Germany). In brief, the labelling process was carried out at 37°C for 2 h. Then, DNase I was added to remove template DNA. The riboprobes were precipitated with LiCl and ethanol. The probe labelling efficiency was confirmed by a dot assay, and the purified probes were stored in diethylpyrocarbonate (DEPC)-treated water at −80°C.

In-situ hybridization of pregnant macaque uterus

In-situ hybridization was performed according to the protocol developed by Braissant and Wahl with slight modifications (Braissant and Wahl, 1998). Frozen sections (10 μm) on poly-L-lysine-coated slides were quickly thawed and fixed in 4% paraformaldehyde (PFA) in DEPC-treated phosphate-buffered saline (PBS) for 15 min at room temperature. The slides were washed for 2 × 15 min in PBS with 0.1% active DEPC before equilibration in 5× standard saline citrate (SSC; DEPC-treated) for 15 min. Prehybridization was carried out at 56°C for 2 h in a buffer containing 50% formamide, 5× SSC and 120 μg/ml SDSNA (Sigma Chemical Co., St. Louis, MO, USA).

In a moist chamber, slides were then hybridized with 400 ng/ml of DIG-labeled probes in prehybridization buffer overnight at 56°C. The sections were serially washed in 2× SSC at room temperature for 30 min, 2× SSC at 65°C for 1 h, and 0.1× SSC at 65°C for 1 h. Slides were rinsed in buffer A (100 mmol/l Tris, 150 mmol/l NaCl, pH 7.5) and then incubated for 2 h with anti-DIG-alkaline phosphatase antibody (1:3000; Roche) in buffer B (buffer A containing 0.5% blocking reagent). The slides were further washed in buffer A for 2 × 15 min before equilibration in buffer C (100 mmol/l Tris, 100 mmol/l NaCl, 50 mmol/l MgCl2, pH 9.5) for 2 min.

The hybridization signal was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) reagents (Roche). To remove non-specific staining, slides were rinsed in 95% ethanol for 30 min. Sense probes were included in each hybridization to monitor the background level. The results were recorded with a SPOT digital camera system (Diagnostic Instruments, Inc., USA), and digital images were processed using Adobe PhotoShop (version 5.5; Adobe, San Jose, CA, USA).

Immunohistochemistry of pregnant macaque uterus

Anti-human MMP-26 polyclonal antibody was developed as detailed elsewhere (Park et al., 2000). Immunohistochemistry was performed using a Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) and diaminobenzidine according to the manufacturer’s instructions. In brief, cryosections (10 μm) were fixed for 10 min in 4% PFA in PBS and washed. The sections were serially incubated with normal blocking serum for 30 min at room temperature, primary antibodies, including mouse anti-vimentin/cytokeratin/actin (4 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and 8 μg/ml rabbit anti-MMP-26 IgG, in PBS containing 0.3% bovine serum albumin for 2 h at 37°C, and biotinylated secondary antibody for 30 min at room temperature. The slides were then treated with hydrogen peroxide (0.3% in methanol) for 30 min to eliminate endogenous peroxidase and incubated with ABC reagent for 30 min at room temperature. Intervening washes were performed after incubation when necessary. Negative controls were slides incubated without primary antibody or with preimmune rabbit serum.

Statistical analysis

The intensity of each band amplified by RT–PCR was normalized to that of β-actin mRNA in corresponding samples, and the MMP-26/β-actin ratios were found to be normally distributed. All values are presented as mean ± SEM. The statistical comparisons among groups were analysed by one-way analysis of variance, followed by Student’s t-test using SPSS (version 10.0.1; SPSS Inc., Chicago, IL, USA). P < 0.05 was considered significant.

Results

RT–PCR analysis of MMP-26 mRNA variation in rhesus monkey endometrium

To gain an overview of the expression and variation of total MMP-26 mRNA in the rhesus monkey endometrium at different stages of early pregnancy, MMP-26 mRNA transcripts were studied on days 12, 18 and 26 of pregnancy using RT–PCR. A representative illustration of the RT–PCR analysis is shown in Figure 1A. A 289 bp fragment was obtained in each sample. No specific band was amplified in the negative control, which lacked cDNA in the PCR system (data not shown). Computer-aided densitometric analysis of the amplified bands showed that the MMP-26 mRNA level was high on day 12 of pregnancy, but significantly lower on days 18 and 26 (P < 0.05, Figure 1B).

The amplified 289 nucleotide fragment was sequenced to confirm its identity (GenBank acc. no. AY090610), and the result showed it had 98% identity to the corresponding fragment of human origin (from nucleotide 191–479 of the human MMP-26 gene, GenBank acc. no. AF248646) (Park et al., 2000; Figure 1C). The nucleotide sequence from number C3 to C287 encodes monkey MMP-26 protein fragment corresponding to the human MMP-26 sequence between His62 and Trp156 (GenBank protein_id = ‘AAF82359.1’) (Park et al., 2000). The protein has the identical PHCGVPDGSD sequence in the prodomain to that in human MMP-26. Four point mutations were observed in the monkey gene fragment compared with its human homologue. They are also shown in Figure 1C and these differences correspond to human/monkey Ser93/Ser, His102/Tyr, Ala119/Thr and Gly145/Glu in their protein sequences.

Immunohistochemical staining of cytokeratin, actin and vimentin in the macaque uterus

Luminal and glandular epithelium were cytokeratin-positive. The arteries in the endometrium and the myometrium were visualized by immunohistochemical staining of actin. Villous mesenchyme was vimentin-positive, whereas synctiotrophicoblast and cytotrophoblast were vimentin-negative (data not shown).

MMP-26 mRNA expression in the pregnant rhesus monkey uterus

In-situ hybridization was performed to obtain a more detailed picture of the spatial and temporal expression of MMP-26 mRNA in the pregnant macaque uterus. Figure 2 shows the restricted expression pattern of MMP-26 mRNA in the glandular epithelium and spiral arteries of the rhesus monkey endometrium on days 12, 18 and 26 of pregnancy. On day 12 of pregnancy, MMP-26 mRNA was specifically and highly expressed in the glandular epithelium (Figure 2A), while on day 18 of pregnancy, the signal intensity in the glandular epithelium was decreased (Figure 2B). MMP-26 mRNA was undetectable in this compartment on day 26 (Figure 2C). Interestingly, MMP-26 mRNA was detected in the walls of some spiral arteries adjacent to the implantation site on days 18 and 26.
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Figure 2. In-situ hybridization of MMP-26 mRNA in the rhesus monkey uterus during early pregnancy. (A–C) Representative illustrations of MMP-26 hybridization signals in the uterine glandular epithelium of the rhesus monkey on days 12, 18 and 26 of pregnancy respectively. (D) Negative control of endometrium tissue from day 12 of pregnancy hybridized with sense probe. (E,F) MMP-26 mRNA expression in the spiral arterioles on days 18 and 26 of pregnancy respectively. (G,H) Placental villi from day 26 of pregnancy hybridized with anti-sense and sense probe respectively. ge = glandular epithelium; a = arteriole; d = decidual cells; pv = placental villi. All the pictures are of identical magnification (×100). Scale bar = 200 µm.

Immunohistochemical localization of MMP-26 protein in the pregnant rhesus monkey uterus

To further examine MMP-26 protein synthesis in the rhesus monkey uterus during early pregnancy, immunohistochemical analysis was included in the study. Representative results of immunostaining with anti-MMP-26 antibody are depicted in Figure 3. Strong MMP-26 immunostaining was visualized in the glandular epithelium on day 12 of pregnancy (Figure 3A). On day 18 of pregnancy, the level of immunoreactive MMP-26 in the glandular epithelium was less intense (Figure 3B), and staining was undetectable on day 26 (Figure 3C). Also in accordance with the result from in-situ hybridization, immunoreactive MMP-26 was detected in the walls of some spiral arterioles adjacent to the implantation site on day 26 (Figure 3D) and was not found in the placental villi (Figure 3E), decidual cells (Figure 3A–D,F) or myometrium (data not shown). Immunoglobulins of
preimmune rabbit serum at an equivalent concentration to that of the primary antibody did not produce significant immunostaining on the representative cryosection of macaque uterus from day 12 of pregnancy (Figure 3F).

Discussion

MMP-26 is a recently discovered member of the MMP family, and it was previously found to be specific to the uterus (Park et al., 2000) and cancer cells of epithelial origin (Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al., 2001). In an attempt to understand the role of MMP-26 in the primate uterus, especially during the period of early pregnancy, we investigated the expression pattern of MMP-26 transcript and protein in the rhesus monkey uterus on days 12, 18 and 26 of pregnancy by using RT–PCR, in-situ hybridization and immunohistochemistry. We here report for the first time, to our knowledge, that the expression of MMP-26 mRNA and protein are both expressed in the monkey endometrial glandular epithelium and spiral arteries during early pregnancy.

Successful implantation of the embryo in the endometrium is a key event in the establishment of pregnancy (Salamonsen, 1999). Mammalian implantation is a very complex process involving invasion of the maternal endometrium by the trophoblast in a spatio-temporally limited manner (Paria et al., 2000). Three families of proteases have been demonstrated to participate in the matrix degradation that is required for implantation: cysteine-, serine- and metallo-proteases (Grevin et al., 1993; Harvey et al., 1995; Alexander et al., 1996; Afonso et al., 1999; Salamonsen, 1999; Fata et al., 2000; Zhao et al., 2002). Among these proteases, MMPs have been recognized to play pivotal roles in embryonic implantation. Our previous research indicates that MMP-2, -9 and -14 are involved in the processes of embryonic implantation in the rhesus monkey (Wang et al., 2001).

In the present study, we found that MMP-26 mRNA and protein are both expressed in the monkey endometrial glandular epithelium on day 12 of pregnancy and decreased with the prolongation of pregnancy. RT–PCR analysis showed that the total RNA transcription level in the endometrium was high on day 12 and significantly down-regulated thereafter, strengthening the data obtained from in-situ hybridization.

It should be noted that although there is relatively low sequence similarity between MMP-26 and -7 (matrilysin), these two MMPs are strikingly similar in their exon–intron structures and minimal number of domains required for secretion and activity (de Coignac et al., 2000; Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al., 2001). Therefore, close relationships may exist between these two MMPs. Although most MMPs are primarily expressed in vivo by stromal cells, the present study observed that the expression of
MMP-26 mRNA and protein are mainly restricted to the epithelium, similar to MMP-7 (Rodgers et al., 1993). MMP-26 seems to be preferentially expressed in epithelial cells. This proteinase is also specifically expressed in cancer cells of epithelial origin, including carcinomas of the endometrium, lung, prostate and breast (Park et al., 2000; Uria and Lopez-Otin, 2000; Marchenko et al., 2001). Taken together, it is expected that the glandular epithelium is the primary site of MMP-26 synthesis in the rhesus monkey uterus during early pregnancy.

Although the significance of MMP-26 in the glandular epithelium is not presently clear, it is reasonable to propose a role for MMP-26 in rhesus monkey uterine glands during implantation because the uterine secretory substances from the endometrial glands are essential for uterine receptivity and embryo implantation (Gray et al., 2001). Considering the similarity between the restricted expression patterns of MMP-26 and MMP-7, the functional role of MMP-7 as an ‘enzymatic pipe cleaner’ (Saarialho-Kere et al., 1995) and MMP-7 expression in rhesus monkey uterine glands (Rudolph-Owen et al., 1998), it is reasonable to hypothesize that MMP-26 may co-operate with MMP-7 in the macaque uterine glands to enhance its activity during early pregnancy. Most importantly, MMP-7 has been shown to be able to activate pro-MMPs such as pro-MMP-1, -2 and -9 (Sang et al., 1995, 1996; Sterrnlicht and Werb, 2001). Hence, MMP-26 may function in tissue reconstruction processes during pregnancy by digesting the extracellular matrix components and/or activating other MMPs such as pro-MMP-9 (Uria and Lopez-Otin, 2000; Zhang et al., 2002).

In addition to the predominant glandular epithelial expression pattern of MMP-26, it was interesting to observe that MMP-26 is also localized in the walls of some arterioles situated adjacent to the implantation site in the endometrial compartment of the rhesus monkey uterus on days 18 and 26 of pregnancy. We have previously demonstrated that MMP-2 and -14 are localized in the walls of the arterioles on day 18 of pregnancy, and on day 26, MMP-2 and tissue inhibitor of metalloproteinases (TIMP)-1 and -2 mRNAs are strongly demonstrated that MMP-26 is localized in the walls of the arterioles on day 18 of pregnancy, and on day 26, MMP-2 and tissue inhibitor of metalloproteinases (TIMP)-1 and -2 mRNAs are strongly expressed in perivascular decidual cells, while TIMP-3 transcripts are only present in the outer membrane of arteries (Wang et al., 2001). The present study extends our previous research and may further imply that co-ordinated and balanced expression of MMP-2, -14 and -26 and TIMP-1, -2 and -3 is essential in maintaining and strengthening blood vessel integrity during early pregnancy in the rhesus monkey.

Although an investigation by de Coignac et al. showed that MMP-26 mRNA expression is relatively high in human placenta (de Coignac et al., 2000), we did not observe any hybridization signal in the placental compartments of the rhesus monkey uterus on days 18 and 26 of early pregnancy. Interestingly, our original study also did not find MMP-26 mRNA in human placenta tested by Northern blot analysis (Park et al., 2000). Recently, Marchenko et al. also did not observe MMP-26 mRNA expression in human placenta (Marchenko et al., 2001). Paradoxically, both MMP-26 mRNA and protein have been shown to be localized in syncytiotrophoblasts and cytotrophoblasts in human placental villi, together with those of TIMP-4 (Zhang et al., 2002). The reason for the discrepancies in the reports of placental MMP-26 expression is not known, but may be due to the different experimental materials applied. The discrepancies further suggest that, in general, MMP-26 expression in the uterus is highly regulated spatially and temporally; only during the selective critical hours of endometrium remodelling, embryo implantation and/or cell invasion are the MMP-26 gene and protein expressed very strictly in certain types of cells, mostly of epithelial origin.

Acknowledgements

The authors wish to thank Professor Rujin Zou and Ms Hua Qin for assistance with tissue collection and data analyses. We are also grateful to Dr Sara Monroe for critical reading of the manuscript. This study was supported by the Special Funds for Major State Basic Research Project (G1999055903) and the Knowledge Innovation Project of the Chinese Academy of Sciences (C.Z.), and by grants from the National Institutes of Health CA78646, the American Cancer Society, Florida Division F01FSU-1, and the Florida State University Research Foundation (Q.-X.A.S.).

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Submitted on April 4, 2002; accepted on June 26, 2002

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