BIOLOGY OF REPRODUCTION **72,** 954–959 (2005)
Published online before print 15 December 2004.
DOI 10.1095/biolreprod.104.036772

Spatio-Temporal Expression of Matrix Metalloproteinase-26 in Human Placental Trophoblasts and Fetal Red Cells During Normal Placentation[1]

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ABSTRACT

The processes of implantation and placentation involve the degradation and remodeling of extracellular matrix, cellular proliferation, apoptosis, and differentiation. Evidence indicates that members of the matrix metalloproteinase (MMP) family play crucial roles in these processes. In the present study, we identified the expression and localization of MMP26/endometase/matrilysin-2 in human placentae at different stages of gestation using methods of reverse transcriptase-polymerase chain reaction, in situ hybridization, and immunohistochemistry. MMP26 was widely localized to villous cytotrophoblast cells, syncytiotrophoblast cells, and to column trophoblasts during early pregnancy. The mRNA and protein level of MMP26 in chorionic villi was highest at Weeks 6–7, and decreased thereafter, reaching its lowest level at the second trimester. The mRNA level was significantly up-regulated in term placenta, while the immunoreactivity remained undetectable. Notably, intense expression of MMP26 was found in fetal nucleated red cells inside the villous capillaries during gestational Weeks 6–9. Strong expression of MMP26 mRNA was also demonstrated in fetal red cells isolated from the whole blood of fetuses at midpregnancy. The expression patterns of MMP26 in human placenta suggests complicated roles for MMP26 during the processes of placentation and hematopoiesis, perhaps working in concert with other members of the MMP family, such as MMP9.

implantation, placenta, pregnancy, trophoblast

INTRODUCTION

Matrix metalloproteinases (MMPs) are a comprehensive family of zinc metalloenzymes involved in the breakdown of extracellular matrix (ECM) proteins [1], and evidence suggests that MMPs are also involved in the processes of cellular growth, apoptosis, migration, and differentiation [2]. So far, more than 20 isoforms of MMPs have been identified. Based on the substrate specificities, MMPs can be classified into four groups: gelatinases, collagenases, stromelysins, and membrane-type matrix metalloproteinases (MT-MMPs).

MMP26, also known as endometase or matrilysin-2, is one of the most recently identified MMPs, and is the smallest member of the MMP family [3, 4]. It has a prodomain structure with a unique cysteine switch sequence, PHCGVPDGSD, and its catalytic domain contains the zinc-binding motif that is characteristic for the MMPs [4]. Evidence shows MMP26 can efficiently cleave fibrinogen and various ECM proteins including fibronectin, vitronectin, and denatured collagen [4–6].

Increasingly, recent data indicate the involvement of MMP26 in reproductive processes. Marchenko et al. [6] and Park et al. [4] demonstrated that MMP26 was not only expressed in cancer cells of epithelial origin, but also in the human placenta and uterus. Data from Chegini et al. [7] showed that MMP26 was present in various types of endometrial cells, with surface and glandular epithelial cells showing the strongest expression, followed by vascular endothelial and endometrial stromal cells. Both Chegini et al. [7] and Pilka et al. [8] demonstrated a cyclic change in MMP26 expression in the human uterus, with peak levels occurring in the endometrial epithelium at the midsecretory phase, indicating a role for MMP26 in endometrial tissue remodeling.

Implantation and placentation are complicated physiological processes involving a series of cellular events that include proliferation and differentiation of both trophoblastic and endometrial cells, adhesion and subsequent invasion of trophoblasts into the endometrium leading to remodeling of uterine tissues and reconstruction of uterine spiral arteries, and placental villi angiogenesis. Evidence indicates that many members of the MMP family exert critical influence over these processes [9], and recent data also indicate the involvement of MMP26 in placentation. Li et al. [10] investigated the expression of MMP26 in the endometrium of pregnant rhesus monkeys, and specifically localized MMP26 to the glandular epithelium and the walls of spiral arterioles on gestational Days 12 and 18. They speculated that the enzyme might be involved in the highly regulated tissue remodeling processes of the glandular epithelium and spiral arteries during early pregnancy in the rhesus monkey. Zhang et al. [11] reported the expression of MMP26 in both

[1]Y.-L.W. was supported in part by NSFC Project grant 30370542, by Special Funds for Major State Basic Research Project grant G1999055903, and by CAS Knowledge Innovation Program grant KSCX-2-SW-201. Y.-G.Z. was supported by a Program Enhancement Grant from the Florida State University Research Foundation, and Q.-X.A.S. was supported by grant DAMD17-02-1-0238 from the Department of Defense Congressionally Directed Medical Research Programs, and by grant CA78646 from the National Institutes of Health.
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Received: 30 September 2004.
First decision: 1 November 2004.
Accepted: 18 November 2004.

ISSN: 0006-3363. http://www.biolreprod.org

normal and choriocarcinoma trophoblast cells in humans, and suggested possible roles for the enzyme in the tissue-remodeling processes associated with placentation and tumor progression. However, little has been elucidated regarding the expression pattern and possible role of MMP26 at the human maternal-fetal interface during normal pregnancy.

In this study we investigated the expression patterns of *MMP26* mRNA and the encoded protein in normal human placentae at different gestational stages to further our understanding of the functions of MMP26 during human placentation. In addition to these findings, we report the first evidence of MMP26 expression in fetal nucleated red cells.

MATERIALS AND METHODS

Human Placental Tissue Preparation

Tissues of human chorionic villi or placentae were obtained in Beijing Haidian Hospital (Beijing, China) from patients who underwent therapeutic termination of pregnancy at 6–12 wk and 26 wk, or normal delivery at term, with informed consent of patients and permission of the Local Ethical Committee. All the patients accepted no special medical treatment before termination of pregnancy, and the placental tissues were pathologically normal. The gestational week of specimens during early pregnancy was defined according to morphological observation of the villi and pathological examination, with the record of menstrual cycles as a reference. Half of each tissue was flash frozen in liquid nitrogen, and the other half was washed twice with PBS buffer and fixed immediately in 4% paraformaldehyde (PFA) at 4°C for 10 h. Fixed tissues were then gradually dehydrated in ethanol and embedded in paraffin. Six μm-thick sections were collected on super Frost+ glass slides (Menzel-Gläser, Braunschweig, Germany). At least three tissue samples were collected from each gestational stage.

Isolation of Human Fetal and Adult Red Cells

Heparin-anticoagulated fetal blood samples were collected in Beijing Haidian Hospital (Beijing, China) from three aborted fetuses at gestational Weeks 25–26, and heparin-anticoagulated adult whole blood samples were collected from three normal, healthy donors in this laboratory. All samples were collected with permission of the Local Ethical Committee and the informed consent of the donors and patients who underwent therapeutic termination of pregnancy. The red cells and lymphocytes were separated from whole blood by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) using the manufacturer's protocol. After washing, resuspension, and centrifuging, cell pellets were subjected to RNA isolation.

RNA Isolation and Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction

Tissue specimens were homogenized with a polytron homogenizer (Kinematika, Switzerland). Total RNA from tissues or cells was isolated using TRIzol reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a 20-μl reaction mixture with random hexamer primers (Promega, Madison, WI) by M-MuLV reverse transcriptase (RT) as specified by the manufacturer (Fermentas, Vilnius, Lithuania). One-microliter aliquots from the reverse transcription were amplified by polymerase chain reaction (PCR) with specific primers (Runbio Biotechnology, Beijing) designed in accordance with cDNA sequences from the National Center for Biotechnology Information database. The primers used were 5′-CCATTTTGACAAGAATGAACA-3′ (antisense) and 5′-CATTAAGGTATGTCAGATGAA-3′ (sense) for *MMP26*, and 5′-ACCACAGTCCATGCCATCAC-3′ (antisense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (sense) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The 25-μl PCR system contained 2 μl of RT products, 200 μmol/l deoxynucleotide triphosphates, 2 mmol/L $MgCl_2$, 1 IU Taq polymerase (Shenengy Biocolor, Shanghai, China), and 10 pmol of each primer. The PCR conditions were denaturing at 94°C for 10 min; running 25–30 cycles at 94°C for 15 sec, 55°C for 20 sec, and 72°C for 20 sec; then elongating at 72°C for 5 min. The cycle number was determined by preliminary experiment to ensure that the amplification was carried out within the exponential phase. The anticipated sizes of the amplified fragments were 232 base pairs (bp) for *MMP26* and 452 bp for *GAPDH*. A PCR system using the total RNA as template was included as a negative control to ensure the absence of genomic DNA contamination. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and analyzed using the Gel-Pro Analyzer (software version 4.0; United Bio., Marlton, NJ).

Preparation of Digoxigenin-Labeled Probes

The *MMP26* fragment obtained by PCR was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), then inserted into the pGEM-T Easy vector (Promega). After confirmation by sequencing, the recombinant plasmid was linearized with restriction enzymes *Sal*I or *Ncol*I (Promega), purified with QIAquick Spin Columns (Qiagen), then used as a template for in vitro transcription reactions to synthesize digoxigenin (DIG)-labeled RNAs using the DIG-RNA labeling system (ENZO Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The transcription mixture (20 μl) was composed of 1 μg template cDNA, 2 μl 10× nucelotide triphosphate labeling mixture, 1 μl RNase inhibitor, and 2 μl SP6 or T7 RNA polymerase. Transcription reactions were performed at 37°C for at least 2 h, and the template cDNA was digested by RNase-free DNase. The reaction was stopped by adding 0.2 M EDTA. The DIG-labeled RNA probes were stored at −80°C at a concentration of 0.1 μg/μl.

In Situ Hybridization

In situ hybridization was performed as previously described [12]. In brief, paraffin sections were routinely deparaffinized and rehydrated. After treatment with 0.2 N HCl, slides were denatured at 70°C in 2× SSC, then digested with 4 μg/μl of proteinase K (Gibco BRL). Postfixation was performed in 4% PFA followed by acetylation in TEA buffer containing 0.5% acetic anhydride. The slides were prehybridized for 4 h at 58°C in prehybridization buffer (50% formamide, 20 mM Tris-HCl, 50 mM EDTA, 0.5 mg/ml tRNA *coli*, and 100 mM dithiothreitol), and further hybridized for 18 h at 58°C in fresh hybridization buffer containing 1 ng/μl antisense probe. After consecutive washes in 2× SSC and 0.1× SSC at 63°C, the slides were blocked with 0.5% blocking reagent (Boehringer, Mannheim, Germany), then incubated with alkaline phosphatase-coupled anti-DIG antibodies (dilution 1:500). Color development was performed in buffer II (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) containing NBT and BCIP (Boehringer Mannheim). Nonspecific staining was removed by rinsing the slides with 95% EtOH. After dehydration with EtOH and xylene, the slides were mounted in resin. Negative control experiments were performed by replacing the antisense probe with the sense probe in the hybridization buffer. Results were assessed by three independent observers.

Immunohistochemistry

Immunohistochemical assays were performed as previously described [12]. Briefly, paraffin sections were deparaffinized and rehydrated, then retrieved in 20 μM EDTA buffer (pH 8.0). After immersion in 1% hydrogen peroxide, the sections were incubated with rabbit anti-human MMP26 immunoglobulin G (IgG; 10 μg/ml) [13] at 4°C overnight. Negative controls were performed by replacing the MMP26 antibody with preabsorbed IgG [13] at the same concentration. Final visualization of positive staining was achieved using DAKO Envision Kits (DAKO Cytomation, DK-2600 Glostrup, Denmark). Counterstaining with hematoxylin was carried out before slide mounting. Results were assessed by three independent observers.

Statistical Analysis

The in situ hybridization, immunohistochemistry, and RT-PCR were repeated three times, each with at least three independent specimens per time point or per developmental stage. The RT-PCR data were measured by comparing the densitometry value of *MMP26* with that of *GAPDH* in the same experimental set. The data were reported as the average ± SD according to results from three independent experiments. Comparison of the relative densities between groups was performed by analysis of variance (ANOVA) and $P < 0.05$ was considered significant.

RESULTS

Expression Pattern of MMP26 in Human Placentae at Different Stages of Pregnancy

To gain an overview of the expression and variation of *MMP26* mRNA in human placentae at different stages of

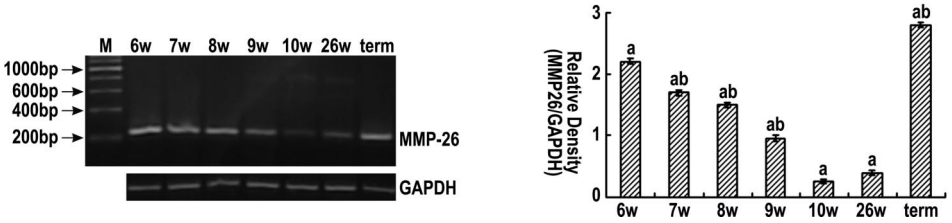

FIG. 1. Semiquantitative RT-PCR of *MMP26* in human placenta tissues at different stages of pregnancy. **a**) The products of a representative semiquantitative RT-PCR were subjected to electrophoresis on a 1.5% agarose gel. Complementary DNAs derived from placenta villi at gestation Weeks 6 (lane 6w), 7 (lane 7w), 8 (lane 8w), 9 (lane 9w), 10 (lane 10w), 26 (lane 26w), and at full term (lane term) were used as templates for PCR, respectively. Lane M, molecular weight standard. **b**) Densitometric analysis of the semiquantitative RT-PCR results. The density of *MMP26* was normalized by that of *GAPDH*, and the relative amount of *MMP26* is reported as the average ± SD according to the results from three independent experiments. Comparison of the relative densities between groups was performed by ANOVA and $P < 0.05$ was considered significant (**a**, compared with the sixth week, and **b**, compared with the 10th week).

pregnancy, semiquantitative RT-PCR for *MMP26* was performed using chorionic villi at Gestational Weeks 6 to 10, Week 26, and full-term. A representative illustration of the RT-PCR analysis is shown in Figure 1a. Statistical analysis (Fig. 1b) demonstrated that *MMP26* mRNA levels were relatively high in villi at the sixth week of pregnancy, then decreased gradually from Week 7 on, reaching the nadir at Week 10. At the 26th week, the level of *MMP26* mRNA in placenta was 25% of that observed at the sixth week. However, the expression was up-regulated significantly at full-term to a level of about 30% more than that at Week 6.

With immunohistochemistry (Fig. 2) and in situ hybridization (Fig. 3), a more detailed picture of the spatial and temporal expression of MMP26 in human placentae was demonstrated. Spatially, both mRNA and protein of MMP26 were observed in villous cytotrophoblast cells, syncytiotrophoblast cells, and column trophoblast. Transcript, but not protein, was present in some villous mesenchymal cells (Fig. 2, A–E and Fig. 3, A–E). Temporally, the signal intensity of MMP26 in trophoblasts was strongest at Week 6–7 (Fig. 2, A–C and Fig. 3, A–C), and then it decreased from Week 8 on (Fig. 2, D and E; Fig. 3, D–E), reaching an undetectable level at Week 26 (Fig. 2F and Fig. 3F). In term placenta, the mRNA level of *MMP26* was significantly up-regulated in both trophoblasts and mesenchymal cells (Fig. 3G), while the immunoreactivity remained undetectable (Fig. 2G).

Notably, during gestational Weeks 6–9, intense expression of MMP26 was exhibited by fetal nucleated red cells (FNRCs) inside the villous capillaries (Fig. 2, B–E; Fig. 3, B–D). The immunoreactivity in FNRCs was even stronger than that in trophoblasts, while the mRNA level was almost the same in FNRCs and trophoblasts. At Week 26 and in term placenta, almost all the red cells in villous capillaries were enucleated, and no signal of MMP26 appeared (Fig. 2, F and G, and Fig. 3, F and G).

MMP26 mRNA Variation in Human Fetal and Adult Red Cells and Lymphocytes

Red cells and lymphocytes in human fetal and adult whole blood were separated by Ficoll-Paque Plus, respectively. More than 90% of the red cells derived from fetal blood at gestational Weeks 25–26 were nucleated. By using RT-PCR, *MMP26* was found to be strongly expressed in fetal red cells, while signals were not detectable in either fetal lymphocytes, adult red cells, or adult lymphocytes (Fig. 4).

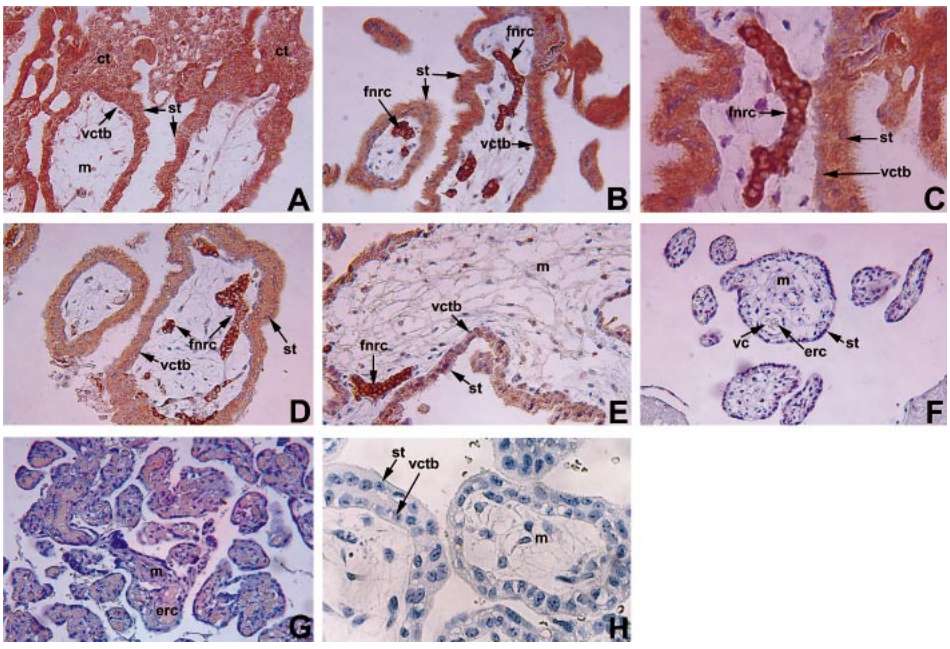

FIG. 2. Immunohistochemical assay to show the distribution of MMP26 protein in human placenta at gestational Weeks 6 (**A**), 7 (**B**, **C**), 8 (**D**), 9 (**E**), 26 (**F**), and at full term (**G**). During Weeks 6–9, immunoreactivity of MMP26 was observed in villous cytotrophoblast (vctb) cells, syncytiotrophoblast (st) cells, and the column trophoblast (ct), but it was nearly undetectable in mesenchymal (m) cells. Intense staining was observed in fetal nucleated red cells (fnrc) inside the villous capillaries. At Week 26 and at full term, immunoreactive MMP26 was undetectable in the placenta villi and enucleated red cells (erc) inside the villous capillaries. **H**) Negative control. Original magnification ×200 for **A**, **B**, and **D**–**G**; ×400 for **C** and **H**.

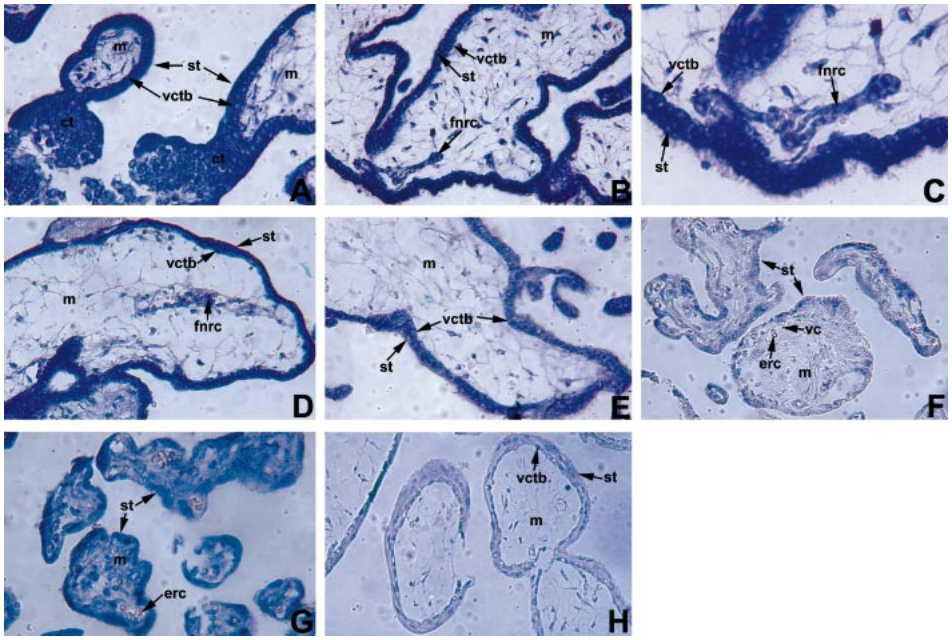

FIG. 3. In situ hybridization to demonstrate the expression of *MMP26* mRNA in human placenta at gestational Weeks 6 (**A**), 7 (**B** and **C**), 8 (**D**), 9 (**E**), 26 (**F**), and at full term (**G**). *MMP26* was expressed in villous cytotrophoblast (vctb) cells, syncytiotrophoblast (st) cells, column trophoblast (ct), and some mesenchymal (m) cells during Weeks 6–9. *MMP26* staining was very strong in fetal nucleated red cells (fnrc) during Weeks 7–9 (**B**, **C**, and **D**), but it was undetectable in enucleated red cells (erc) inside the villous capillaries. *MMP26* expression reached its lowest level at Week 26, but it was up-regulated at full term. **H**) Negative control hybridized with sense probe. Original magnification ×200 for **A**, **B**, and **D**–**H**; ×400 for **C**.

DISCUSSION

One of the most notable observations in the present study is the intense expression of MMP26 in the FNRCs in villous capillaries. By using RT-PCR, the nucleated red cells derived from fetal blood at gestational Weeks 25–26 were also shown to be positive for MMP26. It is well known that ontogenic development of the human hemopoietic system involves a series of coordinated changes in embryonic and early fetal life [14, 15]. At the third or fourth week, the first generation of hemopoietic cells proliferates in the yolk sac and extraembryonic mesenchyme. These elements consist largely of ''primitive'' erythroblasts (megaloblasts), which are present in circulating blood from Week 4 onward. At the fifth or sixth week, the yolk sac is replaced by the liver as the main hemopoietic site where the ''definitive'' erythropoietic cells proliferate and differentiate. The definitive series enter into the bloodstream from Week 8 onward, thus gradually replacing circulating megaloblasts [16]. During gestational Weeks 6–12, the nucleated red cells in placental villous capillaries consist of erythroblasts derived from the yolk sac and then the fetal liver, while circulating nucleated red cells in the fetus are mainly derived from the liver ''definitive'' erythropoietic cells [17]. From our study, it seems that erythroblast cells derived from both the yolk sac and the liver are positive for MMP26. It has been reported that hematopoietic cells in long-term cultures of both normal and leukemic bone marrow could produce MMP2 and MMP9; and tissue inhibitor of MMPs (TIMPs)-1, -2, and -3 [18], suggesting that MMPs and TIMPs might affect hematopoiesis by modifying the structure of bone marrow ECM and regulating diverse cell functions including proliferation, differentiation, adhesion, migration, and survival. Data from Petitfrere et al. [19] and Lambert et al. [20] indicated that TIMP-1 could induce erythroid cell survival, proliferation, and differentiation in the erythroleukemic cell line UT-7. All of this evidence indicates a potential role for various MMPs and TIMPs in hematopoiesis. As a newly identified member of the MMP family, MMP26 has been found to mediate progelatinase B (MMP9) activation [21], and TIMP-2 and TIMP-4 were shown to be potent inhibitors of MMP26 [22]. However, the definite role of MMP26 in hematopoietic cells remains to be clarified.

The wide distribution of MMP26 in both villous trophoblasts and column cytotrophoblasts, as revealed by both immunohistochemistry and in situ hybridization in the present study, seems to indicate its multiple functions in regulating various trophoblast behaviors. During human implantation and placentation, villous tissue remodeling occurs in the placental villi. From the literature, MMP2, -9,

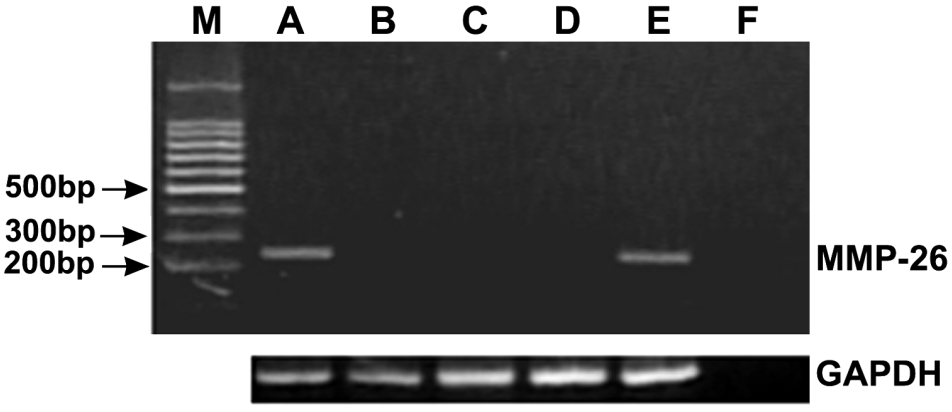

FIG. 4. Semiquantitative RT-PCR of *MMP26* in red cells (lanes A and C) and lymphocytes (lanes B and D) derived from human fetus (lanes A and B) and adult (lanes C and D), respectively. Lane E, positive control using cDNA of human placental villi at Week 6 as a template. Lane F, PCR using total RNA of fetal red cells as a template to eliminate the possible contamination of genomic DNA in the extracted total RNA.

and- 14, as well as TIMP-1, -2, and -3, were found to be produced by villous trophoblast cells [23–25], and they were suggested to participate in the reconstruction of the basement membranes beneath the trophoblast epithelium during villous development. MMP26 can efficiently cleave fibrinogen and extracellular matrix proteins including fibronectin, vitronectin, and denatured collagen [6], most of which were found in villous trophoblast [12]. On the other hand, previous reports have indicated that MMP26 may not be a direct contributor to the degradation of ECM molecules. It can mediate the activation of pro-MMP9 [5, 21], a potent gelatinase that plays a major role in ECM degradation. In our previous work, Bai et al. [26] found a pattern of MMP9 expression similar to that of MMP26 in villous trophoblast cells during the first trimester. Meanwhile, at the maternal-fetal interface of tubal pregnancy, we demonstrated the temporal change of MMP9 expression in villous trophoblast [26], which was the same as that of MMP26 observed in this study. Our previous work in cultured cytotrophoblast cells also showed that production of MMP9 decreased concurrently with the advancement of gestational weeks during the first trimester. These data seem to further indicate a cooperative role for MMP26 and MMP9. Therefore, we proposed that MMP26 might participate in tissue remodeling processes during placentation, either by direct cleavage of ECM, or by working in association with other members of the MMP family such as MMP9.

At the feto-maternal interface, column trophoblast cells are a group of highly proliferative cells and will then differentiate along the invasive pathway. The invasive trophoblasts migrate into the deeper layer of maternal stroma, and as a result, anchor the placenta to the uterine wall [17, 27]. MMP9, MMP26, and TIMP-3 were observed in column trophoblasts, as shown in our previous [26] and present work. Recently, it has been suggested that some MMPs can manipulate cell proliferation, apoptosis, and differentiation by degrading ECM or nonmatrix substrates to release sequestered cell factors [2]. However, it remains unclear whether MMP26 can function this way in the placenta.

Of interest, the mRNA level of *MMP26* in term placenta is significantly up-regulated, while the protein level remains undetectable, so a mechanism for the post-transcriptional regulation of MMP26 expression might exist in the placenta.

It should be noted that some discrepancies exist in the study of MMP26. The investigations of de Coignac et al. [3], Uria et al.[5], and Zhang et al. [11] showed that *MMP26* mRNA expression was relatively high in the human placenta, similar to what we found in the present study. However, neither Park et al. [4], Marchenko et al. [6], nor Li et al. [10] observed *MMP26* mRNA expression in the primate placenta. Similar discrepancies exist regarding the expression of MMP26 in the human uterus. Park et al. [4] identified MMP26 in endometrial tumors, however, Isaka et al. [28] demonstrated that the enzyme was expressed in human endometrium but not in endometrial carcinoma. Recently, Pilka et al. [29] revealed that MMP26 expression was high in hyperplastic endometrial tissues, but decreased during the later stages of tumor expression. Meanwhile, Pilka et al. [8] showed that MMP26 expression is transient and cyclic, being maximal during the periovulatory period. Therefore, tissues collected at different time points may exhibit very different expression patterns of MMP26. This opinion is further demonstrated in recent findings of Zhao et al. [22] regarding the expression of MMP26 in breast cancer. These discrepancies may also reflect the great complexity in the dynamic and transient regulation of MMP26 expression.

In summary, this study is the first to document the spatial and temporal expression patterns of MMP26 in human placental trophoblasts as well as in fetal nucleated red cells. Our findings suggest that MMP26 may be involved in both placentation and hematopoiesis. These data may further our understanding of the complicated functions of the MMP family.

ACKNOWLEDGMENT

We appreciate the editorial assistance of Robert G. Newcomer at Florida State University.

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