Endocrine, *vol. 16, no. 1, 47–54, October 2001* 0969–711X/01/16:47–54/$12.00

Expression of Matrix Metalloproteinase-2, Tissue Inhibitors of Metalloproteinase-1, -3 at the Implantation Site of Rhesus Monkey During the Early Stage of Pregnancy

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We have examined the expression of MMP-2, TIMP-1, and TIMP-3 mRNA at the implantation site of rhesus monkeys during early stage of pregnancy using *in situ* hybridization and Northern blot analysis. The results indicate that MMP-2 mRNA was mainly localized in the chorinoic villi and epithelial plaque, suggesting that MMP-2 may be involved in the process of epithelial plaque and trophoblast invasion. TIMP-3 was specifically expressed in the cells around the spiral arteries and maternal–fetal interface. Therefore, TIMP-3 may be the main inhibitor that restricts the trophoblast invasion. The TIMP-1 mRNA was detected in trophoblast villi and maternal decidua; however, its distribution was not cell-specific, suggesting a general role in the protection of trophoblast villi and maternal decidua from proteolysis by the MMPs secreted by themselves.

Key Words: Implantation site; MMP; TIMP; trophoblast.

Introduction

Implantation of an embryo into the endometrium is a highly controlled event that is critical for establishment of pregnancy. Understanding the molecular basis of this process is therefore important for regulation of fertility *(1)*. In species with hemochorial placentation, such as mouse and primate, the trophoblast penetrates the uterine epithelium and invades deeply into the stroma to make contact with the maternal blood supply. Trophoblast invasion into the maternal tissue is crucial for the success of implantation in the mammalian species that undertake interstitial implantation and provides anchorage for the conceptus and adaptation of uteroplacental circulation. In humans and primates, trophoblast not only invades into the uterine interstitial compartment, but also advances deeply into the spiral arterioles, the blood vessels that penetrate the uterine wall to supply blood for the placenta *(2)*. In macaques such as the rhesus monkey, the endovascular trophoblastic cells can penetrate the spiral arteries as far as the myometrial border *(3)*. Both endometrial and endovascular trophoblast invasion processes are physiologically important. Insufficient endovascular invasion of trophoblast has been believed to be a major pathological component of preeclampsia *(4–6)*.

Trophoblast invasion shares many features of tumor invasion and metastasis, but it is tightly controlled within the uterus by locally produced proteinase inhibitors *(7)*. There are three classes of proteinases that have been postulated to be of importance in trophoblast invasion: cysteine proteinases, serine proteinases, and matrix metalloproteinases *(8)*. We have demonstrated that urokinase plasminogen activator (uPA) was localized in the trophoblast villi, while plasminogen activator inhibitor type 1 (PAI-1)was mainly expressed at the maternal–fetal interface at the implantation site of the rhesus monkey during the early stage of pregnancy *(9)*. Tissue type plasminogen activator (tPA), uPA, PAI-1, and PAI-2 were also detected in the human placenta related to fibrinolysis and angiogenesis during placentation *(10–12)*.

MMPs, which belong to the family of zinc- and calcium-dependent proteolytic enzymes, are the most critical enzymes in matrix degradation as their substrate specificities collectively allow them to degrade virtually all the components of both extracellular matrix and basement membrane *(13, 14)*. MMPs are expressed by a wide variety of reproductive tissues *(14)*. MMP-2 is an important member of the MMP family, and capable of degrading many kinds of extracellular matrix components including type IV collagen *(15)*. It has been reported that MMP-2 was expresed in the invasive trophoblast, which appears to also produce tissue inhibitors of matrix metalloproteinases (TIMPs) *(16)*. The activity of the various MMPs was post-translationally regulated by a family of at least four different TIMPs. The mechanism of the metalloproteinases and their tissue inhibitors involved in human implantation and placentation at the early stage of pregnancy is poorly understood, because it is difficult to obtain the intact human implantation site. Although the embryo of the rhesus monkey does not implant quite as deeply into uterine tissue as the human embryo, the invasive behavior of trophoblast in rhesus monkey is much

Received March 23, 2001; Revised June 23, 2001; Accepted July 2, 2001.

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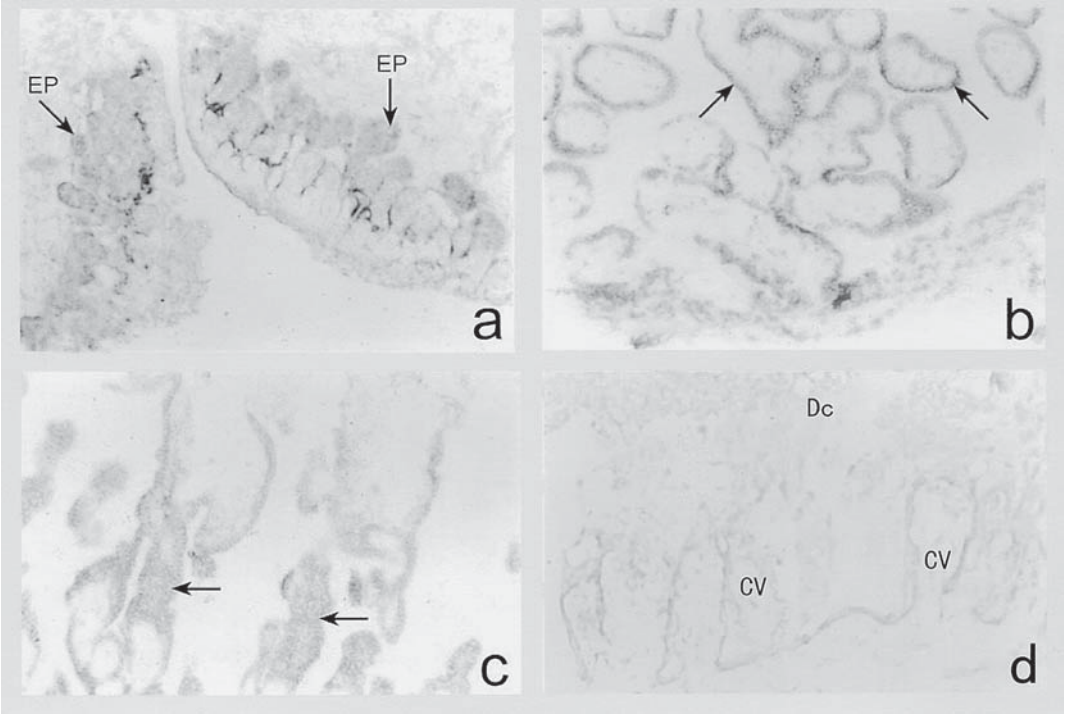

Fig. 1. *In situ* hybridization localization of the MMP-2 mRNA at the implantation site of rhesus monkey during early stage of pregnancy. (**A**) The epithelial plaque (arrow shown) expressed MMP-2 mRNA on d 15 (×200); the placental villi (arrow shown) were labeled by the cRNA probe of MMP-2 on d 21 (**B**), d 32 (**C**) (×400); (**D**) negative control. EP: epithelial plaque; Dc: deciduas; CV: chorionic villi.

more similar to that of humans than that of any other laboratory animals *(3)*.

In order to better understand the interaction between metalloproteinases and their tissue inhibitors during the early stage of placentation in primates, we used the rhesus monkey as a model and examined the expression of MMP-2, TIMP-1, and TIMP-3 at the implantation site in the early stage of pregnancy.

Results

Localization of MMP-2 mRNA in Trophoblast Villi

To examine MMP-2 mRNA expression at the implantation site of the rhesus monkey, *in situ* hybridization was carried out. MMP-2 mRNA was detected mainly in the trophoblast cells from d 15 to d 34 of pregnancy, as shown in Fig. 1B,C. No positive MMP-2 mRNA specific reactivity was noted in the decidual cells. On d 15 of pregnancy, epithelial plaque adjacent to the implantation site (Fig. 1A) was strongly labeled with the MMP-2 cRNA probe.

Localization of TIMP-1 mRNA in the Trophoblast Villi and Decidua

TIMP-1 mRNA was detected in the trophoblast villi (Fig. 2C,D) and epithelial plaque (Fig. 2A). The maternal decidua (Fig. 2B,E) was also positively stained by TIMP-1 cRNA probe. However, the distribution of TIMP-1 mRNA in the decidua was not cell-specific. Almost all of the stromal cells expressed TIMP-1 mRNA, but the tissue around the spiral artery was only weakly labeled. The expression pattern of TIMP-1 was unchanged from d 17 to d 34.

Localization of mRNA of TIMP-3 in the Decidua

TIMP-3 mRNA at the implantation site was cell-specifically distributed. The cells at the maternal–fetal interface were strongly labeled by the TIMP-3 cRNA probe as shown in Fig. 3E–F and the same distribution pattern was noted from d 17 to d 34 of pregnancy. In addition to the maternal–fetal interface, the TIMP-3 mRNA was also detected in the cells around the spiral arteries as shown in Fig. 3A–C, but the expression lasted only a few days in the early stage. A weak expression was observed on d 17 (Fig. 3A) and reached the maximum on d 21 (Fig. 3B), then decreased. After d 26, the signal disappeared. Trophoblast villi did not express TIMP-3 mRNA.

Northern Blot Analysis of TIMP-3 mRNA in the Decidua and Myometrium

Because the material preserved in liquid nitrogen was limited, the most specifically expressed TIMP-3 was checked by Northern blot. As shown in Fig. 4, TIMP-3 mRNA was exclusively localized in the deciduas (also containing trophoblast component), no positive signal was detected in the myometrium during early stage of pregnancy.

Discussion

In the present study, the expression of MMP-2, TIMP-1, and TIMP-3 at rhesus monkey implantation site during

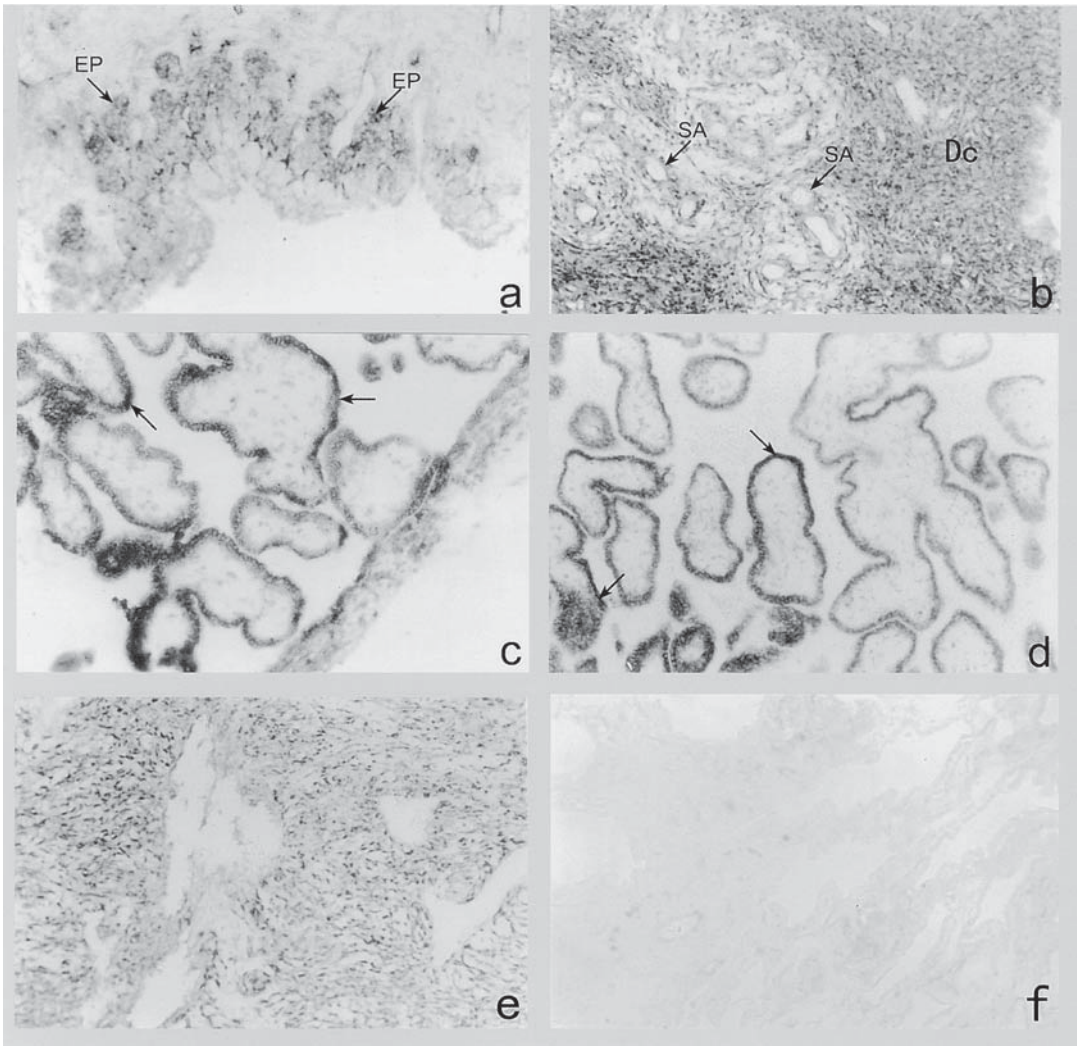

Fig. 2. *In situ* hybridization localization of TIMP-1 mRNA at the implantation site of rhesus monkey during early stage of pregnancy. (**A**) The epithelial plaque expressed TIMP-1 mRNA on d 15 (×200); (**B**) and (**E**) TIMP-1 mRNA was localized in the maternal deciduas on d 19 and d 26 (×200); the trophoblast cells (arrow shown) expressed TIMP-1 mRNA on d 21 (**C**) and d 32 (**D**) (×400); (**F**) negative control. Dc: deciduas. EP: epithelial plaque; SA: spiral artery.

the early stage of pregnancy was examined using *in situ* hybridization and Northern blot. The results showed that the MMP-2 mRNA was mainly detected in trophoblast villi. It has been reported that human first-trimester cytotrophoblast cells could produce MMP-2 in vitro *(17)*. Our experiment further demonstrated that the epithelial plaque cells expressed MMP-2 mRNA at the very early stage on d 15 (six days after implantation) of pregnancy in the rhesus monkey. The epithelial plaque originates from the rapid proliferative uterine epithelial and glandular cells. In normal physiological conditions, the physical contact between fetal trophectoderm and uterine epithelium may trigger this reaction. The epithelial plaque has been shown to be short-lived, and its function in the implantation process remains unclear. The timing of the emergence, the degeneration, and the localization of the epithelial plaque strongly suggests that it may play an important role in implantation. In the present experiment, we have demonstrated that epithelial plaque was strongly labeled by MMP-2 cRNA probe, implying that it may have invasive behavior. Because MMP-2 mRNA was not detected in the decidua, it is possible that the invasion of extravillous trophoblast (EVT) may not be mediated by MMP-2. Our previous study demonstrated that uPA was expressed in proliferating and invasive cytotrophoblast localized in trophoblast villi, as well as in extravillous trophoblast associated with uterine arterioles *(9)*. It is therefore suggested that other proteolytic enzymes, such as uPA, may be involved in this process. The activity of MMPs is tightly controlled physiologically by TIMPs, of which TIMP-1, TIMP-2, and TIMP-3 have been well

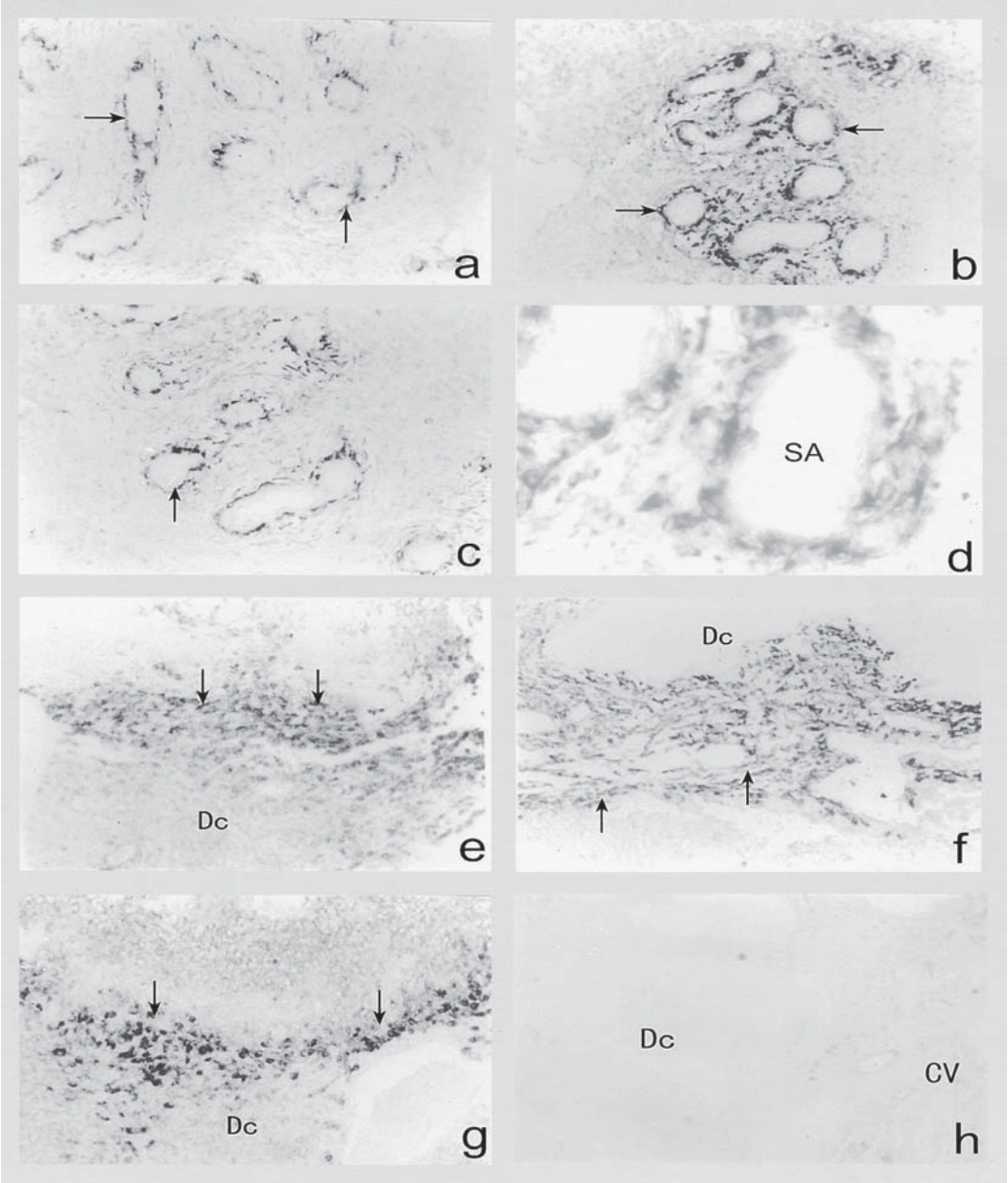

Fig. 3. The localization of TIMP-3 mRNA at the implantation site of a rhesus monkey during early stage of pregnancy. TIMP-3 mRNA was specifically localized in the cells (arrow shown) around the spiral artery on d 17 (**A**), d 21(**B**), and d 26 (**C**) (×200); (**D**) higher magnification of (**B**) (×1000); TIMP-3 mRNA was specifically localized at the maternal–fetal interface (arrow shown) on d 17 (**E**), d 26 (**G**), and d 32 (**F**) (×200); (h) negative control. SA: spiral artery. Dc: deciduas.

documented *(18)*. TIMP-3 was postulated as the inhibitor responsible for limiting the extent of trophoblast invasion in mice *(19–21)*. During placentation, EVT cells invade into the maternal spiral artery and remodel the structure of the blood vessels allowing increased blood flow to the intervillous space *(22)*. In a healthy pregnancy, the invasion is temporally and spatially well controlled, as excessive or insufficient trophoblast invasion into maternal tissue both have the potential to cause damage to the mother. In the present study, TIMP-3 was present at the maternal–fetal interface and the cells surrounding the spiral arteries. It appears that this inhibitor may regulate the extent of invasion of trophoblast and EVT into the maternal tissue. The expression pattern of TIMP-3 at the fetal–maternal junction was not changed during this period, but its expression pattern in the cells around the spiral arterioles was variable. Weak expression was first detected on d 17, reached maximum on d 21, and disappeared after d 26, suggesting that

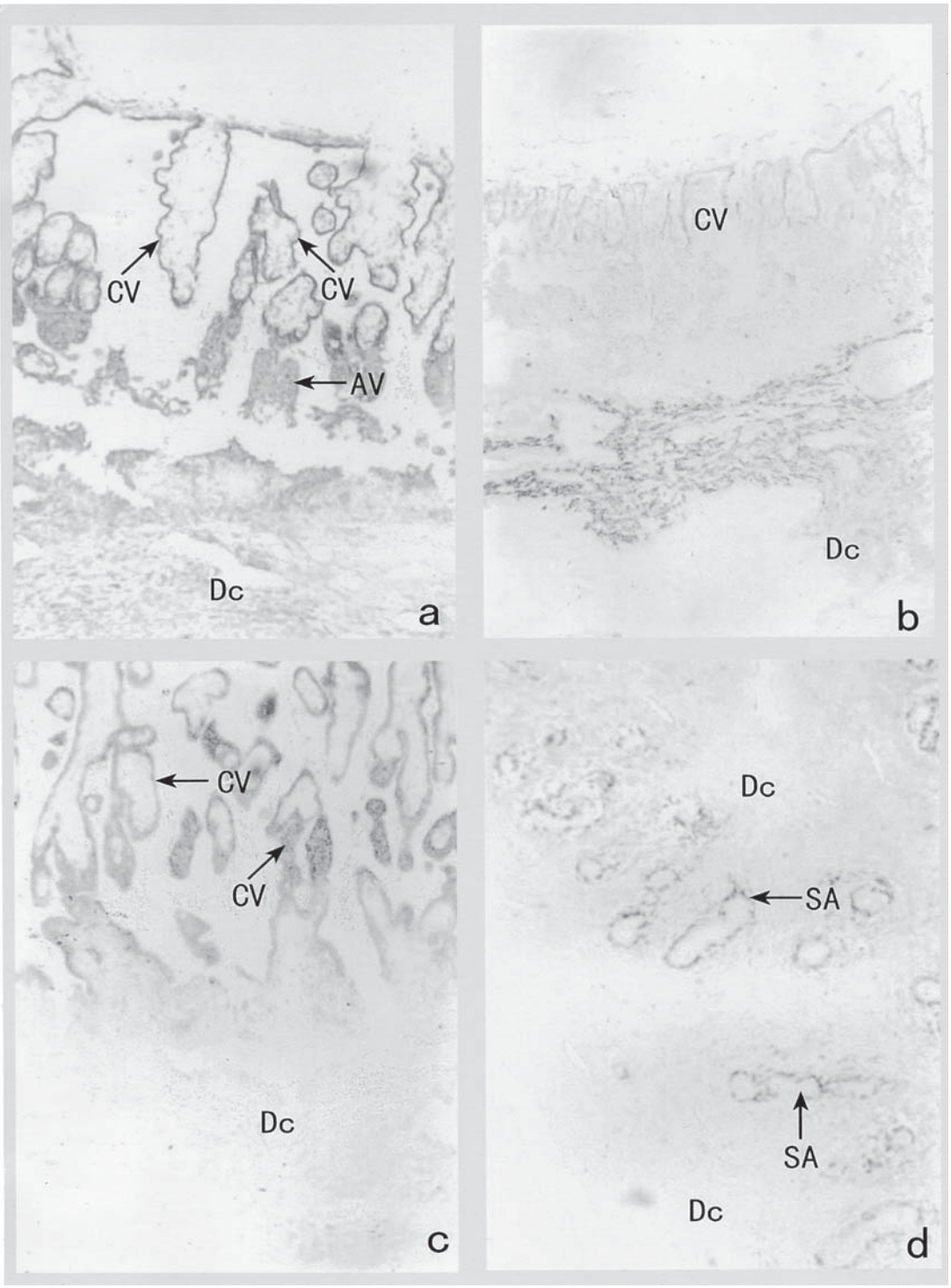

Fig. 4. Low-power magnification of the distribution of TIMP-1, TIMP-3, and MMMP-2 mRNA at the implantation site of a rhesus monkey during the early stages of pregnancy. (**A**) The distribution of TIMP-1 mRNA at the implantation site (×40); (**B**) the distribution of TIMP-3 mRNA at the implantation site (×40); (**C**) the distribution of MMP-2 mRNA at the implantation site (×40); (**D**) the expression of TIMP-3 mRNA in the cells around the spiral arteries (×40). CV: chorionic villi; AV: anchoring villi; Dc: deciduas; SA: spiral arteries.

TIMP-3 is the main inhibitor regulating invasion of trophoblast into the maternal decidua, and has a time-dependent function to limit the invasion of EVT into spiral arterioles.

TIMP-1 is capable of inhibiting almost all the MMPs in their activated form *(18)*. In the present study, we found that the distribution of TIMP-1 was not cell-specific. Therefore

it is possible that the main function of TIMP-1 may be in protecting the trophoblast from degradation by their own MMPs.

In summary, we first investigated the expression of metalloproteinase MMP-2 and the tissue inhibitors of metalloproteinase, TIMP-1 and TIMP-3, at the implantation site of the rhesus monkey at the early stage of pregnancy. The data suggest that the invasion of the EVT may not be mediated by MMP-2, and that TIMP-1 and TIMP-3 may have different functions during the process of monkey implantation. TIMP-1 may maintain the integrity of the trophoblast villi and maternal decidua, whereas TIMP-3 is the main inhibitor regulating the invasion of trophoblast cells.

Material and Method

Reagents

DIG-RNA labeling kit, blocking reagent, alkaline-phosphate-conjugated anti-DIG antibody, 4-nitro blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Beijing, China). Proteinase K was purchased from Merck-Schuchardt (Beijing, China). Restriction enzyme was purchased from Promega Corp (Beijing, China). Diethyl pyrocarbonate (DEPC), Ficoll, dextrasulfate were purchased from Sigma (Beijing, China). The plasmids that contain cDNA of MMP-2, TIMP-1, and TIMP-3 were kindly provided by Dr Ny T (Umeå University, Sweden).

Animals

Twenty-four rhesus monkeys (5–7 yr old)were obtained from the Primate Research Center in Fujian Research Institute of Parenthood and Family Planning. The monkeys used for the experiments have been approved by both the Academic Committee of Zoology, Institute of Chinese Academy of Sciences and the Academic Committee of the Primate Research Center in Fujian Research Institute of Parenthood and Family Planning. Menstrual cycles of the monkeys were monitored, and the animals were permitted to mate over a period of 3 d at the anticipated time of ovulation. The second day of mating was designated as d 0 of pregnancy. The presence of conceptus was confirmed by ultrasound diagnosis examination. The animals were divided into several groups. At various days from d 15 (6 d after implantation) to d 34 of pregnancy, the uterus was taken by surgery and cut carefully in half, allowing the embryo with the amnionic sac to be seen clearly in the uterine lumen. The implantation site (or placenta) contacted with the embryo is the primary implantation site, while the one on the counterside without the direct contact with the embryo is the secondary implantation site. On d 15, the implantation site was only a red point. The embryo and amnionic sac were removed, and the trophoblast villi with maternal decidua from the primary implantation site was cut vertically into small pieces. The samples were fixed in 4% formaldehyde in PBS (pH 7.4), while another part of each sample was preserved in liquid nitrogen. Surgery on the animals was carried out under the standard anesthetized conditions (preoperative 10–15 mg/kg of ketamine hydrochloride supplemented with 0.02 mg/kg of atropine sulfate and sodium pentobarbital 8–12 mg/kg were given).

Synthesis of the DIG-Labeled RNA Probe for MMP-2, TIMP-1, and TIMP-3

The DIG-labeled RNA probe was synthesized as previously reported *(10,23)*. The plasmids that contain the cDNA fragment of MMP-2, TIMP-1, and TIMP-3 were linearized with corresponding restriction enzyme and transcripted with corresponding RNA polymerases in vitro. Transcription was performed using an in vitro transcription system, and cRNA was labeled with digoxigenin using a Dig-RNA labeling kit purchased from Boehringer Mannheim. (Two microliters of RNA polymerase, 4 µL 5X buffer, 2 µL mix, 1 µg linearized plasmid and RNasin were added to an Eppendorf tube and mixed, followed by DEPC-treated water to a total volume of 20 µL. Incubate for 2 hr at 37ºC.) The validation of the labeled probe was evaluated with Dot Blot analysis.

In Situ Hybridization

Paraffin-embedded sections were deparaffinized in fresh xylene (twice each for 10 min), xylene:100% alcohol (1:1, 5 min), 100% alcohol (5 min), 95% alcohol (5 min), 90% alcohol (5 min), 80% alcohol (5 min), 70% alcohol (5 min), then the slides were washed in DEPC-treated PBS (three times each for 5 min), and permeablized with proteinase K (20 µg/mL) in TE buffer (100 m*M* Tris-HCl, 50 m*M* EDTA, pH 8.0) for 20 min. The sections were washed in PBS (twice each for 5 min), and post-fixed with 4% paraformaldehyde in PBS (4ºC, 10 min). The slides were washed with PBS (twice each for 5 min) and DEPC-treated H_2O (5 min). The sections were dehydrated with serial alcohol, air dried, then incubated in prehybridization buffer (2X SSC, 50% deionized formamide, room temperature, 2–4 h).

After prehybridization, the hybridization solution was applied onto the slides and covered with paraffin film. Hybridization solutions were made by mixing DIG-labeled cRNA probes (20–30 ng per slide) with 100 µL hybridization buffer (2X SSC, 50% deionized-formamide, 10 m*M* Tris-HCl, 250 µg/mL yeast tRNA, 0.5% SDS, 1X Denhardt, 10 m*M* DTT, 10% dextran-sulfate). The sections were incubated 16–20 h at 48ºC. At the end of hybridization, the paraffin film was removed from the incubated slides in 4X SSC; subsequently, the slides were washed with 2X SSC (twice, 15 min, room temperature), 1X SSC (twice each for 15 min, 42ºC), 0.1X SSC (twice each for 15 min, 42ºC). Sections were washed by shaking for 10 min with buffer 1 (100 m*M* Tris-HCl, 150 m*M* NaCl), covered with blocking-reagent solution (buffer 1 containing 1% blocking reagent) for 1 h, incubated in humid chamber with blocking solution

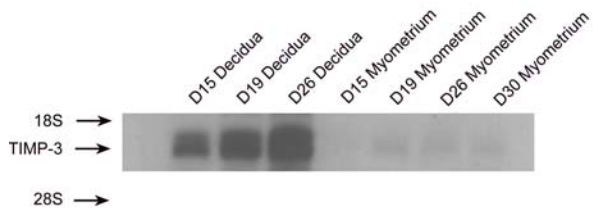

Fig. 5. Northern blot analysis of TIMP-3 mRNA in the decidua and myometrium.

containing anti-DIG alkaline phosphatase antibody (Fab fragment) at a dilution of 1:200, washed by shaking in buffer 1 (three times each for 10 min), and incubated with buffer 3 [100 m*M* Tris-HCl (pH 9.5), 100 m*M* NaCl, 50 m*M* $MgCl_2$] for 5–10 min. Sections were then covered with color generating solution [1 mL buffer 3, 4.5 L NBT solution (75 mg NBT/mL 70% dimethyl formamide), 3.5 L BCIP solution (50 mg BCIP/mL 100% dimethyl formamide)], and incubated in a humid chamber for 2–7 h in the dark. When the color development was optimal, the reactions were stopped by incubating the slides in buffer 1.

RNA Isolation and Northern Analysis

Total RNA was extracted from monkey decidua (containing chorionic villi) and myometrium by a single-step acid guanidine thiocyanate-phenol-chloroform procedure. Twenty micrograms of total RNA was electrophoresed on a formaldehyde denatured 1% agarose gel, vacuum blotted to a piece of Zeta-Probe nylon membrane (Bio-Rad Laboratories) at 45 mbar for 3 h, and crosslinked at 100 mJ by a GS Gene Linker UV chamber (Bio-Rad Laboratories). The membrane was prehybridized in 50% deionized formamide/5X SSC/0.1% N-lauroylsarcosine/0.02% SDS/2% blocking reagent (Boehringer Mannheim) at 68ºC for 2 h. It was then hybridized overnight to a DIG-labeled RNA probe (about 400 ng probe in 10 mL prehybridization buffer). After hybridization, the membrane was washed with 2X SSC twice for 10 min each at room temperature and 0.1X SSC twice for 15 min each at 68ºC. The membrane was then rinsed in DIG buffer I for 5 min, blocked with 1% blocking reagent in DIG Buffer I for 30 min, incubated with alkaline-phosphatase-conjugated anti-DIG IgG (Boehringer Mannheim) diluted 1:10000 in DIG Buffer I containing 1% blocking reagent for 30 min, and washed in DIG buffer I three times each for 10 min. The membrane was then rinsed for 5 min in 0.1 *M* Tris-HCl/0.15 *M* NaCl (pH 9.5), incubated with CDP-Star™ chemiluminescence reagent (Boehringer Mannheim), then exposed with a piece of FUJI medical X-ray film for 2–10 min.

Data Analysis

Each experimental group contained three monkeys, and the data were presented from at least three independent experiments. One of the representative figures from at least three similar results is shown (Fig. 5).

Acknowledgments

This work was supported by WHO/Rockefeller Foundation Network Project, and The "973" Pan-Deng and Chuang-Xin Programs and NSFS (39970284) in China.

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