Effects of vascular endothelial growth factor on MMPs during embryo implantation in mice

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Abstract Vascular endothelial growth factor (VEGF) is a specific mitogen of endothelial cells and plays an important role in vasculogenesis and angiogenesis. In recent years, both our and other laboratories have found that VEGF may be involved in embryo implantation. However, the relationship between VEGF and MMP-2 and MMP-9, the marker molecules of embryo implantation, is still unknown. In the present study, an examination of the effects of VEGF on the expression and secretion of MMP-2 and MMP-9 during implantation in mice was carried out by RT-PCR and gelatin zymography. The results show that VEGF antibody significantly decreased uterine mRNA levels of MMP-2 and MMP-9 during pregnancy, and that VEGF could up-regulate the activities of MMP-2 and MMP-9 secreted by blastocysts cultured *in vitro* in a time- and dose-dependent manner. The results suggest that, in the course of implantation, the ability for metastasis and invasion of blastocysts and the receptiveness of the uterus might be regulated by VEGF through its influence on the transcription and translation of MMPs.

Keywords: vascular endothelial growth factor, matrix metalloproteinase-2, matrix metalloproteinase-9, embryo, implantation.

Vascular endothelial growth factor is a specific mitogen for endothelial cells that exerts its functions through two receptor tyrosine kinases (Flt-1 and Flk-1/KDR). Many studies have shown that VEGF might influence the process of embryo implantation. After the initial attachment reaction between the embryo and endometrium in mice, luminal epithelial and stromal cells immediately surrounding the blastocyst exhibited accumulation of VEGF and its receptor mRNA. On successive days, the accumulation occurred in cells in the decidual bed at both the mesometrial and antimesometrial poles[1], suggesting that VEGF and its receptors could be involved in decidualization and placentation.

Charnock-Jones[2] demonstrated that mRNA encoding specific VEGF receptor, Flt-1, was highly expressed in the cytotrophoblast shell and columns and also by the extravillous trophoblast (EVT) in the maternal deciduas in the first trimester. In addition, the expression and distribution of VEGF and its receptors were observed in embryos during peri-implantation[1,3,4]. VEGF antibody could decrease the number of embryos that implanted and the percentage of blastocysts with attachment and outgrowth on the uterine epithelial cell monolayer in mice[4]. These results indicated that VEGF might induce proliferation and differentiation, as well as be involved in the invasion process of embryos implanting into the endometrium.

After the onset of implantation, trophoblasts penetrate the uterine epithelial basement membrane by the involvement of some proteinases, and finally was implanted in the endometrial stroma. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play a key role in tissue reconstitution during implantation[5,6]. MMPs are a family of zinc-dependent proteinases, which degrade components of the extracellular matrix (ECM), including collagens, glycoproteins, proteoglycans, etc. As MMPs, MMP-2 and MMP-9 play a vital role in the event of embryo implantation, and are regarded as the molecular markers of invasive trophoblasts[7].

Whether VEGF regulates embryo implantation through the involvement of MMPs is still unclear. In this study, effects of VEGF on the expression and secretion of MMP-2 and MMP-9 in the process of mouse embryo implantation were investigated using RT-PCR and gelatin zymography, and the molecular mechanisms by which VEGF acts in embryo implantation were discussed.

1 Materials and methods

（ⅰ）Reagents. Monoclonal anti-human vascular endothelial growth factor (VEGF), fibronectin (FN) and bovine serum albumin (BSA) were manuscriotured by Sigma. Ham's F-12 and TRIzol were purchased from Gibco BRL. Oligo(dT)$_{18}$ and reverse transcriptase MMLV and Taq DNA polymerase were from Promega. GAPDH primer was a product of the Beijing Dingguo Biotechnology Development Center.

（ⅱ）Animals. Adult female mice of the outbred Kunming white strain（5—6 week old, 25—30 g in weight）were supplied by the Experimental Animal Center of the Institute of Heredity Science, Chinese Academy of Sciences and raised at room temperature (25℃) in a constant photoperiod (light：dark cycle, 12 h：12 h). Food and water were freely available.

（ⅲ）Semi-quantitative reverse-transcription chain reaction (RT-PCR). After mating, the morning when a vaginal plug was found was designated the first day of pregnancy (D1). In the afternoon of D4, pregnant mice were given an injection of 3 μg VEGF antibody (diluted in 3 μL PBS) into the lumen of the left uterine horn adja-

cent to the oviduct as the treatment, and 3 μg mouse IgG (also diluted in 3 μL PBS) into the right horn as the control. Total RNA was isolated from uteri on D6, D7 and D8 using TRIzol. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out following the method described in ref. [8]. We used the housekeeping gene, GAPDH, as an internal standard. The gene-specific primers for MMP-2 and MMP-9 were designed according to ref. [9]. All gene sequences are listed in table 1.

Table 1   Details of primers used for RT-PCR

Gene	Primer	Sequence (5′—3′)	Fragment size/bp
GAPDH	sense	GGTCGGAGTCAACGGATTTG	350
	antisense	ATGAGCCCCAGCCTTCTCCAT	
MMP-2	sense	CACCTACACCAAGAACTTCC	332
	antisense	AACACAGCCTTCTCCTCCTG	
MMP-9	sense	TTGAGTCCGGCAGACAATCC	433
	antisense	CCTTATCCACGCGAATGACG	

10 μg RNA were denatured at 72℃ for 5 min, then cooled on ice. The RNA was mixed, in a final volume of 20 μL, with 1.5 μg Oligo (dT)$_{18}$, 0.5 μL Rnasin, 250 μmol/L each dNTPs and 300 U MMLV reverse transcriptase. The mixtures were reacted at 42℃ for 1 h, then the reactions were stopped by putting into 95℃. 1/10 of RT products, 1 μg of each specific primer, 150 μmol/L each dNTPs and 1 U Taq DNA polymerase were added into PCR buffer solution, and the total volume was 100 μL. PCR cycles were as follows: 95℃ for 5 min, followed by 35 cycles of 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min. 10 μL of the PCR products were visualized under ultraviolet light on 1.0% (*w/v*) agarose gels, and quantified by densitometry.

(ⅳ) Culture *in vitro*.   The adult female mice were injected routinely with PMSG-hCG to stimulate superovulation. The day when a vaginal plug was found was designated the first day of pregnancy (D1). The female mice were killed on D4 and their blastocysts were collected. The medium in the control group (C) was Ham's F-12 containing 0.24 mg/mL calcium lactate, 0.3 mg/mL glutamine, 1.6 mg/mL NaHCO$_3$, 400 U/mL gentamycin sulfate, 0.4% BSA and 1 μg/mL estradiol. The media in the treatment groups were Ham's F-12 medium complemented with the following dilutions of VEGF antibody: 0.1 μg/mL (T1) and 1 μg/mL (T2). Culture dishes were precoated with 10 μL FN (1.0 mg/mL). Blastocysts cultured for 24 h that developed consistently were put into 50 μL droplets (1—2 blastocysts per μL culture medium) under mineral oil (37℃, 5% CO$_2$). Culture media from blastcysts treated with VEGF antibody for 6, 24 and 48 h

were subjected to gelatin zymography to examine the activities of MMP-2 and MMP-9.

(ⅴ) Gelatin zymography.   Zymography was carried out according to ref. [10] with some modifications. The samples were standardized according to the protein content. 20 μg of protein was loaded on to each lane for zymography. The medium was mixed in 5︰1 with a sample buffer (27.6 g/L SDS, 70 g/L sucrose and 1 g/L phenol red) and then applied to electrophoresis in a stacking gel containing 2% acrylamide (100 V) and a running gel containing 10% acrylamide and 1 mg/mL gelatin (150 V). After electrophoresis, the gels were washed in 2.5% Triton-X 100 for 1 h (10 min×6) to remove SDS and incubated for 18 h in a buffer (10 mmol/L CaCl$_2$, 50 mmol/L Tris-HCl, pH 7.6) at 37℃. Thereafter, gels were stained with Coomassie Brilliant Blue G-250 and then destained. The gelatinolytic activities were detected as clear bands on a uniform background and quantified by densitometry.

(ⅵ) Statistical analysis.   All results are shown as mean±SE. Differences were evaluated with the Student's *t*-test. Values of $P < 0.05$ were accepted as significant.

2   Results

(ⅰ) RT-PCR.   Total RNA was extracted from pregnant uteri on D6, D7 and D8. Levels of gene expression of MMP-2 and MMP-9 were determined by RT-PCR, and normalized to the GAPDH gene. The results showed that treatment with the uterine injection VEGF antibody could decrease mRNA encoding MMP-2 and MMP-9 in mouse uteri (fig. 1). VEGF antibody produced a maximal inhibition to mRNA of MMP-2 on D6, but subsequently this effect became weaker. However, levels of mRNA encoding MMP-9 were markedly reduced, with maximal suppression occuring on D7 (fig. 2).
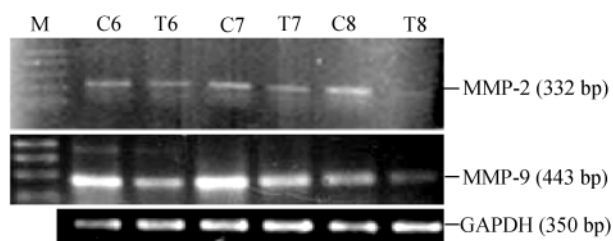

Fig. 1.   Effect of intrauterine injection of VEGF antibody on expression of MMP-2 and -9 mRNA in pregnant mouse uteri. M, PCR molecular mass marker; C, control; T, treatment.

(ⅱ) Gelatin zymography.   Culture media from blastocysts treated by VEGF antibody for 6, 24 and 48 h were subjected to gelatin zymography to examine the activities of MMP-2 and MMP-9. The results showed that

the concentrations of the VEGF antibody used could inhibit gelatinolytic activities of MMP-2 and MMP-9 secreted by blastocysts in a dose-dependent manner (figs. 3 and 4). Furthermore, maximal depression appeared after 24 h treatment, suggesting that the antibodies effect had peaked at this period.
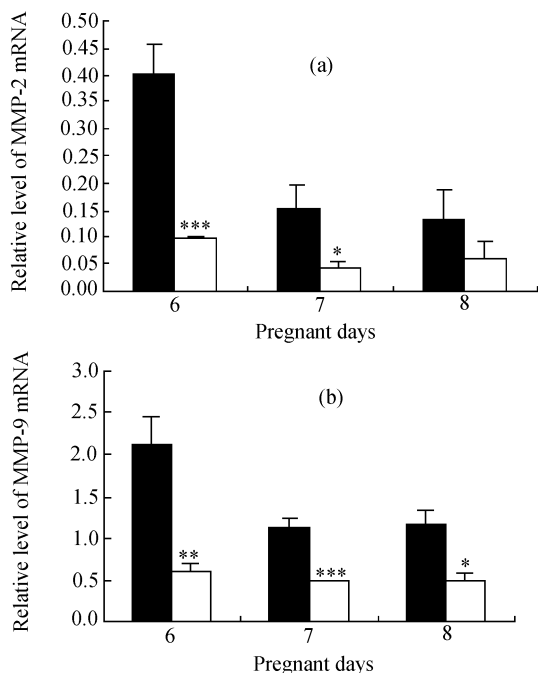

Fig. 2. Effect of intrauterine injection of VEGF antibody on expression of MMP-2 and -9 mRNA in pregnant mouse uteri. ■ Control; □ treatment. $*P < 0.05$; $**P < 0.01$; $*** P < 0.001$. Results are representative of three experiments.

3 Discussion

During implantation, MMP-2 and MMP-9 are believed to play a role in the tissue remodeling that accompanies decidualization in the endometrium and in embryo invasion. MMP-2 may participate in the early phase of decidualization and neovascularization required for placentation, while MMP-9 may regulate trophoblast invasion in the uterus[5,9,11]. Many studies indicate that the expression and activity of the two MMPs might be induced by some cytokines or growth factors, such as leukaemia inhibitory factor (LIF)[8,12] and epidermal growth factor (EGF)[12]. Bany et al.[9] demonstrated that MMP-2 and MMP-9 were mainly localized in the endometrium on D5 and D8 of pregnancy in mice, and continuously varied with pregnancy. MMP-2 and MMP-9 were also localized in the myometrium, and this localization did not change with pregnancy, but no signals were detected in placenta percreta. Changes in the pattern of expression of MMP-2 and MMP-9 in the endometrium during implantation can occur without direct signals from the embryo. Our work shows that VEGF antibody could reduce the levels of MMP-2 and MMP-9 in both uteri and blastocysts, which suggests that it may influence the secretion of these MMPs in endometrium.

VEGF antibody inhibits the transcription and translation of MMP-2 and MMP-9, thereby blocking extracellular matrix (ECM) degradation and tissue remodeling, which, in turn, decreases embryo invasiveness and endometrial receptivity during implantation. This is consistent with the conclusion of Zhang et al.[4]. FN is a major ECM, which could be bound to integrin, activating the Ras/MAPK (mitogen-activated of protein kinase) signal transduction pathway[13—15] and initiating the gene expression[8] and activity[16] of MMP-2 and MMP-9. It has been demonstrated that VEGF could increase the activities of MMP-1 and MMP-9 through its receptor Flt-1 in vascular smooth muscle cells[17], as well as the activity of MMP-2 in dermal microvascular endothelial cells[18]. We infer that the ability of VEGF antibody to inhibit the expression of MMPs in uterus and the activity of MMPs in embryos might be achieved by interrupting its receptors activation and then influencing the transcription and translation of MMPs through the signal pathway of FN-integrin-proteinases, or through the pathway of cytokines-proteinases directly[8]. Shao et al.[19] reported that VEGF might mediate angiogenesis and capillary hyperpermeability in rat uteri during peri-implantation. Therefore, we speculate that VEGF antibody might prevent the angiogenesis necessary for implantation, and then disturb embryo implantation in the endometrium.
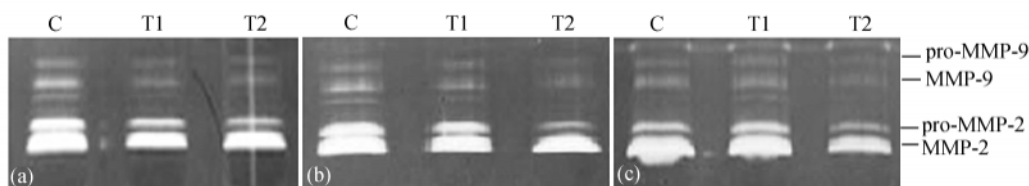

Fig. 3. Effects of VEGF antibody on the activities of MMP-2 and -9 secreted by mouse blastocysts. (a) Cultured for 6 h; (b) 24 h; (c) 48 h. C, Control; T1, 0.1 μg/mL antibody; T2, 1 μg/mL antibody.
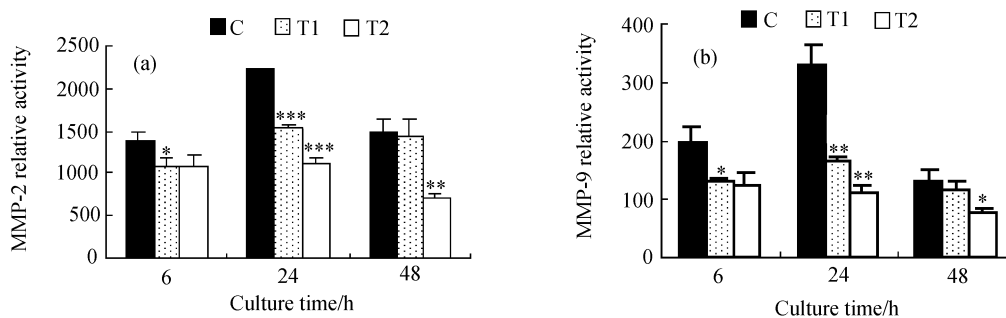
Fig. 4. Effects of VEGF antibody on the activities of MMP-2 and -9 secreted by mouse blastocysts. C, Control; T1, 0.1 μg/mL antibody; T2, 1 μg/mL antibody. All data are the concentrated sums of bands of MMPs and pro-MMPs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Results are representative of four different experiments.

TIMPs form a 1 : 1 stoichiometric complex with activated MMPs in which TIMPs inactivates the latter; TIMPs also bind pro-MMPs in which TIMPs activate zymogen[20]. Therefore, TIMPs have a two-side regulative effect on MMPs. In the normal physiological process, such as embryo implantation, the balance of TIMPs and MMPs is necessary for embryo invasion into endometrium strictly[5,6]. Further work is required to determine whether VEGF antibody only regulates the activities of MMPs, or if it can also affect the expression of TIMPs, and then inactivate MMPs.

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