Expression of vascular endothelial growth factor in rat uterus during peri-implantation

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Abstract **The first distinct mark of rodent implantation is the increased vascular permeability and significant angiogenesis at the sites of blastocyst implantation, but its mechanism is not clearly defined. Vascular endothelial growth factor (VEGF) is the key mediator for angiogenesis during embryogenesis and adult span and also serves as a vascular permeability factor. The aim of this study is to explore VEGF regulation mechanism and the possible role that VEGF plays in implantation by studying the VEGF expression and angiogenesis in the rat uterus during estrous cycle, ovarioectomized and peri-implantation stages using *in situ* message RNA hybridization and confocal laser scanning techniques. The results indicated that VEGF was regulated by ovarian steroid hormones. VEGF expression before implantation was localized at luminal epithelium, shifted to stroma as implantation initiated and extensively located at the decidualizing stroma region after implantation. Bandeiraea simplicifolia-1 (BS-1) agglutinin and antibody against von Willebrand factor (vWF) were used to mark the endothelial cells and blood vessels. The results showed that the active angiogenesis occurred during the implantation process and this effect was probably mediated by VEGF. The results suggest that under the regulation of ovarian steroid hormones, VEGF plays an essential role in angiogenesis and increasing vascular permeability in endometrium, which are necessary for successful implantation.**

Keywords: rat, vascular endothelial growth factor, embryo implantation, angiogenesis.

The process of embryonic implantation is a series of complex events and accounts for an essential tache involved in pregnancy. Parallel to the early attachment between uterine epithelium and blastocyst trophectoderm, the first conspicuous mark during rodent implantation is an increased uterine vascular permeability at implantation sites, which is regarded as the earliest prerequisite[1]. In the rat, the increased uterine vascular permeability happens on the afternoon of D5 (1700—1900) of gestation and lasts for a short period, known as the receptive phase. At this time, vascular bed in endometrium expands markedly; vascular permeability increase in subepithelium stroma leads to local edema; afterwards local decidualization occurs in endometrial stroma and apoptosis emerges in epithelial cells at the sites of blastocyst implantation, which facilitate the invasion of trophoblast cells through the underlying base membrane and stroma[2]. These pro- cesses are accompanied by the remodeling of the extracellular matrix (ECM) and angiogenesis in the endometrial stroma. However, until now, the molecular mechanisms for the focal vascular permeability increase and angiogenesis occur in the uterus at the sites of implantation are poorly defined.

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein, a specific mitogen for endothelial cells and a potent inducer of vascular permeability[3]. VEGF works via binding to the highly affinity tyrosine kinase receptors. Two of the most important receptors are c-fms-like tyrokinase kinase (flt-1, VEGFR-1) and fetal liver kinase-1/kinase domain receptor (flk-1/KDR, VEGFR-2) that selectively express in the vascular endothelium[3]. VEGF can potently induce angiogenesis by stimulating endothelial cells proliferation and migration, vascular permeability increase and invasion into tissues, and is also associated with embryo development[3]. VEGF or its two receptors of genes knockout mouse result in embryo development defect and embryonic lethality during mid-pregnancy[4,5]. Since VEGF is a potent inducer of vascular permeability and angiogenesis, which are critical to rat implantation, we used *in situ* message RNA hybridization and immunofluorescence techniques to examine the expression of VEGF and angiogenesis in rat uterus, tried to explore VEGF regulation mechanism and its effects on implantation.

1 Materials and methods

(ⅰ) Reagents. Estrodiol (E_2) was purchased from Baker Chemical Co., USA; progesterone (P_4), paraformaldehyde, bovine serum albumin (BSA), poly-L-lysine, propidium iodide (PI), trypan blue and FITC labeled Bandeiraea simplicifolia agglutinin (BS-1) were purchased from Sigma, USA; poly-antibody against VEGF was purchased from Santa Cruz Co., USA; poly-antibody against vWF and FITC conjugated goat IgG against rabbit were purchased from Zymed Co., USA; T7 and SP6 RNA polymerases and endonucleases were purchased from Promega, USA; tissue freezing medium was purchased from Triangle Biomedical Sciences, USA; digoxigenis (DIG) conjugated RNA labeling kit and digoxigenis detection kit were purchased from Roche, Germany.

(ⅱ) Animals. Adult Sprague-Dawley (SD) rats (250—280 g) were provided by the Animal Faculity of the Institute of Zoology, the Chinese Academy of Sciences. Rats were divided into 3 groups, 4—5 animals per treatment. (a) E_2 or P_4 was dissolved in sesame oil and injected intraperitoneally (i.p) once into ovarioectomized rats after a 2-week recovery period. The dosages were E_2 (0.1 mg), P_4 (0.5 mg) and E_2+ P_4 (0.1 mg + 0.5 mg). The control rats were injected with vehicle sesame oil (0.5 mL) alone. 24 h later, we killed the animals, collected the uteri, trimmed the extraneous tissues and cut the uteri into 1 cm segments in length. After being fixed with 2% paraformaldehyde in PBS (pH 7.2), the samples were washed with 0.01 mol/L PBS (pH 7.4) and dipped in 30% sucrose overnight until the uterus segments floated. Then, the uteri were coated with tissue freezing medium and stored in −80℃ until used. (b) Rats in different phases of the estrous cycle were ascertained by examining vaginal smears and the uterus samples were collected according to the above protocol. (c) Rats in proestrus or estrus were allowed to mate with the same strain male rats (3∶1) to induce pregnancy and vaginal smear was examined the next morning. The presence of sperm was designated day 1 of pregnancy (D1). Pregnant rats were killed between 0830 and 1000 on D1—D7, 4 animals between 1700 and 1900 on D5.5 were anaesthetized and injected trypan blue dye solution (0.05%) from tail vein and killed 10 min later. The uterus samples (whole uterus on D1—D5 and those blue bands on D5.5 and protuberant sites on D6—D7, i.e. the implantation sites) were collected according to the protocol of the treatment (a). Cryosections (10—12 μm) were made and mounted onto poly-L-lysine pre-coated slides, stored at −80℃ until used.

(ⅲ) Probes labeling and *in situ* hybridization assay. Plasmid for VEGF was generously presented by Prof. S. K. Dey. The VEGF plasmid cDNA was linearlized with *Eco*R Ⅰ and *Hin*dⅢ, purified with saturated Tris-hydroxybenzene, chloroform and isoamyl alcohol and used as templates for antisense and sense DIG-UTP-labeled riboprobe synthesis. The probes synthesis was performed using SP6 and T7 RNA polymerase respectively and a DIG RNA labeling kit according to the kit guide. The nonradioactive procedures of *in situ* hybridization were performed according to Schaeren-Wiemers et al.[6,7]. The hybridization signal was visualized by a DIG detection kit according to the kit guide. The adequately stained slides were treated with TE buffer, dehydrated with gradual alcohol, dried and mounted with gelatin. The control experiment was performed using sense probe instead of antisene probe, and no specific signal was found.

(ⅳ) Immunofluorescence assay. Cryosections at −80℃ were brought to room temperature, dried and fixed for 10 min with cool acetone at 4℃, washed in 0.01 mol/L PBS (pH 7.4) and blocked with 0.5% BSA. Then the sections were incubated with antibody against VEGF (1∶50 dilution) or vWF antibody (1∶100 dilution) overnight at 4℃. After PBS washing, the sections were incubated with FITC conjugated goat against rabbit IgG (1∶100 dilution) for 30 min at 37℃. After PBS complete washing, the sections were coated with PBS: glycerol (9∶1) and mounted with nail oil smeared at the edge of slides. After curdling, the distribution of fluorescence in the sections was observed with a confocal laser scanning microscope (Leica, Germany). For easy observation, some sections were incubated with PI (10 μg/mL) for 10 min at 37℃ to stain the nuclear nucleus. Fluorescence labeling and the afterwards treatment were performed in dark. The confocal laser scanning showed two colors on the computer screen: red indicated PI labeled nuclei and green indicated FITC labeled antigen locations. The negative control was performed according to the same steps as the positive experimental procedures except using PBS in place of primary antibody. The control had no specific staining.

FITC labeled BS-1 was used for direct immunofluorescence. The procedure was the same as the steps above except the secondary antibody steps. Green indicated FITC labeled vascular endothelial cells and red indicated PI labeled nuclei.

2 Results and discussion

Firstly we observed that VEGF mRNA expressed in a cell type and time specific pattern during estrous cycle. In cyclic rat uterus, only weak VEGF mRNA signal expressed in diestrus (fig. 1(a)) but strong signals in proestrus expressed in luminal and glandular epithelium (fig. 1(b)). When at estrus stage, VEGF mRNA shifted to stroma (fig. 1(c)). After that in metaestrus, faint signal was found in luminal and glandular epithelium (fig. 1(d)). This result accorded well with the previous report by Karuri[8]. VEGF expression variations probably result from the variations of ovarian steroid hormones: There is an E_2 and a P_4 peaks in proestrus and a sub-peak in diestrus[9]. However, the intensity of VEGF expression in estrus was higher than that in proestrus and that in proestrus was higher than in diestrus, which implied that the production of VEGF mRNA lagged behind that of ovarian steroid hormines and the hormones secreted in proestrus exert its influence in estrus. Ovarivectomized rats model was used to further study the VEGF mRNA regulation by steroid hormones. The results showed that two weeks after the ovarioectomized operation, the control rat uterus had very faint VEGF mRNA signal, E_2 treatment strongly increased the expression in luminal and glandular epithelium while P_4 had no detectable effect compared with oil treatment (control), while the combined use of both hormones could increase the expression of VEGF mRNA in both stroma and epithelium (fig.1(e)—(h)), which implied that E_2 is the primary regulator for VEGF in two steroids examined. The early scholars had also observed that E_2 significantly increased the VEGF mRNA and protein in human uterine stroma cells and epithelial cells *in vitro* but P_4 had no effect[10]. However, others reported that total VEGF mRNA in uterus of rats treated with P_4 (5 mg/100 g BW) increased significantly determined by Northern blot technique[11]. The possible reasons for the contradiction may lie in the lower P_4 dosages or lower sensitivity of *in situ* hybridization technique used in this study. Besides, we noticed the unreported phenomenon that the combined use of E_2 and P_4 could increase the VEGF mRNA expression in stroma (fig. 1(f)).

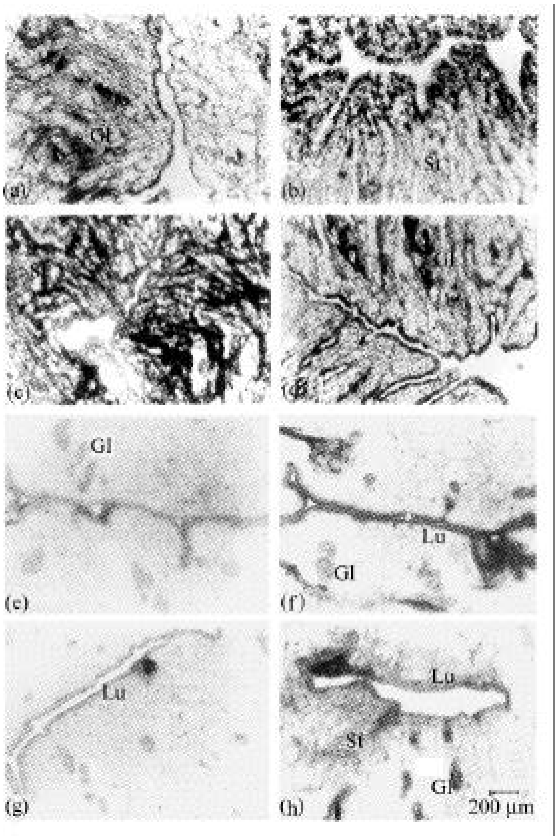

Fig. 1. Pictures of *in situ* hybridization of expression of message RNA for VEGF in uterus of cyclic rats (a)—(d) and ovarioectomized rats (e)—(h). (a) Diestrus; (b) proestrus; (c) estrus; (d) metaestrus; (f) estrodiol; (g) progestone; (h) estrodiol + progestone; (e) control (sesame oil). Lu, Luminal epithelium; Gl, glandular epithelium; St, stroma.

The open of "implantation window", i.e. the acquirement of receptiblity for blastocyst in rat uterus, limits in a fugacious time. During this period occurs the attachment between blastocyst and maternal uterus luminal epithelium and simultaneously happans the increase of vascular permeability at implantation sites, which are believed to be triggered by the timely interplay of the estrogen and progesterone[12,13], but whether or not VEGF mediates this process is unknown. Our results showed that during the early stage before implantation (D1, D5), VEGF mRNA was mainly expressed in luminal and glandular epithelium, only a little signals emerged in stroma (fig. 2(a),(b)). On D5.5, the signals were seen in luminal epithelium, stroma and embryo and also in some spatial arteriole endothelium (fig. 2(c)). The VEGF mRNA was extensively expressed in myometrium and decidualizing regions on D6 and D7 (fig. 2(d), (e)). The

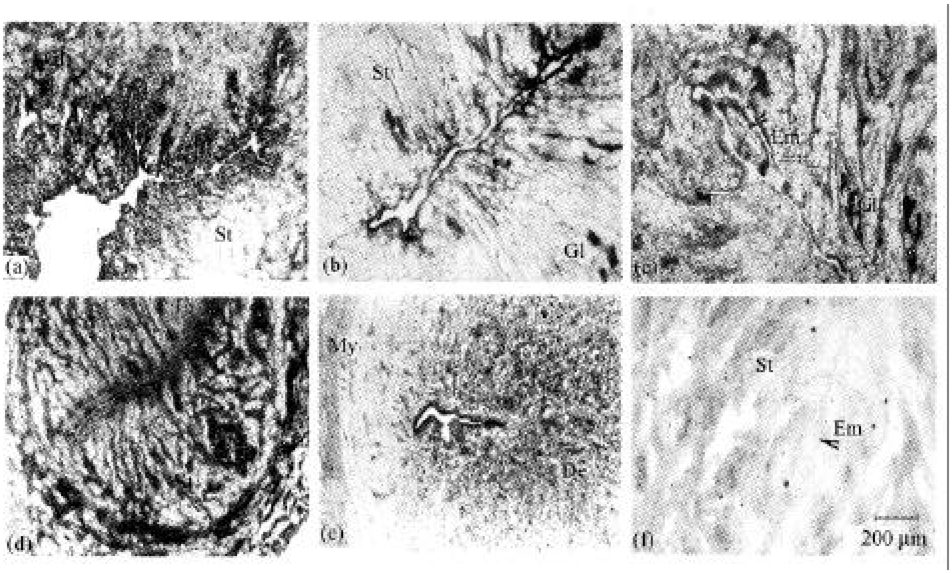

Fig. 2. Pictures of *in situ* hybridization of expression of message RNA for VEGF in rat uterus during early pregnancy. (a) D1; (b) D5; (c) D5.5; (d) D6; (e) D7; (f) D6 sense probe negative control. Gl, Glandular epithelium; St, stroma; Em, embryo; De, decidua; My, myothelium.

similar pattern for VEGF mRNA expression was also found in mouse[13]. The results of VEGF immunofluorescence assay showed that VEGF protein distribution was similar to that of VEGF mRNA: VEGF protein was localized in luminal and glandular epithelium on D3, in luminal epithelium and stroma on D5 and in decidulization region on D7 (Plate Ⅰ-1—3), which means that VEGF protein can be produced by several kinds of cell types during implantation.

The increased vascular permeability and angiogenesis are necessary for implantation. It is well known that the increased vascular permeability occurs at implantation sites. We further studied the angiogenesis in rat endometrium during peri-implantation with Bandeiraea simplicifolia agglutinin (BS-1) and poly-antibody against VEGF. BS-1 agglutinin can specifically bind to the membrane of endothelial cells of rat and mouse species and is a good marker for endothelial cells during angiogenesis[14]. vWF (von Willebrand factor), also named Ⅷ factor related antigen, is a glycoprotein that endothelial cells were secreted into extracellular matrix (ECM), so its specific antibody can be used to recognize blood vessel. Confocal scanning results showed that BS-1 marked cells distribution on D6 was more dense than that on D3, and some endothelial cells are in the division state, which implied that the proliferation of endothelial cells during implantation is more active than that during peri-implantation (Plate Ⅰ-4—6). vWF positively marked microvessels on D6 were more than those of D3 and the vessels were much slimmer in shape (Plate Ⅰ-7—9), which indicated that a number of immature microvessels had formed during implantation.

VEGF mRNA and protein were expressed in a time and space specific pattern and active angiogenesis happened during peri-implantation, and the VEGF expression was regulated by ovarian steroids, which implied that probably it was VEGF that mediated the vascular permeability increase and angiogenesis, and the newly formed endothelial cells could produce VEGF in turn. The accumulation of VEGF in luminal epithelium before implantation was probably due to the result of the estrogen peak during pre-ovulation while peri-implantation (D4—D6) VEGF accumulation in luminal epithelium and sub- epithelial stroma bed may be the result of active endothelial cells induced by a little amount of estrogen and high progesterone level. This guess accords with the results of the ovarioectmized rat and the former study on mouse[13].

Some other studies showed that VEGF participated in trophoblasts cells invasion, migration and endometrium decidualization process during implantation[15,16]. Besides, inhibited attachment and implantation were found in those mice treated with antibody against VEGF[16], which indicates that VEGF plays a multifunctional role in implantation. Due to the high similarity between tumor invasion and implantation, further study of the effects of VEGF and its receptors on implantation not only help elucidate the mechanism of implantation, but also exploit the medicines for contraception or tumor antagonists.

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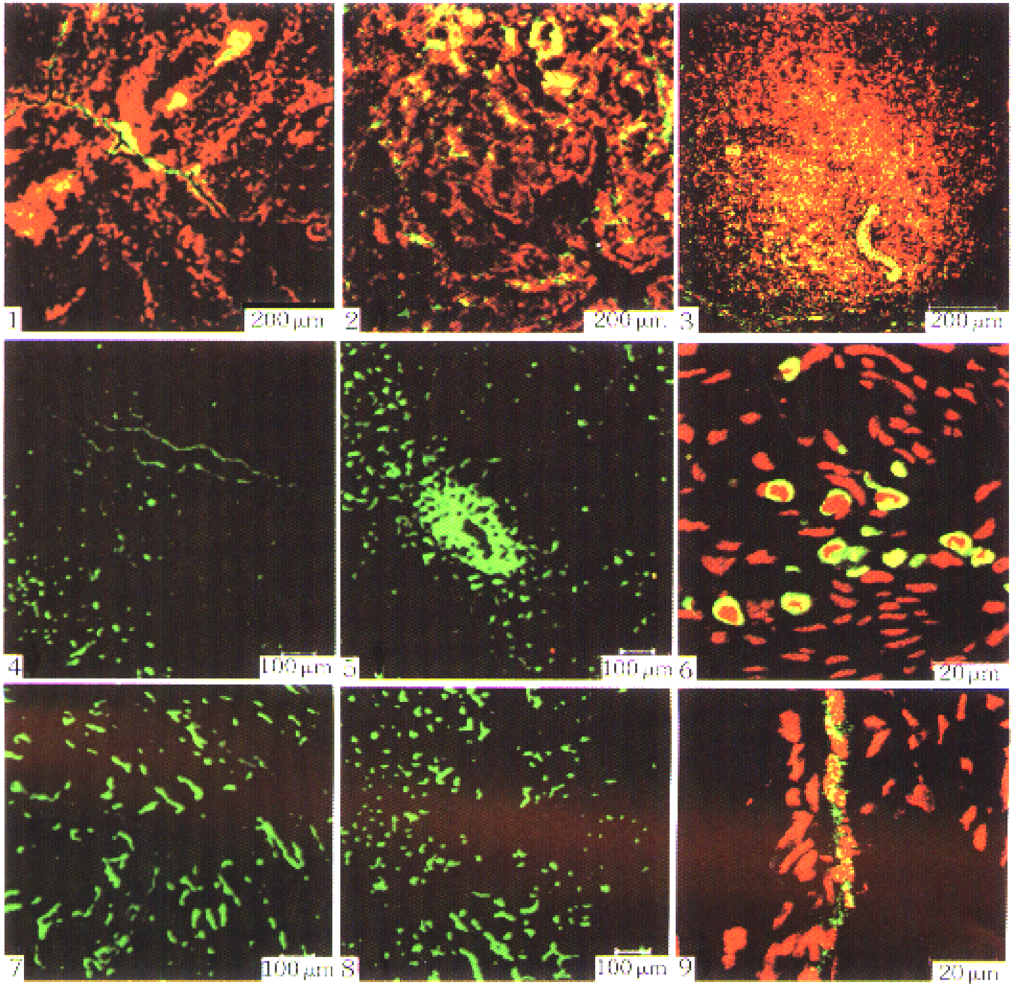

Pictures of immunofluorescence location of VEGF protein, endothelial cells and microvessels in rat uterus during peri-implantation. 1—3, Location of VEGF protein on D3, D6 and D7, respectively; 4 and 5, location of BS-1 agglutinin marked endothelial cells on D3 and D6, respectively; 6, a part of proliferating endothelial cells on D6 with a higher magnification; 7 and 8, location of vWF antibody marked blood vessel in rat uterus on D3 and D6, respectively; 9, a part of microvessel with a higher magnification. Red indicates PI marked nuclei; green indicates FITC marked signal.