PROMOTION OF MOUSE PREIMPLANTATION EMBRYO DEVELOPMENT BY VASCULAR ENDOTHELIAL GROWTH FACTOR

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Abstract

Vascular endothelial growth factor (VEGF) is a regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions. VEGF and VEGF receptors (VEGFRs) have been identified in several reproductive tissues, including the oviducts and uterus. VEGF/VEGFR signaling systems have been shown to play crucial roles during embryo development after implantation. The present study examined expression of VEGFR-1, VEGFR-2 and VEGFR-3 proteins in mouse 2- and 4-cell embryos, morulae and blastocysts during development using immunocytochemistry. In blastocysts, VEGFR-1 and VEGFR-3 proteins were exclusively localized in trophectodermal cells, but not inner cell mass cells. However, VEGFR-2 protein was not observed in all embryonic stages examined. As VEGF expression has been demonstrated in mouse oviduct and uterus, these results indicate paracrine roles of VEGF in the development of preimplantation embryos. *In vitro* analyses showed VEGF promotes the development of mouse preimplantation embryos from early blastocysts to hatched blastocysts in a dose-dependent manner. The effect of VEGF on blastocyst development was suppressed by treatment with a neutralizing VEGF antibody, whereas antibody alone was ineffective. Our findings suggest potential paracrine roles of VEGF in promoting the development of early embryos.

Key words: VEGF; VEGFR; preimplantation embryo

Introduction

The vascular endothelial growth factor (VEGF) family contains several other proteins that are regulators of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions and are highly related structurally: VEGFA; placental growth factor (PLGF); VEGFB; VEGFC; VEGFD; and VEGFE[1,2]. VEGF has 3 transmembrane receptors: VEGF receptor (VEGFR)-1 (flt-1); VEGFR-2 (KDR or flk-1); and VEGFR-3 (flt-4)[3]. VEGFA binds to both VEGFR-1 and VEGFR-2, while PLGF and VEGFB bind to VEGFR-1, VEGFC and VEGFD bind to VEGFR-2 and VEGFR-3[1]. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permea-

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bility-enhancing effects of VEGF, whereas VEGFR-1 inhibits VEGFR-2 signaling during development of the vascular system[3]. VEGFR-3 is expressed exclusively in lymphatic endothelium in adults, and plays a crucial role in lymphogenesis[4].

VEGF and VEGFRs have been identified in several reproductive tissues. VEGF expression has been demonstrated in mouse ovary and uterus[5] and human uterus[6]. Expression of VEGF and VEGFRs has been confirmed in embryonic implantation sites in mice[5], bovine oviduct[7], human oviduct[8,9] and endometrium during the menstrual cycle[10], suggesting important roles in early reproduction.

VEGF mRNA has been detected in 8-cell embryos, morulae and blastocysts, but not in oocytes, by real-time polymerase chain reaction (PCR) in human preimplantation embryos[11], but expression of VEGFRs has not been explored. The present study investigated temporal and spatial expression of VEGFRs in mouse preimplantation embryos and paracrine effects of VEGF in early embryo development.

Materials and Methods

Animals

IVCS mice (Institute for Animal Reproduction, Ibaragi, Japan) were used for all experiments. All procedures involving the care and use of animals were approved by the Animal Research Committee at Akita University School of Medicine.

Collection of mouse preimplantation embryos

Nine-week-old mice were superovulated with a single intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin (Teikoku Hormone Manufacturing, Tokyo, Japan), followed 48 h later by 10 IU of human chorionic gonadotropin (hCG) (Teikoku Hormone Manufacturing). Two-cell embryos were obtained by flushing the oviducts of mated mice at 46-47 h after hCG injection. Embryos were washed 3 times with human tubal fluid (HTF) medium (IS Japan, Saitama, Japan). Groups of 15 embryos were subsequently placed in 30-μl drops of HTF medium, covered by mineral oil, and cultured at 37°C in 5% CO_2 in air. For RT-PCR analysis, 4-cell and 8-cell embryos, morulae and blastocyst-stage embryos were collected from cultures in individual micro-drops at 50-52, 68-70, 90-92 and 118-120 h after hCG injection, respectively.

Immunofluorescence and laser-scanning confocal microscopy

To examine VEGFR protein expression in preimplantation embryos, immunostaining was performed because levels were below the detectable level of Western blot analysis. Collected preimplantation embryos were analyzed by indirect immunofluorescence and laser-scanning confocal microscopy. Primary antibodies were rabbit polyclonal antibody Flt-1/VEGFR-1, rabbit polyclonal antibody Flk-1/KDR/VEGFR-2 (Thermo Fisher Scientific, Fremont, CA, USA) and rabbit polyclonal antibody Flt-4/VEGFR-3 (Santa Cruz, Santa Cruz, CA, USA). Rabbit polyclonal antibody VEGFR-1 and VEGFR-2 were Ready-to-Use for Immunohistochemical Staining. Rabbit polyclonal antibody VEGFR-3 was diluted to 1 : 500 in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (Sigma, St. Louis, MO, USA).

Embryos of each stage were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C after two washes, then washed three more times. For permeabilization, embryos were placed in 2.5% Tween 20 for 5 min at room temperature, then washed three times. Embryos were blocked with 10% normal goat serum (DAKO, Kyoto, Japan) for 30 min at room temperature. Embryos were incubated with each primary antibody overnight at 4°C. After three washes in cold Tris-buffered saline with Tween 20 (TBS-T), embryos were incubated with 1.0 μg/ml of goat antirabbit Cy-3 fluorescein antibody (Zymed Laboratories, San Francisco, CA, USA) in 1% PBS-BSA/10% goat serum for 1 h at room temperature in the dark. To stain nuclei, samples were incubated in 1% Hoechist33342 for 30 min at room temperature after two washes in TBS-T, then washed three times in PBS. Embryos were

placed in a drop of SlowFade Light Antifade Kit (Molecular Probes, Eugene, OR, USA) and analyzed using an LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Negative controls were created by substituting primary antibodies with equivalent concentrations of normal rabbit immunoglobulin (Ig)G serum.

Embryo cultures

Two-cell embryos were collected as described above. Embryos were washed three times with the modified-HTF medium (IS Japan). Groups of 10 randomly selected embryos were placed in 30-μl drops of HTF medium with or without recombinant VEGF (0.1, 1, 10 or 100 ng/ml; PeproTech EC, London, UK) covered by mineral oil. Embryos were cultured over 72 h up to the hatched blastocyst stage at 37°C in 5% CO_2 in air. Embryos without VEGF treatment served as controls. To examine the effects of VEGF on preimplantation embryos, embryos were cultured in HTF medium with 0.2 μg/ml rabbit anti-mouse VEGF (Chemicon International, Temecula, CA, USA) and 10 ng/ml of recombinant VEGF. Embryo development was monitored after 56 and 72 h of culture to determine the proportion of expanded blastocysts and hatched blastocysts, respectively.

Statistical analysis

To analyze the effects of VEGF on development of embryos in a dose-dependent manner, data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference test and trend analysis.

Results

Detection of VEGFR protein in mouse preimplantation embryos

To determine the expression of VEGFR proteins in preimplantation embryos, immunostaining was performed in early embryos at different developmental stages (2-cell, 4-cell and 8-cell embryos, morulae, and blastocysts) (Fig. 1).

VEGFR-1 signal was localized in the cell surface of embryos at all the stages examined. In blastocysts, VEGFR-1 signal was detected in trophoectoderm cells, but inner cell mass cells were not stained with VEGFR-1. In 2-cell, 4-cell and 8-cell embryos and morulae, immunoreactivity for VEGFR-3 was observed on the cell surface, whereas weaker VEGFR-3 signal was detected in blastocysts. Embryos incubated with VEGFR-2 antibody displayed faint staining. Specific signals were undetectable in controls in which VEGFR antibodies were substituted for equivalent concentrations of normal rabbit IgG serum.

All experiments were performed at least three times and a total of at least 10 embryos from each group were surveyed, with representative results shown here.

Effect of VEGF on development of mouse embryos in vitro

Based on the presence of VEGFR proteins in preimplantation embryos, we assumed that VEGF affects the development of preimplantation embryos via VEGFRs. The present study therefore examined the paracrine action of VEGF in preimplantation embryos. Two-cell embryos were cultured in the presence of 0.1, 1.0, 10 or 100 ng/ml mouse recombinant VEGF. With culture up to 48 h, VEGF treatment showed no significant effects on the development of preimplantation embryos into early blastocysts (data not shown). After 56 h of culture, formation of expanded blastocysts from early blastocysts was accelerated by VEGF treatment in a dose-dependent manner ($p<0.005$ control vs. 10 ng/ml; Fig. 2A). After 72 h of culture, the ratio of hatched blastocysts increased with concentrations of 1.0 and 10 ng/ml of VEGF (control vs. 1.0 ng/ml, $p<0.05$; control vs. 10 ng/ml, $p<0.005$; Fig. 2B).

To further examine blocking effects in preimplantation embryos, neutralizing antibody against mouse VEGF was used to block the biological activity of VEGF. Two-cell embryos were cultured with or without 0.2 μg/ml of anti-VEGF and

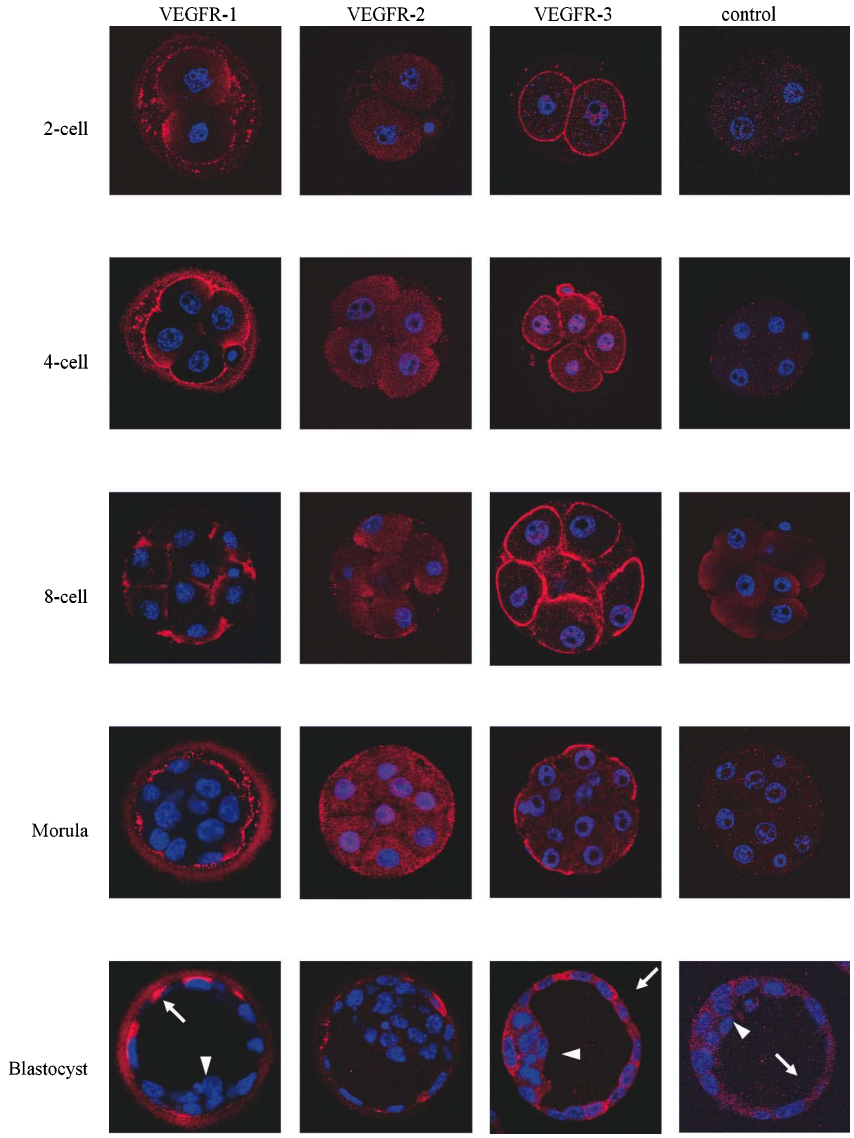

Fig. 1. Immunodetection of VEGF receptors in mouse preimplantation embryos. Two-cell embryos were obtained by flushing the oviducts of mated mice treated with PMSG followed by hCG. Four-cell and 8-cell embryos, morulae and blastocysts were collected from cultures at 50–52, 68–70, 90–92 and 118–120 h after hCG injection, respectively. Embryos were fixed with 4% paraformaldehyde. Immunostaining for VEGFRs was analyzed by laser-scanning confocal microscopy. For negative controls, equivalent concentrations of normal rabbit IgG serum were substituted for the primary antibodies. Signals for VEGFR are shown in red. Nuclei are labeled with Hoechist 33342 in blue. Confocal images are provided at 80 magnification.
VEGFR-1 signals are localized to the cell surface of embryos from 2-cell to blastocyst stages. In blastocysts, the outsides of trophoectoderm cells are stained (arrows), but VEGFR-1 signals are not observed in inner cell mass cells (arrowheads). Embryos incubated with VEGFR-2 antibody showed faint signals. Localization of VEGFR-3 signals in the cell surface of embryos is seen from the 2-cell embryo to the morula, whereas such localization is not apparent in blastocysts.

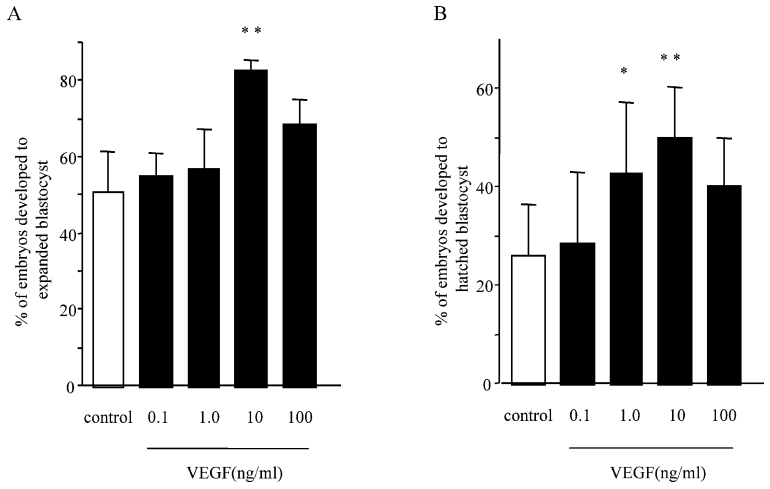

Fig. 2. Dose-dependent effects of VEGF on *in vitro* development of mouse preimplantation embryos. Two-cell embryos were cultured in HTF medium alone (control) or with 0.1, 1.0, 10 or 100 ng/ml of VEGF. Embryo development was monitored after 56 and 72 h of culture to evaluate the proportion of expanded blastocysts (A) and hatched blastocysts (B). Ten embryos were used in each treatment group, consisting of the 12 sets of experiments. A total of 120 embryos were tested in each treatment group. Values represent mean standard error of the mean (SEM). Data were analyzed by one-way ANOVA followed by Fisher's protected least-significant difference test. $^{*}P<0.05$; $^{**}P<0.005$ versus control.

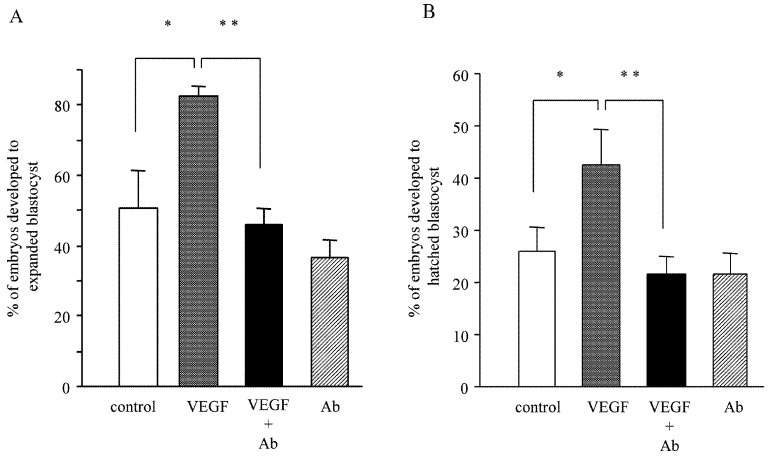

Fig. 3. Antagonistic effect of anti-VEGF antibody on VEGF-induced mouse preimplantation embryo development. Two-cell stage embryos were cultured in HTF medium alone (control) or with 10 ng/ml of VEGF, 10 ng/ml of recombinant VEGF+0.2 μg/ml of anti-VEGF antibody (VEGF+Ab) or 0.2 μg/ml of anti-VEGF antibody alone (Ab). Embryo development was monitored after 56 and 72 h of culture to evaluate the proportion of expanded blastocysts (A) and hatched blastocysts (B). Ten embryos were used in each group, consisting of the 12 sets of experiments. A total of 120 embryos were tested in each group. Values represent mean SEM. Data were analyzed by one-way ANOVA followed by Fisher's protected least-significant difference test. $^{*}P<0.05$; $^{**}P<0.005$ versus control.

10 ng/ml of VEGF. As expected, the accelerated development of embryos incubated with VEGF after 56 h and 72 h was significantly blocked to control levels by anti-VEGF antibody (VEGF vs. VEGF + antibody, $p < 0.005$; Fig. 3A, B). Furthermore, proportions of expanded and hatched blastocysts were not significantly decreased by treatment with anti-VEGF antibody compared to control (Fig. 3A, B). This result suggests that endogenous VEGF does not affect VEGFR actions via an autocrine loop.

Discussion

In the present study, VEGFR-1 and VEGFR-3 proteins were expressed in all stages of embryo development, from 2-cell embryos to blastocysts. In blastocysts, VEGFR-1 and VEGFR-3 were not seen in the inner cell mass and were localized in the trophoectoderm. Expression of VEGFR-2 has been investigated using antibody that was shown to be useful in other cells by fluorescent immunological staining[12], but expression was not seen in mouse preimplantation embryos. Since the ligand, VEGF, is expressed in human and mouse oviducts[5,8,9] and endometrium[5,13], VEGF appears to act on preimplantation embryos via VEGFR-1 and VEGFR-3 in a paracrine manner to regulate embryo development. The present study therefore used an *in vitro* model to investigate the paracrine effects of VEGF on mouse preimplantation embryos. Addition of VEGF was found to significantly promote embryo development, and effects on growth facilitation were marked after the blastocyst stage.

While our findings dealt with embryo development before implantation, a gene-knockout study has clarified that VEGF plays an important role in embryogenesis after implantation. VEGF-deficient mice lack normal vessels and die because vascular endothelial cells do not form during the fetal period[14,15]. Although vascular endothelial cell differentiation occurs in VEGFR-1-deficient mice, severe defects occur in the embryonic vasculature and the mice die during the fetal period[16]. VEGFR-2-deficient mice also die during the fetal period due to early defects in the development of hematopoietic and endothelial cells, and blood vessels are not formed[17]. In VEGFR-3-deficient mice, while vasculogenesis and angiogenesis occur, deformation of large vessels leads to cardiovascular failure, resulting in death during the embryonic period[18]. VEGF thus plays an important role in embryo development before and after implantation, and is an important factor for reproduction.

The facilitative effects of VEGF on embryo development identified in the present study might initially be considered as verifiable in a gene-knockout study. However, since homozygous VEGF- or VEGFR-1- and VEGFR-3-deficient mice die during the fetal period[15-17], VEGFR-1 and -3 (-/-) embryos can only be assessed in the oviduct or uterus. Embryos begin synthesizing proteins using own genes after the 4-cell stage, and protein synthesis using maternal mRNA accumulated in eggs lasts up to the 8-cell stage[19]. As a result, maternal proteins may still function in subsequent developmental stages. Assessing the function of VEGF on embryo development using gene-knockout techniques is thus difficult.

Studies have reported that the VEGF/VEGFR system is involved in the function of monocytes, retinal progenitor cells, hematopoietic cells and renal mesangial cells, all of which are non-vascular endothelial cells[20-23]. In the present study, VEGFR-1 and VEGFR-3 were expressed in the trophoectoderm of blastocysts. After implantation, the trophoectoderm differentiates into the trophoblast to form the placenta. VEGFR-1 mRNA is strongly expressed in the extravillous trophoblast[24], suggesting that VEGF may play an important role in cytotrophoblast development and differentiation during implantation. BeWo cells are trophoblast-like choriocarcinoma cells that express VEGFR-1 and VEGFR-2, and since the addition of VEGF induces phosphorylation of MAP-kinase, BeWo cells express functional receptors[24]. Additionally, in monkey trophoblasts, when VEGF is blocked

using neutralizing antibody, the trophoblast penetrates less deeply into the endometrium, thus increasing the frequency of spontaneous abortion[25]. In rodents, administration of VEGF-neutralizing antibody blocks blastocyst implantation[26,27]. VEGF thus appears to be important for embryo-endometrium interactions during blastocyst implantation.

In the present study, VEGF significantly facilitated embryo development during the blastocyst stage. *In vivo*, during the preimplantation phase, embryos keep dividing and develop into blastocysts upon reaching the uterus from the fallopian tube. Expression of VEGF mRNA in human endometrium is reportedly higher during the secretory phase following ovulation than during the proliferative phase[28]. More VEGF may thus be produced from the endometrium when a preimplantation embryo is in the blastocyst stage. This is associated with significant promotion of embryo development after the blastocyst stage. VEGF mRNA has also been reported in human embryos after the 8- to 16-cell stage[11]. Embryos may therefore produce VEGF themselves and may facilitate embryo development in an autocrine manner. We attempted to suppress the activities of VEGF, which existed in the culture solution and was produced by embryos, by adding VEGF-neutralizing antibody to the culture solution, but embryo development could not be suppressed. The reason for this was that VEGF produced by embryos was diluted by the culture solution, and concentration of VEGF did not reach a sufficient level to promote growth in an autocrine manner. This result also suggests that not only VEGF but other factors may promote the development of early embryos.

In summary, the present study has shown that VEGF acts on murine preimplantation embryos expressing VEGFR-1 and VEGFR-3, and is involved with embryo development. The results suggest that VEGF plays important roles in reproduction as a new paracrine factor regulating the growth of preimplantation embryos.

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