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Original Article

Spatiotemporal expression of Vascular Endothelial Growth Factor-C in mice fetal-maternal tissues during periimplantation (D4–D7)

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ABSTRACT

Objective: Vascular Endothelial Growth Factor (VEGF) is a glycoprotein acting as a potent angiogenic factor. This growth factor initiates vasculogenesis and promotes angiogenesis during early embryogenesis which is crucial for successful pregnancy. The present study determines the cell types expressing VEGF-C and its intensity in mice fetal-maternal tissue during periimplantation (D4–D7).
Settings: Molecular Endocrinology & Reproductive Biology Research Laboratory, Department of Zoology, Rajiv Gandhi University, Arunachal Pradesh, India.
Materials and methods: VEGF-C was localized in the fetal-maternal tissues using anti VEGF-C monoclonal antibody in paraffin embedded sections. Western blot and immunohistochemical studies were performed using DAB for chromogenic reactions to study the expression of VEGF-C. The growth factor transcript was detected by Reverse Transcriptase-PCR (RT-PCR).
Main outcome measures: VEGF-C was localized in the uterine epithelium and glandular epithelium on D4 and D5 of gestation. The decidual cells of primary decidual zone (PDZ) on D5 which expands to secondary decidual zone (SDZ) on D6 showed the expression of VEGF-C. The trophectodermal cells and cells of ectoplacental cone on the embryo showed expression of the growth factor. Intensity of the growth factor was different in different cell types. Western blot and RT-PCR analysis confirmed translation and transcription of the growth factor during this period.
Major conclusion: Programmed spatio - temporal VEGF-C expression in fetal-maternal tissue during periimplantation (D4–D7) in mice could be the key process for growth and development of the embryo. Expression of the growth factor changes quantitatively in different cell types according to the requirements during early embryogenesis.
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1. Introduction

Vascular Endothelial Growth Factor is a homodimeric glycoprotein and unique for its specificity among growth factors for the development of vascular endothelium. It is one of the potent angiogenic factors and prime regulator of uterine vasculogenesis and angiogenesis during the reproductive cycle and gestation. During gestation, tissue remodeling is indispensable for development of conceptus inside the uterine tissue. Formation of new blood vessels in the restructured maternal tissue and newly formed embryo is a must required phenomenon of reproductive process. Recent investigation of angiogenic processes has shown that VEGF plays an essential role in both vasculogenesis and angiogenesis in maternal uterus and developing embryo [1–3]. Our earlier observations showed that abnormal development or death of embryo during periimplantation period is associated with altered expression of VEGF-C in rat fetal maternal tissue. VEGF-C fails to express in the fetal maternal tissues spatiotemporally in response to exogenous agent(s) during D4–D7 causing deleterious effects to the embryo [4]. Under physiological conditions in the adult female, angiogenesis occurs primarily in the uterus and ovary during the reproductive cycle and pregnancy [5]. It has been reported that VEGF and its receptors critically regulate the uterine vascular permeability at the blastocyst attachment site and endometrial decidualization [6]. It also involves in the differentiation and invasion of trophoblast during periimplantation. Research on VEGF for the last two decades has revealed that VEGF-C is primarily involved in both vasculogenesis and lymphangiogenesis during early embryogenesis [7–11].

Despite the growing interest of investigation on the role of VEGF in embryo development and implantation, the precise function is still significantly unaddressed. The role of estrogen in

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regulation of growth factors' effects on gestation is yet to be elucidated. A number of growth factors including VEGF play their respective roles in making the whole process of pregnancy successful. The process of gestation starts with the communication between free floating blastocyst and the endometrium during preimplantation period and the endometrium. The intimate interaction of endometrial epithelium and embryonic trophoblasts finally leads to invasion of the blastocyst into maternal tissue. The coordinated process of blastocyst implantation is governed by intimate cross talk between intrinsic signals of embryo and extrinsic signals of receptive uterus [12]. In these events, sex steroids (estrogen and progesterone), growth factors, cytokines, adhesion molecules, extracellular matrix proteins and prostaglandins play important role [13]. The temporal and cell specific coordination of estrogen and progesterone mainly regulates the process of implantation [14]. However, time and tissue specific effects of these gonadal hormones on expression of genes in fetal and maternal tissues during early embryonic development are yet to be elucidated.

It has been well established that at the site of blastocyst attachment, endometrial vascular permeability increases which is one of the earliest prerequisites for implantation [15,16]. Along with the vascular permeability, apoptosis of luminal epithelium and decidualization of stromal cells at the implantation site lead to subsequent adhesion and penetration of trophoblast for further development of the embryo. In mice, the increased uterine vascular permeability happens on the afternoon of D5 of gestation and lasts for a short period. During this time, vascular bed in endometrium expands markedly. Sequential events of increased vascular permeability, decidualization in endometrial stroma and subsequent apoptosis in epithelial cells at the site of blastocyst implantation facilitate the invasion of trophoblast cells through the base membrane and stroma [17]. At the fetal-maternal interface, angiogenesis is characterized by uterine vascular permeability and subsequent development of maternal wall separating a fluid filled cavity where growth and development of the embryo take place. In the developing embryo, generation of new vessels is governed by the splitting of new capillaries from the pre-existing vasculature [18]. The detail of road map of embryo implantation and role of various factors including cytokines, growth factors, gonadal hormones during periimplantation is yet to be studied. It has been speculated that expression of VEGF-C during periimplantation is the *sine qua non* for cross talk in between fetal-maternal tissues. VEGF-C could play a role in the nutritional supplement to the embryo through generation of new blood vessels as well as space within maternal tissue for the growing embryo. To meet the objective, VEGF-C expressed spatiotemporally in fetal-maternal tissue during periimplantation.

The present study was undertaken to elucidate the spatial and temporal expression of VEGF-C in fetal-maternal tissue during the periimplantation period (D4 - D7) in mice. It is expected that the findings of this research will help to address many unfold enigmas toward the female reproductive health especially female fertility regulation.

2. Materials and methods

2.1. Experimental animals

All animal procedures were approved by Institutional Animal Ethical Committee, Rajiv Gandhi University and conducted according to the institutional guidelines for the care and use of laboratory animals. Adult cyclic female Swiss albino mice of LACA strain (25 ± 10 gm body weight, 6–8 weeks old) were reared in the central animal facility, maintaining natural light and dark period under standard husbandry conditions. Animals were fed with routine diet (Bengal gram, corn and vitamin supplement) and water *ad libitum*. Estrous cycle of the adult females was studied by observing the cell types in the vaginal smear prior to the experiment to ensure normal cycle. The females showing normal estrous cycle were considered for *in vivo* studies. Group of 10 animals were selected for each experiment. Adult cyclic females were kept for mating with the male at the ratio of 2:1 (2 females:1 male). The morning on which copulatory plug detected was considered as day 0.5 of gestation. The implantation sites were visualized from day 4 (D4) to day 7 (D7) of gestation by intravenous injection of 1% Chicago sky blue dye (Sigma Aldrich, cat no. C8679-25G) prepared in 0.9% saline into the tail vein (0.1 ml) 15 min before necropsy. Implantation sites were appeared as discrete blue bands at the uterine horns following the injection of Chicago sky blue dye.

2.2. In situ localization of VEGF-C

VEGF-C immunohistochemical localization was carried out in paraffin embedded uterine sections using HRP conjugated secondary antibody. Briefly, pregnant females were sacrificed every morning (07:00–09:00 am) on D4 to D7 of gestation for tissue sample collection. Uterine horns were collected and fixed in Bouin's fluid for 72 h. Fixed tissues were then washed for 4 h in running tap water and dehydrated passing through alcohol gradient (30–100%) and finally embedded in paraffin for block preparation. Sections were cut in 5 μm thickness using a rotary microtome and mounted on poly-L-Lysine (Sigma. Cat. No. P8920) coated glass slides. Sections were dried and incubated at 37 °C overnight. Immunolocalization of VEGF-C was carried out following the standard protocol [19]. For staining and binding of antibody, tissue sections were deparaffinized in xylene for 5 min; rehydrated in alcohol gradient (from 100% to 30%) and subsequent three times wash in TBS buffer (25 mM, pH 8) for 5 min each. The quenching of endogenous peroxidase activity was done by putting the slides in 3% H_2O_2 in methanol for 30 min. The sections were treated with blocking serum (Normal goat serum, Santa Cruz Biotech, U.S.A. sc-2043) in a humidified chamber for 3–4 h to prevent the non-specific binding. In the present study, the mouse monoclonal VEGF-C antibody (Santa Cruz Biotech, U.S.A. sc-7269) was used. Primary antibody was diluted in TBS-BSA at the concentration of 2.5 μg/ml. Sections added with primary antibody were incubated overnight at 4 °C. Sections were washed thrice in TBS buffer for 5 min each and subsequently treated with goat antimouse IgG-HRP secondary antibody (Santa Cruz Biotech, U.S.A. sc-3697) at a concentration of 1 μg/ml in a humidified chamber for 2 h. The slides were washed with TBS buffer and antibody-antigen reaction sites were detected by using diaminobenzidine (DAB) substrate. The DAB substrate was dissolved in TBS with 0.3% H_2O_2 at the concentration of 1 mg/ml. Tissue sections were counterstained with hematoxylin, dehydrated and mounted with DPX for observation. Negative control has been performed using Normal IgG instead of VEGF-C antibody. Photomicrography of the sections was taken at different magnifications using a DM5000B microscope (Leica Microsystems, Germany). Quantification of the VEGF-C signal was calculated using ImageJ software (NIH, USA).

2.3. VEGF-C Western blotting

Total protein was extracted from the uterine horn excised from the experimental animals and washed with PBS. Horn was minced in ice and homogenized with SDS-sample buffer using pestle and mortar. The ground tissue was boiled for 10 min and immediately kept in ice followed by centrifugation at 12,000*g* at room temperature for 10 min. The supernatant containing the denatured pro-

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