Seasonal Changes in Immunoreactivity of Vascular Endothelial Factor and its Receptors VEGFR1 and VEGFR2 in the Uterus of Wild Ground Squirrels (*Citellus dauricus* Brandt)

Jiaju WENG¹⁾, Ben LI²⁾, Xia SHENG²⁾, Haolin ZHANG²⁾, Xiao HU^{2,3)}, Jiao ZHOU²⁾, Meiyu XU²⁾, Qiang WENG^{2,4)}, Gen WATANABE^{4,5)} and Kazuyoshi TAYA^{4,5)}

¹⁾School of Basic Medical Science, Peking University, Beijing 100083, PR China

²⁾College of Biological Science and Technology, Beijing Forestry University, Beijing 100083, PR China

³⁾Department of Biology, University of Ottawa, Ontario K1N 6N5, Canada

⁴⁾Laboratory of Veterinary Physiology, Cooperative Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

⁵⁾United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

Abstract. In this study, we investigated the immunoreactivity of vascular endothelial growth factor (VEGF) and its receptors flt-1 (VEGFR1) and the kinase domain receptor (KDR/Flk-1, VEGFR2) in the uteri of the wild ground squirrels during the estrous period, early pregnancy and nonbreeding period. Cellular localizations of VEGF, VEGFR1 and VEGFR2 were detected by immunohistochemistry, and total proteins were extracted from uterine tissue in the estrous period, early pregnancy and nonbreeding period for Western blotting analysis. In addition, plasma estradiol-17 β and progesterone concentrations were measured by radioimmunoassay. Stronger positive staining of VEGF was found in luminal epithelial cells and glandular cells, and its receptors (VEGFR1 and VEGFR2) were observed in stromal cells in the estrous period and early pregnancy compared with the nonbreeding period. The protein levels of VEGF, VEGFR1 and VEGFR2 were significantly higher in the estrous period and early pregnancy as compared with the nonbreeding period. Besides, plasma estradiol-17 β and progesterone concentrations were higher in the estrous period and early pregnancy than in the nonbreeding period, suggesting that the immunoreactivities of VEGF, VEGFR1 and VEGFR2 were correlated with changes in plasma estradiol-17 β and progesterone concentrations. These results suggested that VEGF and its receptors may be involved in the regulation of seasonal changes in the uterine functions of wild female ground squirrels.

Key words: Uterus, VEGF, VEGFR1, VEGFR2, Wild ground squirrel

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During the reproductive cycle, the uterine endometrium undergoes a precisely timed complex sequence of physiological and morphological changes in preparation for implantation. These changes are controlled primarily by the ovarian steroid hormones 17 β -estradiol (E₂) and progesterone [1]. During pregnancy, blood flow to the uterus is increased dramatically to meet the rising demands of the growing fetus. Endocrine control of this phenomenon is complex but includes in part the action of many growth factors [2, 3]. Vascular endothelial growth factor (VEGF) is a protein with angiogenic activity and a potent stimulator of microvascular permeability [4, 5]. It plays an important role in physiological and pathological neovascularization via its receptors Flt1/VEGFR1 and kinase insert domain containing region (KDR)/VEGFR2, both of which have tyrosine kinase activity [6]. In reproductive organs, VEGF is required for normal ovarian angiogenesis and endometrial growth throughout the ovulatory cycle in humans [7, 8] and rodents [9, 10]. The expression of VEGF and

its receptors has been demonstrated in the endometrium in humans and in experimental and domestic animals throughout the menstrual cycle, with an upregulation in the late proliferative and luteal phases [11–15], periods that correspond to angiogenesis and an increase in vascular permeability [16]. However, the expression of VEGF and its receptors VEGFR1 and VEGFR2 in the endometrium of a seasonal cycle breeder has not been clarified. To study the basic mechanisms of VEGF regulation of uterine function changes during the breeding and nonbreeding seasons, the wild female ground squirrel may offer a useful model without any manipulations.

The wild ground squirrel (*Citellus dauricus* Brandt) is a typical seasonal breeder that has a strict and extremely compressed breeding period (for females, it consists of the estrous period and pregnancy) from April to May and a long period of sexual dormancy from June to the following March that includes a 6-month hibernation period [17]. The wild female ground squirrel exhibits estrus immediately after emergence from hibernation in spring, and has a gestation period of 28 days [18, 19]. Although many observations have been reported recently concerning the regular roles of VEGF and its receptors on uterine function, there are still limitations in understanding the mechanisms of uterine function, especially the roles of VEGF and its receptors VEGFR1 and VEGFR2 in the seasonal uterine function

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Correspondence: Q Weng (e-mail: qiangweng@bjfu.edu.cn)

changes. The aim of our study was to investigate the immunoreactivity of VEGF and its receptors VEGFR1 and VEGFR2 in uteri of wild ground squirrels during the estrous period, early pregnancy and nonbreeding period, to gain a better understanding of the relationship between VEGF system and uterine function changes in the wild ground squirrels.

Materials and Methods

Animals

All the procedures on animals were carried out in accordance with the Policy on the Care and Use of Animals of the Ethics Committee, Beijing Forestry University, and were approved by the Department of Agriculture of Hebei Province, PR China (JNZF11/2007). Wild female ground squirrels that were regarded as adults according to their body weights (242–412 g) were captured on April 13 after emergence from hibernation ($n=5$), on May 2 in the pregnancy period ($n=5$) and on June 9 in the nonbreeding period ($n=5$) in 2009 in Hebei Province, PR China. The animals were euthanized and decapitated, and blood samples were collected for radioimmunoassay. The uterus of each animal was quickly removed and dissected into 2 portions. One portion was fixed in 0.05 M phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde (Sigma, St. Louis, MO, USA) for histological and immunohistochemical observation; the other portion was immediately frozen in liquid nitrogen and stored at -20°C for Western blotting detection.

Histology

Uterine samples were dehydrated through ethanol series and embedded in paraffin wax. Serial sections ($4\text{ }\mu\text{m}$) were mounted on poly-L-lysine (Sigma) coated slides and stained with hematoxylin-eosin (HE) for general histological observations. The number of uterine glandular nuclei was assessed with the NIH ImageJ software using the method described by Kirby *et al.* [20].

Immunohistochemistry

Uterine sections were blocked with 10% normal goat serum to prevent the nonspecific binding of the second antibody. The primary antibodies used were all purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA): rabbit polyclonal antibody against VEGF (sc-152, 1:100), rabbit polyclonal antibody against VEGFR1 (sc-316, 1:200) and mouse monoclonal antibody against VEGFR2 (sc-6251, 1:100). After 12 h of primary antibody incubation at 4°C , the sections were then incubated with the corresponding secondary antibody, goat anti-rabbit IgG for VEGF and VEGFR1 and goat anti-mouse IgG for VEGFR2 conjugated with biotin and peroxidase with avidin, for 1 h at room temperature. The sections were visualized using a rabbit ExtrAvidinTM staining kit (Sigma) in 150 ml of 0.05 M Tris-HCL buffer containing 30 mg 3,3'-diaminobenzidine (Wako, Tokyo, Japan) plus $30\text{ }\mu\text{l}$ H_2O_2 . Finally, the sections were counterstained with hematoxylin (Merck, Tokyo, Japan). Control sections were treated with normal bovine serum (Sigma) instead of the primary antisera. The immunostained slides were scanned using the Image-Pro Plus 4.5 software (Media Cybernetics, MD, USA) at $20\times$ magnification. The background of each section in the different periods was used as the internal control

during evaluation of the intensity of the immunostaining. Similar to our previous studies [21, 22], immunoreactivity was shown as – for negative staining, + for the positive staining, ++ for the strongly positive staining and +++ for very strongly positive staining.

Western blotting

Uterine tissues were dissected into small pieces using a clean razor blade. The intersection tissues between each embryo were homogenized in a tissue homogenizer containing $300\text{ }\mu\text{l}$ of 10 mg/ml PMSF and incubated for 30 min on ice. Homogenates were centrifuged at $12,000\text{ g}$ for 10 min at 4°C . Protein extracts ($25\text{ }\mu\text{g}$, measured by the Lowry protein assay) were mixed with equal volumes of $2\times$ Laemmli sample buffer. Equal amounts of proteins from each sample were loaded onto a 12% SDS-PAGE gel, and electrophoretically separated at 18 V/cm and transferred to a nitrocellulose membrane using a wet transblotting apparatus (Bio-Rad, Richmond, CA, USA). The membrane was blocked in 3% BSA for 1 h at room temperature. Primary incubation of the membrane was carried out using VEGF, VEGFR1 or VEGFR2 antibody (all at 1:500 dilution) for 1 h at room temperature. Secondary incubation of the membrane was then carried out using an IRDye (1:5000 dilution, Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. Finally, the membrane was washed in 25 ml Tris-buffered saline with Tween-20 (TBST wash buffer, 0.02 M Tris, 0.137 M NaCl and 0.1% Tween-20, pH 7.6) plus $3\text{ }\mu\text{l}$ H_2O_2 and visualized with an Odyssey infrared imaging system. Water, instead of primary antisera, was used as a negative control. β -actin was selected as an endogenous control. The intensities of the bands were quantified using the Quantity One software (Version 4.5, Bio-Rad Laboratories), and expression ratios were calculated.

Hormone assays

Plasma concentrations of estradiol- 17β (E_2) and progesterone (P_4) were determined by a radioimmunoassay (RIA) kit (kit 02010306021 for E_2 and kit 02010305021 for P_4 , China Diagnostics Medical, Beijing, China). The plasma samples from each animal were sent to the Beijing North Institute of Biological Technology for the assay. The intra-assay variation was less than 10% for E_2 and P_4 . The detection sensitivity was 5 pg/ml for E_2 and 0.2 ng/ml for P_4 .

Statistical analysis

Means and standard deviations were calculated. Data were analyzed using a one-way ANOVA, and the means were compared for significance using Tukey's test ($P=0.05$) and the SPSS computer software package.

Results

Histology

Morphological and histological features of the uterus were observed in the wild female ground squirrel during the estrous period, early pregnancy and nonbreeding period (Fig. 1). Uteri of 5 wild female ground squirrels captured in April were regarded as being from the estrous period since no fetuses were detected in them (Fig. 1a). The 5 squirrels from May were categorized as being in early pregnancy, as small fetuses were found in their uteri (Fig. 1b). The uteri of 5 wild ground squirrels captured in June were regarded as being

from the nonbreeding period (diestrous period) (Fig. 1c). Marked histological changes in the uterus were also observed in the estrous period, early pregnancy and nonbreeding period (Fig. 1d, e, f). The uterine lumen went through remarkable enlargement during the early pregnancy period and atrophy during the nonbreeding period. As shown in Fig. 1g, h and i, and calculated in Table 1, the uterine glands were bigger and contained more nuclei in early pregnancy, whereas fewer nuclei were observed in the glands from the estrous and nonbreeding periods.

Immunohistochemistry

Representative sections for immunohistochemical localization of VEGF and its receptors VEGFR1 and VEGFR2 are shown in Fig. 2,

and the results are summarized in Table 2. VEGF was strongly present in luminal epithelial cells and glandular cells in the estrous period and

Table 1. Mean cell density of glandular cell nuclei $\pm$ SE/mm² $\times 10^2$ in the uteri of wild ground squirrels during the estrous period, early pregnancy and nonbreeding period

Period	Number of glandular cell nuclei
Estrous period	63 $\pm$ 7
Early pregnancy	93 $\pm$ 9*
Nonbreeding period	51 $\pm$ 9

*Indicates significant difference ($P < 0.05$).

Fig. 1. Morphological and histological features of uterine tissues of the wild ground squirrels during the estrous period, early pregnancy and nonbreeding period. Morphological features of uteri were observed in the wild ground squirrels during the estrous period (a), early pregnancy (b) and the nonbreeding period (c). The myometrium and endometrium of uterine tissues were observed in the wild ground squirrels during the estrous period (d), early pregnancy (e) and the nonbreeding period (f), respectively. Uterine glands were found in the endometrium of the wild ground squirrels during the estrous period (g), pregnancy (h) and the nonbreeding period (i). E, the estrous period; EP, early pregnancy; NB, the nonbreeding period; Mm, myometrium; Em, endometrium; Ug, uterine gland. The scale bars represent 1 cm (a, b, c), 100 μ m (d, e, f) and 30 μ m (g, h, i).

Table 2. Relative abundance of VEGF, VEGFR1 and VEGFR2 in uteri of the wild ground squirrels during the estrous period, pregnancy and nonbreeding period

	VEGF			VEGFR1			VEGFR2		
	E	EP	NB	E	EP	NB	E	EP	NB
Stromal cells	+	+	–	++	+++	+	+	+++	+
Luminal epithelial cells	++	+++	+	–	–	–	–	–	–
Glandular cells	++	+++	+	–	–	–	+	–	–

The immunohistochemical staining was determined as positive (+), strongly positive (++) , very strongly positive (+++), and negative (–). Staining that was weak but higher than that of the control was set as positive (+). The highest intensity staining was set as very strongly positive (+++). A staining intensity between + and +++ was set as strongly positive (++) . E, estrous period; EP, early pregnancy; NB, nonbreeding period.

Fig. 2. Immunohistochemical localization of VEGF and its receptors VEGFR1 and VEGFR2 in uterine tissues of the wild ground squirrels during the estrous period, early pregnancy and the nonbreeding period. The first column (a, e, i) represents negative control samples stained by normal rabbit IgG in the estrous period, early pregnancy and nonbreeding period; the second column (b, f, j), the third column (c, g, k), and the fourth column (d, h, l) represent the samples immunostained with antibodies against VEGF, VEGFR1 and VEGFR2, respectively. E, the estrous period; EP, early pregnancy; NB, the nonbreeding period; Ec, epithelial cell; Sc, stromal cell; Ug, uterine gland. The scale bar represents 50 μ m.

early pregnancy (Fig. 2b, f) but weakly observed in luminal epithelial cells and glandular cells during the nonbreeding period (Fig. 2j). Little to no signal of VEGF in stromal cells was detected throughout the three reproductive periods. On the other hand, cytoplasmic staining of both VEGFR1 and VEGFR2 was mainly found in stromal cells during the estrous period, early pregnancy and nonbreeding period, respectively (VEGFR1, Fig. 2c, g, k; VEGFR2, Fig. 2d, h, l), and the intensities of the immunohistochemical signals for VEGFR1 and

VEGFR2 were significantly higher in early pregnancy than those in either estrous or the nonbreeding period. No immunostaining was detected in negative control sections when normal rabbit serum was substituted for the primary antibody (Fig. 2a, e, i).

Western blotting

Western analysis of proteins extracted from the wild female ground squirrel uterine tissue samples revealed immunoreactive

Fig. 3. Western blotting for the detection of VEGF, VEGFR1 and VEGFR2 in uterus tissues of the wild ground squirrels during the estrous period (lane E, n=5), early pregnancy (lane EP, n=5) and nonbreeding period (lane NB, n=5), respectively. Water was used as the negative control (lane NC). β-actin blots were used as controls to correct for loading in each lane. The expression levels of VEGF (A), VEGFR1 (B) and VEGFR2 (C) in uteri of wild female ground squirrels in different periods were determined by densitometric analysis. Bars represent means + SD for five independent experiments. Means within the columns marked with different letters are significantly different from each other ($P < 0.01$).

VEGF monomer, VEGFR1 and VEGFR2 proteins at 21 kD, 180 kD and 230 kD respectively during the estrous period, early pregnancy and nonbreeding period (Fig. 3), which are in accordance with the manufacturer's specifications. The results were normalized to the expression level of β-actin. The proteins extracted from water in the estrous period were used as a negative control (Fig. 3, lane NC). The expressions of VEGF, VEGFR1 and VEGFR2 were significantly

Fig. 4. Plasma estradiol-17β (A) and progesterone (B) levels in wild female ground squirrels during the estrous period (E), early pregnancy (EP) and nonbreeding period (NB), respectively. Bars represent means + SD for five independent repetitious experiments. Means within the columns marked with different letters are significantly different from each other ($P < 0.01$).

higher in the estrous period and early pregnancy as compared with the nonbreeding period (Fig. 3A, B, C).

Plasma concentration of estradiol-17β and progesterone

The profiles of plasma estradiol-17β and progesterone are shown in Fig. 4. The plasma estradiol-17β concentration was high in the estrous period, and declined significantly during pregnancy and then to the minimum level in the nonbreeding period (Fig. 4A). The plasma progesterone concentration remained relatively high in the estrous period and reached its peak in pregnancy, but then declined precipitously in the nonbreeding period (Fig. 4B).

Discussion

This is the first study to investigate the immunoreactivity of VEGF and its two receptors VEGFR1 and VEGFR2 in uterine tissues of the wild ground squirrel, and it showed that the immunoreactivities of VEGF, VEGFR1 and VEGFR2 are correlated with the changes in the plasma concentrations of estradiol-17β and progesterone during the estrous period, early pregnancy and nonbreeding period. These findings suggest that VEGF and its receptors may be involved in the regulation of seasonal changes in the uterine functions of the wild ground squirrel.

The endometrium undergoes cyclic proliferation and differentiation of both the glandular epithelial and stromal cells for preparation of embryo implantation. Ovarian steroids are the prime modulators of these changes, and they interact with local growth factors to regulate growth and differentiation of the endometrium [23]. In the present study, VEGF and its receptors VEGFR1 and VEGFR2 were immunolocalized in uterine tissues during the estrous period, early pregnancy and the nonbreeding period, showing the capability to synthesize and secrete proteins of VEGF and its receptors in the wild ground squirrel. Previous studies have shown that VEGF binding to VEGFR2 induces endothelial cell recruitment and proliferation [13], and the interaction between VEGF and VEGFR1 stimulates the endothelial cells to form tubules with the induction of tight junctions [12]. In the mare, VEGF, VEGFR1 and VEGFR2 together facilitate the development of maternal and fetal vascular networks for the interchange of gases, nutrients and waste products throughout gestation [24]. In the bovine endometrium, VEGF and its receptors

were regulated throughout the estrous cycle [25]. A study in the rhesus monkey during early pregnancy also indicated that VEGF-VEGFR pairs were involved in the process of trophoblast invasion, maternal vascular transformation and fetoplacental vascular differentiation and development [26]. In the uterus of the wild ground squirrel, VEGF was mainly found in luminal glandular cells, and its receptors (VEGFR1 and VEGFR2) were observed in stromal cells, suggesting that VEGF release by secreting cells might stimulate different endothelial functions in a paracrine fashion [27] and that uterine VEGF and its receptors might play a paracrine/autocrine role in seasonal changes in growth and differentiation of the uterine endometrium of wild female ground squirrels.

Growing evidence indicates that ovarian steroid hormones regulate primarily uterine functions by stimulating uterine production of cytokines and growth factors [28], which have been implicated in mediating the action of steroid hormones [29, 30]. Previous studies have demonstrated that estrogen induces uterine epithelial cell proliferation and that estrogen withdrawal results in cell death [31, 32]; other studies have shown that the luminal and glandular epithelium as well as stromal cells proliferate and degenerate in response to cyclic changes in serum steroid hormones [33, 34]. Besides, it has been suggested that steroid actions affect the local production of growth factors and their receptors [29, 35]. VEGF mediates the vascular effects of estrogens in target tissues such as the uterus, a response attributed to the effect of an estrogen response element on the VEGF gene expression [36]. The present study showed a higher plasma estradiol-17 β concentration in the estrous period and early pregnancy as compared with the nonbreeding period. Also, similar patterns of VEGF, VEGFR1 and VEGFR2 protein expression were observed in the uterus of the wild female ground squirrel. These findings are similar to those observed in other mammals. VEGF mRNA levels were elevated by estrogen *in vivo* in the mouse [37], rat [38, 39] and ovine [40] uterus and in cultures of human endometrial glandular epithelial cells [36] and stromal cells [41, 42]. In addition, Nayak and Brenner also showed that estrogen stimulated endometrial VEGF expression *in vivo* in ovariectomized rhesus monkeys [43]. Therefore, combined with the present study, evidence supports that estrogen has a significant role in stimulating VEGF system expression by glandular, luminal epithelial and stromal cells of the endometrium to promote its angiogenesis and growth [44].

The VEGF/VEGFR pathway plays a key role in the maintenance of early pregnancy through its regulation of peri-implantation angiogenesis in the uterine decidua [45]. The presence of VEGF mRNA and protein has been demonstrated in the human and mammalian endometrium throughout the menstrual cycle, with an increase in the late proliferative and luteal phases [38, 41, 46]. A previous study suggested that endometrial proliferation was stimulated by estrogen, while endometrial angiogenesis was stimulated by progesterone, with estrogen acting as a cell mitogen and progesterone, in contrast, acting as a differentiation factor [47]. In the present study, VEGF protein was observed in luminal epithelial cells and glandular cells in early pregnancy, whereas VEGFR1 and VEGFR2 were detected in stromal cells of the endometrium. At the same time, the plasma concentration of progesterone reached the highest level in this period. In the rhesus monkey, VEGF, VEGFR1 and VEGFR2 mRNA and proteins were localized in the glandular epithelium on day 12 of

pregnancy, suggesting that VEGF mediated through the regulation of VEGFR1 and VEGFR2 may serve as a mediator of cellular growth and differentiation in addition to its function as an endothelial mitogen [26]. Other studies have shown that progesterone alone increased VEGF mRNA expression *in vivo* in the rat uterus [48], in the uterus of rhesus monkeys suppressed with gonadotropin-releasing hormone agonist [49] and *in vitro* in human endometrial cells [41]. Taken together, the present results are compatible with the broader concept that the expressions of VEGF and its receptors in the wild ground squirrel uterus are progesterone-dependent, which may contribute to events either leading to or associated with the cellular growth and differentiation of the endometrium as well as peri-implantation angiogenesis in early pregnancy.

In summary, the correlation between the changes in the immunoreactivity of VEGF, VEGFR1 and VEGFR2 and plasma estradiol-17 β and progesterone concentrations in this study suggests a broader concept that estrogen and/or progesterone may act synergistically with the VEGF system on seasonal regulation of the uterine functions of wild female ground squirrels.

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