Manipulation of ovarian follicle development by injecting vascular endothelial growth factor (VEGF) gene

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SUMMARY

The genetic and molecular mechanisms that control the development of capillary blood vessels during follicular development are beginning to be elucidated. Ovarian follicles contain and produce angiogenic factors that may act alone or in concert to regulate thecal angiogenesis. These factors are ultimately controlled by endocrine, paracrine and autocrine regulation in the ovary. Our recent study indicated that vascular endothelial growth factor (VEGF) plays an important role in the thecal angiogenesis during follicular development. In this review, we focus on the vasculature and the expression of angiogenic factors during follicular development in a mammalian ovary. Reproductive Biology 2005 5 (3):257-268.

Key words: VEGF, gene injection, ovary, follicle development

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INTRODUCTION

The genetic and molecular mechanisms that control the development and remodeling of capillary blood vessels are beginning to be elucidated. Two processes are responsible for the development of new blood vessels. The first is vasculogenesis, which is the \textit{de novo} differentiation of blood vessels from mesodermal precursor cells. Vasculogenesis is mainly found during the prenatal period. The second is angiogenesis, which is defined as the formation of new blood vessels by migration and proliferation of endothelial cells from preexisting vessels. Angiogenesis is found in pathological situations such as tumor growth and metastasis in the adult. Physiologic angiogenesis in the adult is prominent only in the female reproductive system. It takes place in the uterus, placenta, mammary gland and ovaries. In the ovaries of mammals, the transition of the follicle into the growth phase and subsequent follicle development result in massive growth of blood vessels induced by relationships between angiogenic factors and related receptors [28]. This review will discuss the current state of knowledge regarding the physiology of angiogenic processes and their regulation in the mammalian ovary.

DIFFERENCE IN VASCULAR FORMATION IN SEVERAL MAMMALIAN OVARIES

The angiogenesis of ovarian follicles plays an important role in folliculogenesis. A recent study using corrosion casts for scanning electron microscopy (SEM) revealed the structure of the microvasculature in the ovary [16, 17]. Porcine ovaries have coiled arteries in the hilus and spiraling branches in the cortex (fig. 1). In addition, small arterioles originating from the cortical coiled arteries straighten before entering the vascular complexes of the follicles. The follicles of 150-300 µm in diameter were surrounded by a polygonal meshwork of a few large capillary meshes, but no basket-like structure was visible (fig. 2). Some follicles of 500-700 µm in diameter had a spherical meshwork of a few capillaries, and the capillary network was arranged as a thin single layer of capillaries. The microvascular architecture
Shimizu & Sato

Fig. 1. Artery configurations in the cortex of the a, b/ porcine ovary and c, d/ follicles. Insert: higher magnification view of the selected area in figure 1a (small coiled artery); b, c/ large arrowheads: straight arterioles from coiled arteries before entering follicles; d/ higher magnification view of the selected area in figure 1c (asterisk: dichotomy of an arteriole in follicles); connections between arteriole-derived capillaries (AC) and vein-derived capillaries (VC) were also observed. Bars: 1.38 mm (a, c), 300 µm (insert), 400 µm (b), 120 µm (d). (reproduced with permission from [16])

Fig. 2. Microvasculature of a/ primordial or primary follicles (stars), b/ small antral and c, d/ medium antral follicles in the porcine ovary; b/ small arrowheads: necks of drainage capillaries that connect to venular vessels (v); d/ higher magnification view of the selected area from figure 2c. Budding (arrowheads) and sprouting (arrows) images indicate angiogenesis of venular capillaries (vc). Capillaries that contact each other in parallel are indicated by open rectangles. Bars: 300 µm (a, b), 1.38 mm (c), 200 µm (d). (reproduced with permission from [16])
of follicles of 1,000-2,000 µm in diameter was composed of three layers of vascular plexus (fig. 3). The inner plexus consisted of a small, spherical, basket-like capillary network that was not well developed. The middle layer consisted of small arterioles and venules, and the outer layer was a coarse capillary plexus.

*Fig. 3.* Freeze-fractured vascular casts of the medium antral follicles in porcine ovaries. Higher magnification views of the selected areas in figure 3a are shown in figures 3b and 3c. The three-layer microvasculature (b) of the medium antral follicles consists of an inner capillary network (*large arrowheads*), medium vessels (*black asterisks*), and an outer capillary network (*arrows*). Irregular distributions of capillaries in the inner plexus are shown in figure 3c. Angiogenic and degenerative figures of capillaries are indicated by *large white arrowheads* and *small white arrows* (avascular areas in the inner wall), respectively. *Bars:* 1.38 mm (a), 200 µm (b, c). (reproduced with permission from [16])
The microvascular architecture and its changes in the follicles of pigs are different from those in rats, but similar to those in rabbits and cows, especially those of the large antral follicles. In the rat, the vascular bed of developing and mature follicles remains in a single-layered wreath configuration during development [18]. However, in ovaries of the rabbit [18, 19] and cow [34], the single-layered capillary wreath in the small-sized follicles becomes a multilayered structure in the thickened theca interna as the follicles develop into larger and more mature Graafian follicles. These morphological changes in follicular microvasculature support the need for an increase in blood supply as follicles develops.

ANGIOGENIC FACTORS AND THEIR ROLES IN MAMMALIAN OVARY

A recent study reported that vascular endothelial growth factor (VEGF), known as a potent mitogen for endothelial cells [6] and as a stimulator of vascular permeability [5, 27], was expressed in granulosa cells isolated from pig follicles [1, 28]. Moreover, it has been demonstrated that the short-term inhibition of angiogenesis after anti-VEGF antibody administration during the later growth phase of the dominant follicle interferes with its normal development [35]. It was also shown that the blocking of VEGF action by treatment with a soluble truncated form of the fms-like tyrosine kinase (Flt-1) receptor resulted in an 87% decrease of proliferation in the theca cells of secondary and tertiary follicles, a reduction in endothelial cell area and a marked decline in Flt-1 mRNA expression [33]. These studies suggested that VEGF is associated with follicular development and vasculature in the mammalian ovary.

Angiopoietins (Ang) are relatively highly expressed in the ovary, uterus and placenta [20]. Since they appear to play a major role in both stabilization of blood vessels and endothelial cell death, the angiopoietins are attractive candidates as regulators of these divergent process as they occur in the female reproductive tract. When active angiogenesis is occurring in the presence of VEGF, Ang-2 acts in concert to enhance angiogenesis, while Ang-1 is involved in the process of maturation and stabilization of new
blood vessels [20]. Paradoxically, in circumstances of reduced VEGF expression, Ang-2 may act to destabilize blood vessels and induce vascular regression by competitive inhibition of Ang-1 [20]. Previous studies have reported that Ang-1 and Ang-2 mRNA expression was detected in the ovary of the rat [20], human [32], monkey [12, 13], pig [29] and cow [8]. These results suggested that angiopoietins might be associated with thecal angiogenesis during follicular development in the mammalian ovary.

Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) also have angiogenic action in the ovary [9, 22, 25]. bFGF mRNA was predominantly localized to the granulosa cells of the dominant follicles of rat [10] and pig [28] ovary. In the bovine ovary, bFGF mRNA expression in the theca interna increased significantly during the final growth of follicles, whereas its expression in granulosa cells was very weak [2]. Previous studies indicated that EGF is an angiogenic factor [26], and it was shown to enhance the proliferation of vascular endothelial cells in vitro [3] and affect neovascularization in vivo [9]. EGF is soluble in tissue fluid, can be translocated in tissues and induces endothelial cells to proliferate and form capillaries [3]. Immunocytochemical studies showed that EGF peptide was localized in the cumulus cells and granulosa cells [31], and in thecal and interstitial cells around growing follicles [4, 22]. In addition, mRNA of EGF was expressed in the granulosa cells in pig follicles [28]. These results suggested that bFGF and EGF are not only associated with follicular angiogenesis but also inhibit apoptosis promoting the production of angiogenic factors by granulosa cells, and stimulate granulosa cell proliferation [29].

**VASCUlATURE, DOMINANCE AND ATRESIA OF FOLLICLES: MANIPULATION OF OVARIAN ANGIOGENESIS IN MAMMALS**

In primates, rats and pigs, dominant follicles develop only during the follicular phase and are thus destined for ovulation [7]. In contrast, in cows, sheep and horses, recruitment, selection and dominance of follicles

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occur at regular intervals and only the dominant follicle present during the follicular phase ovulates. Dominance of follicles may be influenced by vascularization and it has been proposed that increased vascularity may be a primary determinant of follicular dominance. A previous study [24] demonstrated that dominant follicles are more vascular than non-dominant follicles. Dominant follicles have a more vascular theca compared with other antral follicles, and as a result they display an increased uptake of serum gonadotropins [21].

The number of oocytes drops steadily after birth and out of hundreds of thousands of oocytes present at puberty, a species-specific number, but less than 1%, will reach full maturation and ovulation. The rest of the follicles will degenerate in a process termed atresia. It has been reported that decreased proliferation of thecal capillary endothelial cells leads to reduced thecal vasculature, one of the earliest events during follicular atresia. Atresia of primordial follicles may be due to reduced blood supply [15].

The local administration of angiogenic inhibitors (TNP-470 or angiostatin) or VEGF antagonist (soluble VEGFR1) into the preovulatory follicle of rhesus monkeys during spontaneous menstrual cycle indicated that VEGF antagonist impairs ovulation and attenuates subsequent luteal function possibly by altering normal steroidogenic-nonsteroidogenic cell interaction or differentiation without dramatically changing cell numbers [14]. These findings may raise the possibility that the enhancement of circular commitment of VEGF around the follicles could positively affect their development process (follicle selection).

We tested this hypothesis in pigs by inducing extra-production of VEGF by direct ovarian injection of its gene fragments. We performed in vivo injection of VEGF gene fragments to enhance the thecal angiogenesis associated with follicular development [32]. Histological examination revealed that the vascular density in the theca interna of follicles in the VEGF-treated ovary increased two-fold compared with that in the VEGF-untreated ovary (fig. 4). The pattern of perifollicular angiogenesis in the VEGF-treated group was essentially the same during follicular development after eCG treatment. Large follicles (larger than 5 mm in diameter) protruding on the ovarian surface were observed in the ovaries of eCG-treated gilts with
Manipulation of ovarian follicle development

Fig. 4. VEGF gene injection promotes development of the capillary network in the theca interna of follicles. 

A/ Hematoxylin-eosin staining indicating blood vessels (arrows) in the thecal cell layer of the preovulatory follicle in eCG-primed gilt with VEGF gene administration. 

B/ Vascular density was expressed as the number of follicular capillaries (10 µm in diameter) per theca interna area (mm²). Data are represented as means ± SEM. Significant differences between means were analyzed by one-way ANOVA followed by the Fisher’s LSD test as a multiple comparison test. Different superscripts denote significantly different values (p<0.05). (reproduced with permission from [30])
or without VEGF gene fragment injection, but the mean number of large follicles significantly increased in the VEGF gene fragment-treated ovaries (14.8±1.89/ovary vs 8.3±0.75/ovary, fig. 5A). In the VEGF gene fragment-treated ovaries, in particular, the large follicles protruding from the surface of the ovary were rich in visible blood vessels with erythrocytes. The average weight of the VEGF gene fragment-treated ovaries was significantly heavier than those of the control and the eCG alone groups (fig. 5B). These changes clearly indicated stimulatory signs of thecal angiogenesis associated with a

Fig. 5. A/ Morphology and B/ weight of ovaries from control (n=4), eCG alone (n=4) and VEGF+eCG (n=3) treated animals. VEGF gene injection increased the ovarian weight (g per 10 kg body weight). Data are represented as means ± SEM. Significant differences in the ovarian weight were analyzed by one- way ANOVA followed by the Fisher’s LSD test as a multiple comparison test. Different superscripts denote significantly different values (p <0.05). (reproduced with permission from [30])
significant amount of relative tissue areas covered by vessels. Since peri-follicular angiogenesis supplies gonadotropins and many growth factors to the granulosa cells of follicles, the progress in peri-follicular angiogenesis is associated with the promotion of follicular development.

Importantly, the increase in vascular density surrounding the antral follicles contributes to the inhibition of atresia. Early atretic follicles can regenerate when placed in culture, suggesting that the follicle remains in the atretic state due to a decrease in vascularity that limits access to nutrients, substrates and trophic hormones [23]. Our findings demonstrated that promotion of thecal vascularization by VEGF contributed to the decrease in atretic follicles [30]. In pig ovaries, as in those of other species, apoptotic cell death is a mechanism which induces follicular atresia [11]. Thus, VEGF may suppress the granulosa cell apoptosis and inhibit the follicular atresia.

CONCLUDING COMMENTS

The ovary offers an excellent system for studying physiologic angiogenesis and vascular regression. The study of VEGF gene fragments injection has provided new insights into the relationships between angiogenesis and follicle growth (selection and atresia). However, much remains to be discovered about the function of vascular specific growth factors, as well as the pleiotropic angiogenic factors present during angiogenesis and vascular regression of follicles. Thus, new research is necessary to learn more about the role of the extracellular matrix and cell-to-cell interactions among endothelial cells, granulosa cells, theca cells and immune cells.

REFERENCES


Manipulation of ovarian follicle development


