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The expression of angiogenic growth factors and their receptors in ovarian follicles throughout the estrous cycle in the ewe

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Abstract

Healthy follicles are highly vascularized whereas those undergoing atresia have poor vascularity, suggesting a relationship between follicular vascularization and follicular function. Vascularization is regulated by angiogenic factors, and among them vascular endothelial growth factor (VEGF) and angiopoietin-Tie (Ang-Tie) systems are of central importance. The objectives of this study were to determine if VEGF, VEGF receptor-2 (VEGFR-2), and components of the Ang-Tie system are expressed in ovarian follicles at both the protein and mRNA levels and to explore if their expression is related to the stage of the estrous cycle in the ewe. Ovaries from cyclic ewes were collected during the luteal phase (n = 5) or before (n = 5), during (n = 4), and after (n = 4) the preovulatory luteinizing hormone (LH) surge. After fixation, ovaries were wax-embedded, serially sectioned, and analyzed for both protein and mRNA expression of VEGF, VEGFR-2, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie-1 (mRNA only), and Tie-2. mRNA was studied by *in situ* hybridization using digoxigenin-11-UTP–labeled ovine riboprobes. A similar pattern of expression was observed for mRNA and protein for all of the factors. Both mRNA and protein expression of VEGF, VEGFR-2, Ang-1, Ang-2, Tie-1 (mRNA only), and Tie-2 in the granulosa and theca cells of follicles ≥2 mm in diameter was significantly different among the stages of the estrous cycle, with the highest expression detected at the post-LH surge stage. Theca cells expressed significantly greater levels of the six angiogenic factors compared with granulosa cells at all stages of the estrous cycle. Expression levels in granulosa and theca cells were comparable between small (2.0 to 2.5 mm) and medium (2.5 to 4.0 mm) follicles, but large follicles (>4.0 mm) expressed higher mRNA and protein levels (all $P < 0.05$) for all factors at all stages of the estrous cycle. These data show (i) that VEGF, VEGFR-2, and the Ang-Tie system are present in both granulosa and theca cells of the ovarian follicle, (ii) that thecal cells consistently express greater levels of all of these factors compared with granulosa cells, and (iii) that their levels of expression are related to the stage of the estrous cycle and to follicle size.

Keywords: Angiopoietins; Estrous cycle; Ewe; Ovarian follicle; Vascular endothelial growth factor (VEGF)

1. Introduction

Angiogenesis is essential for normal tissue growth [1,2] and is regulated by complex interactions among the angiogenic growth factors; of these vascular endothelial growth factor (VEGF) and angiopoietins 1 and 2 (Ang-1 and Ang-2) are the most significant [3–6]. The cellular actions of VEGF are mediated through two major receptors, VEGFR-1 (Flt-1 or *fms*-like tyrosine kinase-1) and VEGFR-2 (Flk-1 or fetal liver kinase-1), belonging to the receptor tyrosine-kinase family of membrane receptors [7,8]. Both VEGFR-1 and VEGFR-2 undergo ligand-dependent tyrosine phosphorylation, although VEGFR-1 has weak or

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undetectable cellular responses to phosphorylation [9,10]. Angiopoietins are responsible for the maturation and stabilization of blood vessels [11]. Angiopoietin-1 binds specifically to Tie-2 [12] and is required for recruitment of perivascular cells that stabilize newly formed capillaries [13]. It also promotes blood vessel maturation [12] and acts as a survival factor for endothelial cells [14,15]. In contrast, Ang-2 is a Tie-2–dependent antagonist of Ang-1 and a competitive antagonist for Ang-1–mediated stabilization of blood vessels [11]. In the absence of survival signals such as VEGF, Ang-2 promotes destabilization and eventually regression of blood vessels. Thus, the ratio of Ang-2 to Ang-1 is critically important for both angiogenesis and regression of blood vessels [16]. In the presence of high VEGF concentrations, a high Ang-2 to Ang-1 ratio leads to active angiogenesis and the formation of new blood vessels, whereas a high Ang-2 to Ang-1 ratio in the presence of low VEGF results in vessel stabilization [17].

The ovarian follicle is a site of active angiogenesis. Up to the small preantral stage, follicles are avascular, and angiogenesis is initiated at this time and continues throughout follicular growth [18]. The vascular network in the follicle is confined to the thecal layer, and thecal capillaries do not penetrate the basement membrane so that the granulosa compartment remains avascular until the basement membrane breaks down at ovulation. The ovulatory follicle had a high degree of vascularity compared with subordinate follicles [19], and regression of the thecal vascular network of was observed in nonovulatory follicles [20,21] and during atresia [22]. These findings suggest a strong association between follicular development and the network of follicular blood vessels.

Patterns of angiogenesis and of angiogenic factors in the follicle have been widely studied in several species over the past 10 to15 yr, and several comprehensive reviews have been published [17,23–27]. Patterns of VEGF and its receptors and their RNAs have been described in ovarian follicles from rodents [28,29], humans [30–34], pigs [35], and cattle [36,17]. *In situ* hybridization studies have shown that the mRNA for VEGF was expressed in the thecal layer of preantral and antral follicles in rat ovaries [37], and its receptor, VEGF-R2, was present on endothelial cells in the theca of growing follicles [38]. Of the angiopoietins, Ang-1 was uniformly expressed in the thecal layer during follicular development in cattle [17], and in the rat, Ang-2 mRNA was increased in the granulosa cells of atretic follicles in association with reduced expression of VEGF [11]. In the marmoset, Ang-1 mRNA was detected in the theca of tertiary follicles, and in contrast with the rat, Ang-2 mRNA was not detected [39], and mRNAs for the angiopoietins, Tie-1, and Tie-2 were detected in ovarian follicles of the rat [29], the primate [40], and the cow [36].

Functional studies have shown that VEGF induced rapid vascular growth in follicles [41,42] and increased vascular permeability [43,44] and the survival of newly formed blood vessels [45,46]. The use of VEGF antagonists suppressed folliculogenesis in marmoset monkeys [39]. Furthermore, recent studies have suggested that Ang-1 and Ang-2 have important roles in the modulation of vascular growth and regression in the follicle [13,15,16]. From the above-mentioned studies, it is evident that angiogenic growth factors in the follicle have a significant role in preparing the follicle for ovulation and formation of a functional corpus luteum.

The ewe has been an important and widely used model for mammalian folliculogenesis [47], but surprisingly little is known about the role of VEGF and the angiopoietins in ovine folliculogenesis, and the pattern of expression of the angiogenic factors across the estrous cycle has not been reported. Thus, the objectives of this study were to determine the patterns of VEGF, VEGFR-2, Ang-1, Ang-2, Tie-1, and Tie-2 and of their respective mRNAs in ovarian follicles collected from ewes at different times in the luteal and follicular phases of the estrous cycle and to determine if the follicular cell type and/or the follicle size was related to these patterns.

2. Materials and methods

2.1. Animal and tissues

Eighteen Welsh Mountain ewes were used in this study. All procedures were conducted with Home Office authorization and in compliance with the U.K. Animal (Scientific Procedures) Act of 1986. Estrus was synchronized to a common day in all ewes using intravaginal progestogen sponges (Chronogest; Intervet UK Ltd, Milton Keynes, Northampton, UK) for 12 d followed by 250 IU pregnant mare serum gonadotropin (Folligon; Intervet UK Ltd) at the time of sponge removal. Estrus was detected with a vasectomized ram, and all the ewes showed estrus within 24 h of sponge removal. The day of estrus was considered to be Day 0 of the synchronized cycle. On Day 9 of the synchronized cycle (the luteal phase), five animals were killed by captive bolt and their ovaries collected. On Day 11 of the synchronized cycle, remaining ewes