Human follicle fluid vascular endothelial growth factor concentrations are correlated with luteinization in spontaneously developing follicles

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Introduction

The process of luteinization is induced by the luteinizing hormone (LH) surge, and this leads to a sequence of events resulting in the transformation of granulosa cells into luteal cells and the formation of a corpus luteum (Doody et al., 1990). However, granulosa cells from large pre-ovulatory follicles may also undergo spontaneous luteinization in vitro (Ledwitz-Rigby et al., 1977). Thus, it has been suggested that large pre-ovulatory follicles may produce factors locally that mediate the luteinization process (Adashi, 1991).

If luteinization mediators are indeed produced locally by granulosa cells, one would naturally expect to find much higher concentrations in the pre-ovulatory follicle fluid than in the serum. One would also expect to find a positive association between follicle fluid concentrations of the putative mediator and markers of luteinization, such as the concurrent serum LH and follicle fluid progesterone concentrations.

Angiogenesis is a prominent histological component of the luteinization process (Findlay, 1986) and angiogenic factors exist in the follicle fluid of human ovarian pre-ovulatory follicles. In fact, this fluid stimulates angiogenesis when injected into rabbit eyes (Frederick et al., 1984). Neovascularization initially occurs within the thecal layer of the dominant follicle and, in response to the LH surge, this neovascularization spreads across the follicle basement membrane into the granulosa layers (Findlay, 1986). This neovascularization, induced by LH stimulation, might be mediated by local production of vascular endothelial growth factor (VEGF).

The cytokine VEGF, a basic 45 kDa dimeric glycoprotein, is an endothelial cell-specific mitogen (Ferrara et al., 1992; Ferrara and Davis-Smyth, 1997). VEGF induces angiogenesis by stimulating the proliferation of vascular endothelial cells (Dvorak et al., 1995; Ferrara and Davis-Smyth, 1997).

Interestingly, VEGF expression can be demonstrated immunohistochemically in granulosa cells of human Graafian follicles (Kamat et al., 1995; Gordon et al., 1996). Also, there is a positive linear correlation between granulosa cell VEGF messenger ribonucleic acid (mRNA) expression and serum progesterone concentrations in women undergoing oocyte retrieval for in-vitro fertilization (IVF) (Doldi et al., 1997). Furthermore, human chorionic gonadotrophin (HCG) induces a dose-dependent enhancement of mRNA expression in cultured human luteinized granulosa cells obtained from women undergoing oocyte retrieval for IVF (Neulen et al., 1995). Follicle fluid VEGF concentrations in cycles stimulated by exogenously administered gonadotrophins are indeed 100-fold greater than serum concentrations; however, this is an artificial situation in which the normal feedback mechanisms controlling gonadotrophin effect are missing (Krasnow et al., 1996; Abramov et al., 1997; Lee et al., 1997).

These findings support the hypothesis that local VEGF production by granulosa cells, stimulated by the LH surge, indeed may be a luteinization mediator in women. To investigate this hypothesis in spontaneously developing dominant follicles in cycling normal fertile women, we examined whether there is a gradient in VEGF concentration between the dominant pre-ovulatory follicle fluid and the serum. We also investigated...
the hypothesis that there is a positive correlation between human pre-ovulatory follicle fluid VEGF concentrations and the luteinization markers serum LH and follicle fluid progesterone concentrations.

Material and methods
We measured VEGF concentrations in the serum and fluid from the dominant follicle of healthy multiparous regularly cycling women undergoing laparoscopic sterilization. This protocol was approved by the Institutional Review Board of the National Institute of Child Health and Human Development.

We recruited healthy women desiring sterilization, who were between 21 and 40 years of age. Each patient underwent a complete history and physical examination.

Follicle size was monitored serially by transvaginal ultrasound starting on day 8 of the menstrual cycle (GE 3000 5 MHz or Ultramark 9.5–9 MHz probe). Patients were scheduled for laparoscopic sterilization and follicle aspiration when the dominant follicle reached a diameter of 11 mm or greater. Aspirations were accomplished transabdominally using an 18 gauge single lumen needle (Cook, Spencer, IN, USA). Peripheral blood was collected from an antecubital vein concurrent with follicle aspiration. Sera and follicle fluids were immediately centrifuged for 10 minutes at 1000 g and the clear supernatants were stored at −70°C until assays were performed. VEGF concentrations were measured following one freeze–thaw procedure (we found that four freeze–thaw cycles had no measurable effect on VEGF determination by this method).

Surgical procedures were performed under general anaesthesia using endotracheal intubation.

Immunoreactive VEGF was measured by a solid phase enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (R&D Systems Inc., Minneapolis, MN, USA). The assay employs recombinant human VEGF165 standards and antibodies raised against the recombinant protein. There was no significant cross-reactivity or interference between VEGF and other cytokines (over 90 cytokines tested, R&D Systems). Intra- and interassay coefficients of variation (CV) were less than 10%. The assay remained parallel up to 1:16 dilution and the minimum detectable dose was found to be 5 pg/ml.

Progesterone and LH were measured by radioimmunoassay using previously described techniques (Odell et al., 1967; Abraham et al., 1971). Progesterone extraction assay had intra- and interassay CV of less than 20%, and LH (LH standard AFP 1713A) had intra- and interassay CV of less than 10%.

Follicle vascularization occurs early in the process of follicle luteinization. Therefore, we were particularly interested in examining the correlation between follicle fluid VEGF and progesterone concentrations during the early phase of luteinization. To do this we defined the early phase as those follicles that had a progesterone concentration to be free of bloody contamination.

On average, follicle fluid VEGF concentrations were 100 times greater than serum concentrations. The mean pre-ovulatory follicle fluid VEGF concentration was 6900 pg/ml (SEM = 644 pg/ml, range 1200–17 100 pg/ml), whereas the mean serum VEGF concentration was only 62 pg/ml (SEM = 19.4 pg/ml, range 5–590 pg/ml, P < 0.0001, Figure 1). The mean follicle fluid progesterone concentration was 10 176 nmol/l (range 636–66 780 nmol/l) and the mean serum progesterone concentration was 3.65 nmol/l (range 0.76–31.48 nmol/l).

Follicle fluid VEGF concentrations from the 31 women were correlated positively with their follicle fluid progesterone concentrations (r = 0.62, P = 0.0002). In the earlier stages of follicle luteinization (n = 22) this correlation was even tighter (r = 0.87, P < 0.0001, Figure 2). Furthermore, in these women serum LH concentrations (mean = 18.1 mIU/ml, range 8.2–26.7 mIU/ml) were also positively correlated with follicle fluid VEGF concentrations (r = 0.51, P = 0.02, Figure 3).

Results
Thirty one healthy regularly menstruating multiparous women with a median age of 33 years (range 21–40 years) completed the study. Follicle aspiration was performed at a median of 13 days from the last menstrual period (range 11–17 days). The median follicle diameter was 16 mm (range 11–23 mm). All aspirated follicle fluids were clear to inspection and appeared to be free of bloody contamination.

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Discussion

Here we demonstrate that VEGF concentrations in the fluid of human pre-ovulatory ovarian follicles of natural cycles are greater than serum concentrations by more than two orders of magnitude. Also, these concentrations of VEGF associate positively with markers of luteinization, such as serum LH and follicle fluid progesterone concentrations. Our findings are consistent with the hypothesis that local VEGF production, in response to LH, is a mediator of the neovascularization taking place during luteinization.

Luteinization of the granulosa cells, triggered by LH, is characterized by increased progesterone synthesis. In fact, during the late follicular phase the dominant follicle contains 20 times higher progesterone concentrations than any other follicle throughout the cycle (McNatty et al., 1975). Interestingly, follicle fluid from large porcine follicles enhances granulosa cell luteinization (Tsafiri and Adashi, 1994) by increasing the number of LH receptors (Osteen et al., 1982), amount of progesterone secretion (Ledwitz-Rigby and Rigby, 1979), as well as the morphologic changes characteristic of luteinization (McLean et al., 1986).

Neovascularization is also a hallmark of the luteinization process (Findlay, 1986). Shweiki et al. (1993) investigated neovascularization of ovarian follicles using follicle-stimulating hormone-primed female rats as a model. They found that VEGF message was expressed in the interstitial tissue and the theca layers of ovaries containing follicles undergoing neovascularization (i.e., preantral follicles and follicles with small antrum). Subsequently, with further growth and maturation of follicles, high concentrations of VEGF mRNA were detectable in granulosa cells as well (Shweiki et al., 1993). Similar results were obtained from primate ovaries, where intense expression of VEGF mRNA was found in the developing follicle during the late follicular phase (Ravindranath et al., 1992). In addition, it was demonstrated that LH increases VEGF mRNA concentrations of ovarian bovine granulosa cells in vitro (Garrido et al., 1993). In fact, immunofluorescent analysis of granulosa cells, retrieved from women undergoing oocyte retrieval for IVF, revealed the presence of a morphologically specific granulosa cell subpopulation containing VEGF protein (Antczak et al., 1997). Furthermore, in women undergoing oocyte retrieval for IVF, evidence suggests that VEGF concentrations and the perifollicular vascularity may play a role in maintaining intrafollicular oxygen content necessary for follicle and oocyte health (Van Blerkom et al., 1997).

In women undergoing oocyte retrieval for IVF follicle fluid VEGF concentrations are also 100-fold greater than serum concentrations (Krasnow et al., 1996). Furthermore, there is a positive correlation between follicle fluid VEGF and progesterone concentrations from the largest follicles obtained at oocyte retrieval (36 h after the administration of 7500 IU of HCG) (Lee et al., 1997). Interestingly, high serum, peritoneal fluid, and follicle fluid VEGF concentrations have been correlated with the development of ovarian hyperstimulation syndrome (Krasnow et al., 1996; Abramov et al., 1997; Rizk et al., 1997).

It is noteworthy that primates luteinized granulosa cells produce eight-fold greater VEGF concentrations than non-luteinized granulosa cells (Christenson and Stouffer, 1997). Furthermore, in-vitro treatment with HCG increases VEGF production by primate non-luteinized granulosa cells to similar concentrations of those of luteinized granulosa cells (Christenson and Stouffer, 1997). It is tempting to speculate that local VEGF production in response to the LH surge mediates early luteinization of the dominant follicle by inducing angiogenesis. This neovascularization may lead to increased concentrations of substrates in the theca layer of the dominant follicle and subsequently to increased progesterone production by the granulosa cells.

It is well known that appropriately timed luteinization is essential for normal follicle function. High serum LH concentrations have been associated with infertility and increased miscarriage rates (Regan et al., 1990; Balen et al., 1993; Watson et al., 1993). Furthermore, inappropriate luteinization of follicles, due to increased LH concentrations, appears to contribute to the follicle dysfunction in some women with karyotypically normal spontaneous premature ovarian failure (Nelson et al., 1994). Further research is needed to investigate the ovarian physiologic and pathophysiologic mechanisms mediated by VEGF, possibly using a VEGF inhibitor (Kendall and Thomas, 1993; Aiello et al., 1995).

In conclusion, VEGF is present in the fluid of human pre-ovulatory follicles in natural cycles at concentrations 100 times that found in serum. Furthermore, VEGF concentrations are positively associated with two markers of luteinization, serum LH and follicle fluid progesterone. Our findings demonstrate the close dynamic relationship between VEGF production and early luteinization in human follicles during normal non-stimulated menstrual cycles.

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