In Controlled Ovarian Hyperstimulation, Steroid Production, Oocyte Retrieval, and Pregnancy Rate Correlate with Gene Expression of Vascular Endothelial Growth Factor

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Purpose: Whether the gene expression of vascular endothelial growth factor (VEGF) in human granulosa cells is a predictor of fertilization was evaluated in patients participating in an in vitro fertilization program.

Methods: Fifty patients with normal ovaries who were participating in an in vitro fertilization program at the University of Milan, San Raffaele Scientific Institute, were included in the study. We correlated E₂ and P serum levels on the day of oocyte collection, the number of follicles, oocytes collected, and fertilized, and pregnancies with mRNA for VEGF of luteinizing granulosa cells obtained at the time of oocyte retrieval.

Results: Comparing E₂ and P serum levels, the number of follicles, oocytes collected and fertilized, and pregnancies with gene expression for VEGF, we found a positive correlation. E₂ and P serum levels were higher in patients with increased VEGF ($P < 0.01$). Furthermore, there were more follicles, oocytes collected and fertilized, and pregnancies in patients with maximum expression of VEGF, and the difference was statistically significant ($P < 0.05$).

Conclusions: Our results suggest that VEGF may be important for vascular development during follicular growth and luteal differentiation, oocyte maturation, and fertilization.

KEY WORDS: ovarian hyperstimulation; in vitro fertilization; vascular endothelial growth factor; granulosa cells.

INTRODUCTION

Gonadotropins play a major role in the regulation of growth and maturation of the ovarian follicle in the

process of controlled ovarian hyperstimulation (COH). Indeed, the variable fate of follicles exposed to comparable gonadotropic stimulation suggests the existence of additional intraovarian modulatory mechanisms. Increasing evidence from animal and in vitro studies suggests that local growth factors have important paracrine or autocrine effects in granulosa cell proliferation and differentiation. In particular, angiogenic factors play a role in the vascular changes associated with follicular and luteal development (1,2). 17 β -Estradiol (E₂) is elevated in hyperstimulated patients, and it has been shown to increase angiogenesis and capillary permeability in animal models (3). Furthermore, several substances have been proposed as possible mediators of capillary permeability, such as prostaglandins (4), histamine (5), serotonin (6), transforming growth factors α (TGF α) (7) and β (TGF β) (8), and vascular endothelial growth factor (VEGF). VEGF is a recently identified secreted protein that has the potential to play a dynamic role in the physiological and pathologic regulation of vascular endothelial growth, angiogenesis, and capillary permeability (9–15). In the course of follicular growth, the theca interna becomes richly vascularized. After ovulation, the thecal vessels grow into the ruptured follicle and form a complex network, which brings about the development of the corpus luteum.

VEGF has been detected in human ovarian granulosa and theca cells late in follicle development and, subsequent to ovulation, in granulosa and theca lutein cells (16). These findings suggest that the growth factor may be in direct contact with the oocyte until the time of fertilization or implantation and may play a physiological role in the regulation of the development and function of the corpus luteum.

In the present study we examined if the gene expression of VEGF in human granulosa cells is a predictor

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of fertilization in patients participating in an in vitro fertilization program.

MATERIALS AND METHODS

Patients

This study includes 50 patients with normal ovaries participating in an in vitro fertilization program at the University of Milan, San Raffaele Scientific Institute, Reproductive Endocrinology Center, OB/GYN Clinic. All patients had a tubal factor, and all of the male partners had normal semen quality according to World Health Organization criteria. Follicular development had been stimulated by folliculotropin (Metrodin; Serono, Rome) in the presence of a GnRH analogue (Decapeptyl; Ipsen, Paris) until an adequate response was achieved. Final maturation of the oocytes was effected with 10,000 IU human chorionic gonadotropin (hCG; Serono).

Granulosa Cells

Oocyte retrieval was accomplished 35 hr after hCG injection. After removing the oocytes, the remaining cells from each patient were pooled and washed twice with medium 199 (Flow Laboratories, Milan, Italy). Granulosa cells and red blood cells were transferred to a 12-ml tube containing 3.5 ml of lymphocyte separation medium (Flow Laboratories) and separated by centrifugation at 600 *g* for 5 min. Granulosa cells were washed twice with medium 199 and then frozen immediately at -80°C until RNA extraction.

RNA Analysis

Undegraded total RNA was prepared from frozen granulosa cells by guanidine thiocyanate/phenol chloroform single-step extraction (RNAfast; Molecular Systems, San Diego, CA). For dot-blot hybridization,

5 μg of total RNA from each sample was denatured in 6% formaldehyde and 50% deionized formamide in $1\times$ standard saline citrate (SSC; 0.75 M NaCl and 0.075 M Na-citrate). The RNAs were then transferred to nitrocellulose and nylon membranes (Hybond-N; Amersham). Hybridizations with cDNA probe (VEGF) were performed in Northern hybridization buffer (Celbio, Milan, Italy) in 50% formamide and 100 $\mu\text{g}/\text{m}$ salmon sperm DNA. Hybridizations were performed at 42°C for 16 hr with $2-4 \times 10^6$ cpm/ml labeled with [α - ^{32}P]dCTP cDNA probe. After hybridization the blots were washed twice with $2\times$ SSC with 0.1% sodium dodecyl sulfate (SDS), then with $0.1\times$ sulfate SSC/ $0.1\times$ SDS for 15 min at room temperature. After washing, the blots were exposed to X-ray film (Kodak XAR 5; Eastman Kodak, Rochester, NY) at -80°C with intensifying screens.

The relative amount of mRNA examined was determined by reprobing all blots for mRNA encoding actin. The relative intensity of signals for VEGF on the dot blots was compared to that for actin using computer-assisted densitometry.

Statistical methods included the paired *t*-test and analysis of variance. Significance was assumed at $P < 0.05$.

RESULTS

Table I shows the correlation of VEGF gene expression with E_2 and P serum levels on the day of oocyte collection. There was a significant difference between the group with maximum (VEGF 100) and the group with minimum (VEGF 25) expression of VEGF mRNA ($P < 0.01$). Comparing the number of follicles, oocytes collected and fertilized, and pregnancies, we again found a positive correlation with mRNA expression for VEGF. Table II shows the difference between the groups. There were more follicles, oocytes collected and fertilized, and pregnancies in patients with

Table I. Comparison of VEGF Expression and E_2 and P Serum Levels on the Day of Oocyte Retrieval

	VEGF expression (%)			
	25	50	75	100
Serum E_2 (ng/ml)	$0.74 \pm 0.21^*$	1.83 ± 0.41	1.34 ± 0.22	$2.11 \pm 0.31^*$
Serum P (ng/ml)	$0.37 \pm 0.12^*$	0.99 ± 0.25	0.63 ± 0.05	$2.01 \pm 0.45^*$

* $P < 0.01$.

Table II. Comparison of VEGF Expression and Numbers of Follicles, Oocytes Collected and Fertilized, and Pregnancies

	VEGF expression (%)			
	25	50	75	100
Follicles ≤ 16 mm (day of hCG)	1.5 (0–3)*	2.5 (0–4)	3.5 (0–7)	6.5 (2–15)*
Follicles ≥ 16 mm (day of hCG)	3.5 (2–7)*	6.5 (3–11)	5 (3–6)	8.5 (4–18)*
Oocytes collected	2.5 (2–4)*	5 (2–9)	4 (2–8)	10 (2–16)*
Oocytes fertilized	0*	0.25 (0–1)	0.5 (0–2)	1.5 (0–4)*
Pregnancies	0	2	2	6

* $P < 0.05$.

maximum expression of VEGF (VEGF 100) and the difference was statistically significant ($P < 0.05$).

DISCUSSION

In the present study we examined the gene expression of VEGF as predictor of fertilization in 50 patients participating in an in vitro fertilization program. We found that in human luteinizing granulosa cells, on the day of oocyte retrieval, there was a positive correlation of VEGF gene expression with E_2 and P serum level. Furthermore, the numbers of follicles, oocytes obtained and fertilized, and pregnancies were higher when mRNA encoding VEGF was highly expressed. VEGF and other angiogenic factors play a role in the vascular changes associated with follicular and luteal development (16). VEGF mRNA and protein were previously detected in human ovarian granulosa and theca cells late in follicle development and, subsequent to ovulation, in granulosa and theca lutein cells. Therefore, VEGF is ideally positioned to provoke the increased permeability of thecal blood vessels that occurs shortly before ovulation (17). VEGF likely also contributes to the angiogenesis and connective tissue stroma generation that accompany corpus luteum/corpus albicans formation (16,18). Because the granulosa and corpus luteum vasculature consists exclusively of new capillaries, while the theca interna contains numerous arterioles, we expect an increase in VEGF mRNA in granulosa and luteal cells, where the processes of growth and remodeling are rapid.

The elevated number of capillaries in the follicle and the corpus luteum due to VEGF secretion from granulosa and luteal cells induces an increased level of gonadal steroids. We found that VEGF mRNA correlates positively with E_2 and P. Recent studies found an augmentation in the permeability rate of both follicular fluid and peritoneal fluid from patients undergoing

ovarian hyperstimulation, who responded with an increasing number of eggs compared with controls (19). The authors postulated that VEGF may increase capillary permeability, leading to ovarian hyperstimulation syndrome (20). Our results confirm that VEGF may play an important role in the regulation of vascular development during follicular growth and luteal differentiation.

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