Regulation of vascular endothelial growth factor-A and its soluble receptor sFlt-1 by luteinizing hormone in vivo: implication for ovarian follicle angiogenesis

Guy Gutman, M.D.,[a,b] *Vivian Barak, Ph.D.,*[c] *Sharon Maslovitz, M.D.,*[a,b] *Ami Amit, M.D.,*[a,b] *Joseph B. Lessing, M.D.,*[a,b] *and Eli Geva, M.D.*[a,b,d]

[a] Sara Racine IVF Unit, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv; [b] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv; [c] Immunology Laboratory for Tumor Diagnosis, Oncology Department, Hadassah University Hospital, Jerusalem; [d] Present address: Department of Obstetrics and Gynecology, Edith Wolfson Medical Center, Holon, Israel

Objective: To determine in vivo whether LH supplementation during the late follicular phase induces ovarian follicle angiogenesis in humans, as reflected by vascular endothelial growth factor (VEGF)-A, its soluble receptor sFlt-1, and placental growth factor (PlGF) expression.

Design: Randomized, double-blind, placebo-controlled study.

Setting: Academic tertiary care medical center.

Patient(s): Twenty infertile, healthy women (aged 18–39 years) undergoing IVF.

Intervention(s): Administration of recombinant FSH after down-regulation and equal randomization of subjects to receive recombinant LH 75 IU/day or placebo when two or more follicles reached a mean diameter of 14 mm.

Main Outcome Measure(s): Serum and follicular fluid (FF) VEGF-A, sFlt-1, and PlGF protein levels were measured.

Result(s): Recombinant LH increased both the FF VEGF-A/sFlt-1 ratio statistically significantly and PlGF/sFlt-1 insignificantly. Recombinant LH did not affect the serum VEGF/sFlt-1 ratio. Plasma levels of PlGF were undetectable.

Conclusions: This in vivo study demonstrates for the first time in humans that LH induces ovarian follicular angiogenesis via modulation of VEGF-A and its soluble receptor sFlt-1 expression. A constant VEGF-A/sFlt–serum ratio may prevent adverse effects of VEGF-A. Because angiogenesis is essential during the periovulatory period, recombinant LH supplementation during the late follicular phase may improve ovulation induction outcome. (Fertil Steril® 2008;89:922–6. ©2008 by American Society for Reproductive Medicine.)

Key Words: Angiogenesis, LH, PlGF, ovary, VEGF-A

Angiogenesis is a fundamental process by which new capillary blood vessels from preexisting vessels are regulated by vascular endothelial–specific growth factors and inhibitors. The female reproductive system undergoes a number of programmed angiogenic processes during the menstrual cycle and decline of ovarian and endometrial function (1, 2). Folliculogenesis, ovulation, and corpus luteum formation and maintenance are processes that are critically dependent on angiogenesis (3).

Vascular endothelial growth factor (VEGF)-A is a basic, heparin-binding homodimeric glycoprotein that was initially defined, characterized, and purified on the basis of its ability to induce vascular permeability, as well as for its ability to promote vascular endothelial cell proliferation (4). Vascular endothelial growth factor-A is a potent mitogen and survival factor for endothelial cells that initiates vasculogenesis and angiogenesis by inducing endothelial cell proliferation, migration, and sprouting activity, as well as by promoting endothelial cell formation of tubulelike structures. Vascular endothelial growth factor-A exerts its effects by binding with high affinity to two endothelial cell–specific tyrosine kinase receptors: VEGFR-1/c-fms–like tyrosine kinase (Flt-1) and VEGFR-2/kinase domain receptor (KDR). Angiogenesis is stimulated primarily by activating the VEGFR-2 receptor (4), whereas VEGFR-1 appears to play a later role in the angiogenic process (5). Vascular endothelial growth factor R-1 might function as a "decoy" by binding VEGF-A and thereby regulating the availability of VEGF-A for activation of VEGFR-2 (6). This decoy function might be subserved by soluble (s)Flt-1, an alternatively processed form of VEGFR-1 that contains the extracellular ligand-binding domain but lacks the signaling tyrosine kinase domain (7). Soluble Flt-1 has strong antagonistic activity and neutralizes the effects of VEGF-A and placental growth factor (PlGF).

Placental growth factor is a member of the VEGF family of growth factors and displays 53% homology with VEGF-A. Although the PlGF proteins bind with high affinity to VEGFR-1, they fail to bind to VEGFR-2. Purified PlGF isoforms have little or no direct mitogenic or permeability-enhancing activity. They can, however, significantly

potentiate the action of low concentrations of VEGF-A in vitro and, more strikingly, in vivo, either directly (VEGFR-1) or by modulation of VEGF-A activity (VEGFR-2) (6).

In situ hybridization studies in rat ovaries have shown that VEGF-A messenger RNA (mRNA) is expressed in the interstitial tissue and theca layers of preantral and small antral follicles (3). Vascular endothelial growth factor-A receptors were detectable on endothelial cells interspersed in the stroma and on capillaries arranged in the peripheral theca layers of growing follicles (4). Concomitantly with further follicular growth and maturation, an additional compartment expresses VEGF-A mRNA, the cumulus cells engulfing the oocyte (3). In the granulosa cells, high levels of VEGF-A mRNA were detectable only at the immediate preovulatory stage. Shortly after ovulation, the predominant site of VEGFA mRNA expression was the granulosa-lutein cells (3). There is, therefore, a dynamic pattern of VEGF-A mRNA expression that parallels LH stimulation. Several studies have suggested that LH and hCG modulate expression of VEGF-A (8–10). Analysis of cultured granulosa-lutein cells obtained at oocyte retrieval during IVF and treated with hCG stimulated VEGF-A mRNA expression in a concentration-dependent manner (11). These data support the concept that the midcycle gonadotropin surge in the periovulatory phase promotes posttranscriptional regulation of VEGF-A production by gonadotropins in luteinizing granulosa cells (12).

We investigated in vivo in humans whether LH supplementation (recombinant LH [rLH]) during the late follicular phase induces ovarian follicle angiogenesis, as reflected by VEGF-A, sFlt-1, and PlGF expression.

MATERIALS AND METHODS

This in vivo, prospective, randomized, double-blind, placebo-controlled study was conducted in infertile, premenopausal, and apparently healthy women between 18 and 39 years of age who were undergoing IVF and who had discontinued any hormonal treatment at least 1 month before screening for study entry. The etiology of infertility was mechanical, unexplained, or male factor infertility, without any known endocrinopathy (e.g., hyperprolactinemia, hypothyroidism and hyperthyroidism, Cushing's disease, adrenal hyperplasia, androgen-secreting tumor, or diabetes). Candidates were also screened by hematologic, biochemical, and urine analyses to ensure that there were no clinically relevant abnormalities. An endovaginal ultrasound scan was performed to rule out structural abnormalities, such as uterine malformations, intracavitary lesions, ovarian tumors or cysts, and follicles >10 mm in diameter. Other exclusion criteria included chronic systemic disease, pregnancy, a history of ovarian hyperstimulation syndrome, previous or current hormone-dependent tumor, substance abuse, eating disorder, treatment with psychotropic agents, central nervous system pathologic conditions, and past failure of more than two IVF cycles. Body mass indices (BMIs) of the participants were between 18 and 30. Participants provided informed consent, as ap-

proved by the institutional review board, to undergo study-related procedures. The assignment to rLH (Luveris; Serono Inc., Rockland, MA) or placebo (sucrose) treatment was by a computer-generated randomization list (Serono Inc., Geneva, Switzerland). Recombinant LH/placebo vials were labeled with a kit randomization number. The treatment protocol included administration of a GnRH agonist (GnRH-a) (buserelin acetate, SC 0.2 mg/day) on day 2 of the menstrual cycle and then daily for at least 14 days. After 2 weeks of GnRH-a treatment, inhibition of the hypothalamic-pituitary-ovarian axis was confirmed by E_2 level <70 pg/mL, and an endovaginal ultrasound examination was performed to exclude ovarian cysts. Recombinant FSH (rFSH) (Gonal F; Serono Inc.) was subsequently administered at a dosage of 150 to 225 IU/d, and serial ultrasonography and E_2 and P concentrations were used to monitor follicular development. Recombinant LH 75 IU/d or placebo were added to the treatment regimen once two follicles >14 mm in diameter were sonographically detected. Human chorionic gonadotropin 10,000 IU (Profasi; Serono) was given on the day after the last administration of the study drug when at least one follicle had reached a mean diameter of 17 mm. Oocyte pickup procedures were carried out according to local protocols, after which IVF and intracytoplasmic sperm injection could be carried out. Luteal phase support was given by daily P starting on the day of embryo transfer and continuing for at least 2 weeks, or until menses. Sera and follicular fluid (FF) levels of VEGF-A, sFlt-1, and PlGF were measured in all women by ELISA: VEGF-A, minimal sensitivity 5 pg/mL, intra-assay coefficient of variation percentage (CV) 5.1%–6.7% interassay CV 6.2%–8.8%; sFlt-1, minimal sensitivity 5.01 pg/mL, intra-assay CV 2.6%–3.8%, interassay CV 7%–8.1%; PlGF, minimal sensitivity 7 pg/mL, intra-assay CV 3.6%–7%, interassay CV 10.9%–11.8% (Quantikine; R&D Systems Inc., Minneapolis, MN).

STATISTICAL ANALYSIS

Data are expressed as mean $\pm$ SD. Statistical analysis was performed with use of Student's t-test. Because of the relatively small number of participants and the large variability of the data, we additionally used nonparametric tests for comparison, namely, Wilcoxon two-sample test. A P value of <.05 was considered statistically significant. All analyses were performed with use of SAS software version 9.1 (SAS Institute Inc., Cary, NC).

RESULTS
Patient Characteristics

Twenty infertile women fulfilled the study entry criteria and were randomly assigned to two study groups: rLH 75 IU/d (n = 10) or placebo (n = 10). No statistically significant differences were found between patient's age, BMI, and number of previous cycles. There were no statistically significant differences in these parameters between the women who received treatment with rLH and those who received placebo, nor in the basal levels of hormones, the total dose and

duration of rFSH treatment, or the number of oocytes that had been retrieved (Table 1).

Plasma Levels of VEGF-A, sFlt-1, and PlGF

Plasma levels of VEGF-A, sFlt-1, and PlGF were measured on the day of rLH or placebo administration and 48 hours later on the day of hCG administration. The differences in VEGF-A concentrations in the sera were not statistically significant between the rLH and the placebo groups on either the rLH administration day (311.60 ± 78.50 pg/mL and 375.00 ± 139.91 pg/mL, respectively) or the hCG day (289.00 ± 81.80 pg/mL and 337.00 ± 52.34 pg/mL, respectively).

No difference was found between serum sFlt-1 in either the rLH or the placebo groups on the rLH administration day (39.10 ± 3.20 pg/mL and 33.60 ± 3.67 pg/mL, respectively) or on the hCG day (35.30 ± 6.49 pg/mL and 30.90 ± 4.60 pg/mL, respectively). Plasma levels of PlGF were undetectable.

Because the sFlt-1 molecule binds to VEGF-A protein and, therefore, modulates its signaling pathway, we reasoned that the plasma VEGF-A/sFlt-1 ratio would be a better indicator of VEGF-A action. Our data demonstrated that there was no statistically significant difference in the sera VEGF-A/sFlt-1 ratio between the rLH and the placebo groups on the hCG administration day (11.38 ± 4.41 pg/mL and 12.96 ± 4.05 pg/mL, respectively).

Follicular Fluid Concentrations of VEGF, sFlt-1, and PlGF

Follicular fluid levels of VEGF-A, sFlt-1, and PlGF were measured on the day of oocyte retrieval. Vascular endothelial growth factor-A concentrations in the FF were not statistically significant between the rLH and the placebo groups (2,414 ± 306 pg/mL and 2,269 ± 460 pg/mL, respectively). No difference was found in either the FF sFlt-1 (2,891 ± 309 pg/mL and 3,453 ± 446 pg/mL) or the PlGF (47 ± 10 pg/mL and 42 ± 7 pg/mL) levels between the rLH and placebo groups, respectively.

Soluble Flt-1 is known to antagonize the proangiogenic molecules VEGF and PlGF by binding to them and preventing their interaction with their cell-surface receptors, Flt1 and KDR. Excess sFlt-1 causes widespread endothelial dysfunction by interfering with the effects of VEGF and/or PlGF. The VEGF/sFlt-1 ratio reflects the free VEGF fraction. As such, increased levels of "free" or unbound VEGF should increase the angiogenesis drive (7).

We found that rLH induces follicular angiogenesis by increasing the VEGF/sFlt-1 ratio (rLH 1.01 ± 0.19, placebo 0.59 ± 0.08: $P<.05$) (Fig. 1) but not the PlGF/sFlt-1 ratio (rLH 0.02 ± 0.00, placebo; 0.01 ± 0.00: $P=.22$) (Fig. 2). Similar results were also found in nonparametric comparison.

DISCUSSION

Our in vivo study demonstrates for the first time in humans that LH induces ovarian follicular angiogenesis, most likely via modulation of VEGF-A and expression of its soluble receptor, sFlt-1. Understanding the molecular mechanisms that regulate these divergent processes is a major challenge in human reproductive biology, with widespread clinical implications. Enhanced ovarian angiogenesis will facilitate oxygen, nutrients, and hormone precursor delivery to the developing follicle.

The female reproductive system undergoes a number of programmed vascular modulations coupled with cyclic evolution and decline of ovarian and endometrial structures. Ovarian biology is gonadotropin dependent (2, 3). The cyclic pattern of gonadotropin secretion leads to ovulation and steroidogenesis within the ovary (2, 3). Therefore, it is likely that the angiogenic waves within the reproductive system are coordinated by gonadotropins and/or by locally produced steroids in a paracrine and autocrine manner. This implies that the expression of the vascular endothelial–specific growth factors is hormone dependent. Vascular endothelial growth factor-A is the principal angiogenic factor controlling follicular angiogenesis, and its expression is controlled by gonadotropin and ovarian steroids (2, 8–10).

Several studies suggest that LH and hCG modulate expression of VEGF-A in vitro and in vivo (8–12). Therefore, we hypothesized that administration of LH will modulate the
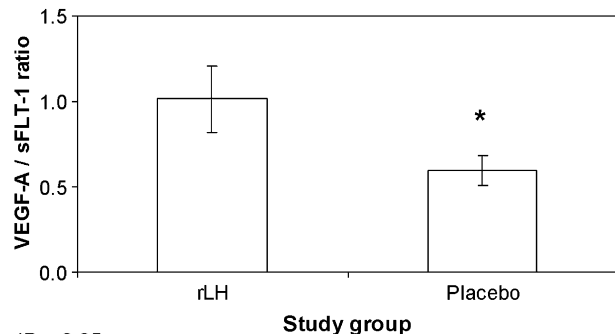
TABLE 1

In vitro fertilization–embryo transfer data.

	rLH	Placebo
Basal FSH levels (mIU/mL)	6.1 ± 2.1	5.8 ± 2.4
Basal LH levels (mIU/mL)	4.4 ± 2.4	4.5 ± 2.8
Peak E_2 levels (pg/mL)	1,556 ± 1,003	1,601 ± 983
Total rFSH dosage (IU)	2,090 ± 903	2,136 ± 1,095
Duration of rFSH (d)	10.4 ± 2.6	10.8 ± 2.1
Retrieved oocytes (n)	8.7 ± 3	8.1 ± 2.4

Note: Values are given as mean ± SD. NS = not significant; E_2 = 17β-estradiol. *P*=not significant for all values.

Gutman. Vascular endothelial growth factor in ovarian follicle angiogenesis. Fertil Steril 2008.

FIGURE 1

Follicular fluid VEGF-A/sFlt-1 ratio.

*P = 0.05

Gutman. Vascular endothelial growth factor in ovarian follicle angiogenesis. Fertil Steril 2008.

ing and after ovulation. The perifollicular capillary network of the theca interna shows marked changes after the LH surge: there is an increase in vascular luminal size that results in an increase in both blood flow and vascular permeability in the capillary walls, resulting in edema of the entire follicle, a condition that persists throughout the time of follicular rupture (14). These processes are concordant with VEGF-A expression and localization (3). After ovulation, LH supports and maintains corpus luteum function. During this period of intense angiogenesis, blood vessels from capillaries surrounding the granulosa cell compartments grow into the newly forming gland and are accompanied by a second wave of angiogenesis and vessel stabilization (15, 16).
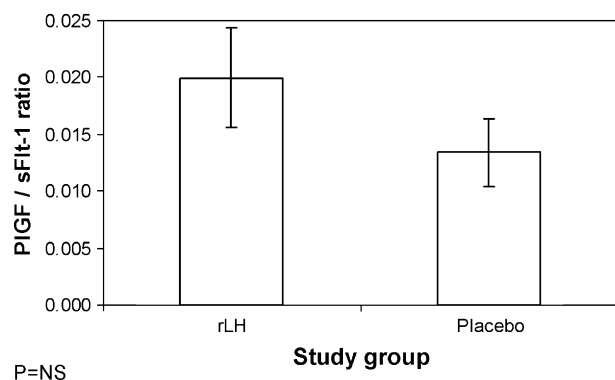
The principal regulatory molecule of this cyclic endocrine process is VEGF-A (2, 3). Vascular endothelial growth factor-A, a potent stimulator of microvascular endothelial cell proliferation and migration, as well as a promoter of vascular permeability, is probably the principal angiogenic factor controlling follicular angiogenesis (2, 3). Numerous studies have shown that the manipulation of the VEGF-A system can alter follicular development at all stages (17–19). The specific mechanisms that regulate VEGF-A expression during folliculogenesis are not completely understood, although a number of studies have shown that gonadotropins and ovarian steroids can stimulate VEGF-A transcription and translation both in vivo and in vitro (2, 3). This would suggest that inadequate gonadotropin support could lead to decreased follicular vascularization and subsequent impaired endocrine function.

Our study provides evidence that LH supplementation supports follicular angiogenesis by modulation of VEGF-A and its soluble receptor sFlt-1 ratio but not by PlGF. Therefore, we hypothesize that rLH supplementation during the late follicular phase may benefit ovulation induction by improved follicular maturation, steroidogenesis, ovulation, and corpus luteum function.

expression of VEGF-A and/or PlGF and their soluble receptor, sFlt-1.

The results of the present randomized double-blind, placebo-controlled study have shown that rLH supplementation during the late follicular phase induces ovarian follicle angiogenesis via modulation of VEGF-A and sFlt-1 expression but not by PlGF.

The principal action of LH in preantral and small antral follicles is the stimulatory effect on theca cells to induce the formation of the androgen substrate needed for granulosa cell conversion to estrogen. The LH midcycle surge is undoubtedly a major event in the mechanism of ovulation. The LH surge is responsible for [1] the resumption of meiosis in the oocyte, [2] the activation of enzymes that will determine the timing of follicular rupture and subsequent ovulation, and [3] steroidogenesis, namely P production (13). In addition, LH plays a critical role as an angiogenic factor dur-

REFERENCES

1. Geva E, Jaffe RB. Role of angiopoietins in reproductive tract angiogenesis. Obstet Gynecol Surv 2000;55:511–9.
2. Geva E, Jaffe RB. Role of vascular endothelial growth factor in ovarian physiology and pathology. Fertil Steril 2000;74:429–38.
3. Geva E, Jaffe RB. Ovarian angiogenesis. In: Leung P, Adashi E, eds. The ovary. 2nd ed. San Diego (CA): Elsevier Science, 2004.
4. Ferrara N. Endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
5. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995;376:66–70.
6. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575–83.

FIGURE 2

Follicular fluid PlGF/sFlt-1 ratio.

P=NS

Gutman. Vascular endothelial growth factor in ovarian follicle angiogenesis. Fertil Steril 2008.

7. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 1996; 226:324–8.

8. Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ. Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. Endocrinology 1992;131:254–60.

9. Neulen J, Yan Z, Raczek S, Weindel K, Keck C, Weich HA, et al. Human chorionic gonadotropin-dependent expression of vascular endothelial growth factor/vascular permeability factor in human granulosa cells: importance in ovarian hyperstimulation syndrome. J Clin Endocrinol Metab 1995;80:1967–71.

10. Christenson LK, Stouffer RL. Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. J Clin Endocrinol Metab 1997;82:2135–42.

11. Laitinen M, Ristimaki A, Honkasalo M, Narko K, Paavonen K, Ritvos O. Differential hormonal regulation of vascular endothelial growth factors VEGF, VEGF-B, and VEGF-C messenger ribonucleic acid levels in cultured human granulosa-luteal cells. Endocrinology 1997;138:4748–56.

12. Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 1999;5:1115–21.

13. Shoham Z, Schacter M, Loumaye E, Weissman A, MacNamee M, Insler V. The luteinizing hormone surge—the final stage in ovulation induction: modern aspects of ovulation triggering. Fertil Steril 1995;64:237–51.

14. Miyabayashi K, Shimizu T, Kawauchi C, Sasada H, Sato E. Changes of mRNA expression of vascular endothelial growth factor, angiopoietins and their receptors during the periovulatory period in eCG/hCG-treated immature female rats. J Exp Zoolog A Comp Exp Biol 2005;303: 590–7.

15. Wulff C, Dickson SE, Duncan WC, Fraser HM. Angiogenesis in the human corpus luteum: simulated early pregnancy by HCG treatment is associated with both angiogenesis and vessel stabilization. Hum Reprod 2001;16:2515–24.

16. Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Munstedt K, Rao CV, et al. Characterization of human chorionic gonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab 2002;87: 5290–6.

17. Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF, Fraser HM. Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor trap(A40). J Clin Endocrinol Metab 2001;86: 3377–86.

18. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicular growth in the rat ovary. Biol Reprod 2003;68:1736–41.

19. Shimizu T, Jiang JY, Iijima K, Miyabayashi K, Ogawa Y, Sasada H, et al. Induction of follicular development by direct single injection of vascular endothelial growth factor gene fragments into the ovary of miniature gilts. Biol Reprod 2003;69:1388–93.