Bone Morphogenetic Protein 7 Increased Vascular Endothelial Growth Factor (VEGF)-A Expression in Human Granulosa Cells and VEGF Receptor Expression in Endothelial Cells

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Abstract

The formation of an individual capillary network in the theca cell layer is required for ovarian folliculogenesis. Although vascular endothelial growth factor (VEGF) is critical for this process, the regulation of VEGF has been unclear. In the present study, the relationship between VEGF and intraovarian cytokine, bone morphogenetic protein 7 (BMP-7) was investigated. Granulosa cells (GC), obtained from in vitro fertilization patients, were cultured with BMP-7 followed by RNA extraction. Human umbilical vein endothelial cells (HUVECs) were also cultured with BMP-7 followed by RNA extraction, tube formation assay, or cell count analysis. The BMP-7 stimulated VEGF messenger RNA (mRNA) and protein expression in GC significantly. In HUVEC, BMP-7 increased an approximately 1.8-fold in the cell number and induced the tube formation significantly compared to control. The BMP-7 also induced a 2-fold increase in VEGF receptor mRNA transcript relative abundance in HUVEC. The BMP-7, a theca cell-derived factor, may stimulate endothelial cell to form vasculature in the follicle via 2 distinct mechanisms, induction of VEGF expression in GC and increased sensitivity of endothelial cells to VEGF.

Keywords

BMP-7, VEGF, angiogenesis, folliculogenesis

Introduction

In the female reproductive system, as in a few adult tissues, angiogenesis occurs as a normal process and is essential for normal tissue growth. In the ovary, new blood vessel formation facilitates delivery of many substances, including oxygen, nutrients, and follicular-stimulating hormone (FSH) to follicles.¹ The formation of an individual capillary network around each follicle is required for follicles to grow beyond the secondary stage, which contains multiple layers of granulosa cells (GC) and theca cells.¹ As the follicle continues to develop, endothelial cells are recruited to the theca cell layer from the blood vessels in the adjacent ovarian stroma.² Robust endothelial cell proliferation is maintained in healthy tertiary follicles to support the ongoing expansion of the theca vasculature. In contrast, follicular atresia is associated with inadequate development and/or regression of the theca vasculature.³ These observations support the notion that angiogenesis plays a crucial role in follicular growth and in selection of the ovulatory follicle.

In many species, vascular endothelial growth factor (VEGF), which is detected in the granulosa and theca layer of secondary follicles,^{4,5} is recognized as an important factor in recruitment of a vascular network to the theca layer.^{5,6} In 1 study, administration of VEGF stimulated the development of secondary follicles in cows.⁵ Moreover, administration of

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VEGF inhibitor in primates reduced the endothelial cell area of secondary follicles and inhibited the formation of antral follicles.⁶ The factors responsible for stimulation of VEGF expression during folliculogenesis have not been identified. However, it is likely that intraovarian factors, which are derived from theca cells, might induce VEGF expression.

Recently, bone morphogenetic protein (BMP) cytokines have attracted much attention in the field of ovarian physiology. The BMPs function as luteinization inhibitors by suppressing luteinizing hormone (LH) receptor expression in GC.⁷ Additionally, the BMP cytokine system was shown to play a crucial role in folliculogenesis in humans.⁸⁻¹⁰ Recent observations also emphasize the emerging role of BMPs in angiogenesis *in vivo*¹¹ and *in vitro*.¹²⁻¹⁴ Of the BMP cytokines, BMP-7 is most highly expressed in the theca cell layer in the ovarian follicles.⁷ Thus, in the present study, we used human GC obtained from *in vitro* fertilization (IVF) cycles and human umbilical vein endothelial cell (HUVEC) as experimental models and investigated the function of BMP-7 in regulating angiogenesis in the human ovary.

Materials and Methods

Reagents and Materials

Hyaluronidase, fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM)/F12, and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St Louis, Missouri). Recombinant human BMP-7 was purchased from R&D Systems (Minneapolis, Minnesota). Matrigel (growth factor reduced) was purchased from Becton Dickinson (Franklin Lakes, New Jersey).

Cell Culture of Human GC

The method to obtain and culture human GC was described previously.⁸⁻¹⁰ The experimental procedures were approved by the institutional review board, and signed informed consent for use of GC was obtained from each patient. Follicular fluids with GC were aspirated from 77 patients undergoing oocyte retrieval for IVF. The clinical indications for IVF in these patients were primarily male factor or tubal factor infertility. Patients with ovarian dysfunction were excluded from the study. Controlled ovarian stimulation was carried out by daily injection of 150-300 IU human menopausal gonadotropin from day 3 of the menstrual cycle. Gonadotropin-releasing hormone antagonist, cetrorelix acetate (Merck Serono, Tokyo, Japan) at 0.25 mg was given daily when the leading follicle had reached a size of 15 mm until the day of human chorionic gonadotropin (hCG) injection. When the size of the leading follicle reached 18 mm in mean diameter as measured by transvaginal ultrasound, 10 000 IU of hCG was administered. Oocytes were retrieved transvaginally with ultrasound guidance 35 hours after hCG injection. All of the follicular aspirates from each patient were mixed and centrifuged at 200g for 5 minutes, resuspended in phosphate-buffered saline (PBS) with 0.2% hyaluronidase, and incubated at 37°C for 30 minutes. The

suspension was layered onto Ficoll-Paque (GE Healthcare, Tokyo, Japan) and centrifuged at 150g for 20 minutes. The GC were collected from the interphase, washed with PBS, and cultured in DMEM/F12 media supplemented with 5% FBS and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL amphotericin B) for 15 minutes at 37°C, in order to remove contaminating macrophage cells from GC. Using this method, GC were collected in the supernatant, while macrophages remained attached to the culture dish. The collected GC were cultured in DMEM/F12 containing 5% FBS and antibiotics in 12-well plates at a density of 2×10^5 cells/mL and kept at 37°C in a humidified 5% CO₂/95% air. After 24 hours, culture medium was changed with DMEM/F12 containing 5% FBS to remove inviable cells. The viability of attached GC at this point was 93%, which was confirmed with Trypan-blue (Sigma) staining method. To evaluate the effect of BMP-7, human GC were cultured with or without BMP-7 for up to 24 hours. Recombinant BMP-7 was dissolved in 0.1% BSA + 4 mmol/L HCl as a vehicle. The same amount of vehicle was used as a control. The dilution magnification of recombinant BMP-7 or vehicle to the culture medium was 1000×, and in the pilot study, we confirmed that there was no effect of vehicle on experiments.

In a dose-response study of BMP-7, GC were cultured with various concentrations of BMP-7 (1, 10, and 100 ng/mL) for 24 hours and in a time course study, we cultured GC with or without BMP-7 (100 ng/mL) for 6, 12, and 24 hours.

Cell Culture of HUVEC

Normal HUVECs were obtained from Kurabo (Tokyo, Japan) and cultured in HuMedia containing 2% FBS. The HUVECs were plated on 12-well tissue culture plates and cultured with or without BMP-7 (100 ng/mL) for 24 hours followed by RNA extraction.

To examine the effect of BMP-7 on the cell number of HUVEC, confluent HUVEC at the cell concentration of 3×10^4 /mL in 96-well plates were treated with vehicle or BMP-7 (100 ng/mL) in DMEM/F12 without serum for 48 hours. After 48 hours stimulation, cell numbers were measured using a cell counting kit (CCK-8; Dojindo, Kumamoto, Japan), based on the colorimetric assay method. Briefly, media was replaced with DMEM/F12 without serum and 10 µL of the CCK-8 solution was added to each well and incubated for 1 hour in the cell culture incubator. Absorbance was measured at 450 nm using a microplate reader.

Tube Formation Assay

A matrigel tube formation assay was performed to assess the ability of HUVEC to form endothelial cell vascular structures. Matrigel (growth factor reduced) was spread onto 48-well chamber slides. The HUVECs (3×10^4 /well) were plated and incubated with or without BMP-7 (100 ng/mL). After 18 hours, the number of tubes formed was counted in 3 fields.

Figure 1. Bone morphogenetic protein (BMP)-7 induced vascular endothelial growth factor (VEGF) A expression in human granulosa cells (GCs). Cultured human GCs were stimulated with BMP-7 (1, 10, and 100 ng/mL) for 24 hours (A) or with BMP-7 (100 ng/mL) for 6 to 24 hours (B). Total RNA was extracted from the cells and subjected to real-time polymerase chain reaction (PCR) to determine the VEGF-A messenger RNA (mRNA) levels. Data were normalized by GAPDH mRNA levels to show the relative abundance. C, VEGF-A concentration in cultured supernatant stimulated with BMP-7 (100 ng/mL, 24 hours) was measured using enzyme-linked immunosorbent assay. Data from at least 4 different experiments were combined and shown as the mean $\pm$ standard error of the mean relative to an adjusted value of 1.0 for the mean value of the each control. * $P < .01$ (vs control).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from GC and HUVEC, using the RNeasy mini kit, in which RNA is purified with spin column-based purification method (Qiagen, Hilden, Germany). Total RNA of around 6 μ g was obtained, and in the pilot study, the quality of RNA, which was checked on agarose gel, revealed no sign of degradation.

Reverse transcription (RT) was performed using Rever Tra Dash (Toyobo, Tokyo, Japan). In brief, incubate 1 μ g of RNA solution at 65°C for 5 minutes and keep on ice afterward. Add Master Mix and nuclease-free water to RNA template and incubate at 37°C for 5 minutes. Add RT Master Mix and incubate at 37°C for 15 minutes, 50°C for 5 minutes and heat at 98°C for 5 minutes to obtain 20 μ L of complementary DNA (cDNA).

The quantification of the polymerase chain reaction (PCR) was done using SYBR-Green I as the fluorescent dye in a Light cycler system and relying on an external standard curve, according to the manufacturer's instructions (Roche Diagnostic

GmbH, Mannheim, Germany). Briefly, in a reaction tube, prepare the PCR mixture per 20 μ L reaction by adding water, PCR primer, Master Mix, and 1 μ L of cDNA template. Place the samples in the Light Cycler instrument (Roche Diagnostic GmbH) and start the program to amplify PCR products.

The PCR primer sets were designed to span introns to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences were as follows, VEGF-A (NM_1025370.2: 1233-1255 and 1477-1459), VEGFR (NM_002019.4: 2491-2510 and 2719-2700), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NM_002046: 628-648 and 1079-1060). The PCR conditions were as follows, VEGF-A: 33 cycles of 95°C for 10 seconds, 65°C for 10 seconds, and 72°C for 12 seconds; VEGFR: 35 cycles of 95°C for 10 seconds, 59°C for 10 seconds, and 72°C for 10 seconds; and GAPDH: 35 cycles of 95°C for 10 seconds, 64°C for 10 seconds, and 72°C for 18 seconds. Following amplification, melting curve analysis was performed. The transcript relative abundance of each messenger RNA (mRNA) was normalized by GAPDH mRNA.

Enzyme-Linked Immunosorbent Assay

The concentration of VEGF-A in conditioned media of GC culture was measured using a specific enzyme-linked immunosorbent assay kit (R&D). The limit of sensitivity of the kit was 5.0 pg/mL. The intra-assay and interassay coefficients of variation were <5% in the assays.

Statistical Analysis

All results are shown as mean $\pm$ standard error of the mean of data from at least 3 separate experiments, each performed in triplicate. Data were analyzed by Student *t* test for paired comparison and 1-way analysis of variance with post hoc test for multiple comparisons using Statview software (SAS Institute Inc, Cary, North Carolina). A *P* value of less than .05 was considered statistically significant.

Results

The Effect of BMP-7 on VEGF Expression in GC

As shown in Figure 1A, BMP-7 stimulated VEGF-A mRNA transcript relative abundance in a dose-dependent manner. Therefore, we utilized 100 ng/mL of BMP-7 in the subsequent experiments. In a time course study, VEGF-A mRNA was induced significantly following 6 hours of stimulation (Figure 1B). The addition of 100 ng/mL of BMP-7 for 24 hours resulted in a 1.3-fold increase in the concentration of VEGF in the culture supernatant compared to the control (Figure 1C; *P* < .01).

The Effect of BMP-7 on Endothelial Cells

To investigate the effect of BMP-7 on endothelial cells, HUVEC were cultured with or without BMP-7 up to 48 hours. In the cell proliferation assay, there was an approximately 1.8-fold increase in the number of HUVEC treated with BMP-7 compared to the control (Figure 2; *P* < .01). Additionally, BMP-7 treatment resulted in a significant acceleration of tube formation in HUVEC when compared to the control, as evidenced by the tube formation assay (Figure 2).

The BMP-7 also induced a 2-fold increase in VEGF receptor mRNA transcript relative abundance in HUVEC (Figure 3; *P* < .05).

Discussion

In the present study, we found that BMP-7 induced VEGF-A mRNA and protein expression in human GC. In endothelial cells, BMP-7 not only increased the number of cells but also accelerated tube formation and upregulated the relative abundance of VEGF receptor mRNA.

Several studies have reported that the dominant follicle contains a more vascularized theca layer than the other antral follicles in the cohort.¹⁵ Increased vascularity is a determining factor in the establishment of follicular dominance.¹⁵ Consequently, angiogenic factors are of increasing interest in ovarian physiology. In

Figure 2. The role of bone morphogenetic protein (BMP)-7 in human umbilical vein endothelial cells (HUVECs). A, Preconfluent-cultured HUVEC in 96-well plates were treated with vehicle (0.1% BSA + 4 mmol/L HCl) or BMP-7 (100 ng/mL) in DMEM/F12 without serum. After 48 hours of treatment, HUVEC number was determined using a cell counting kit. B, Tube formation assay; Matrigel (growth factor reduced) was spread onto 48-well chamber slides. HUVEC (3×10^4 /well) were plated and incubated with or without BMP-7 (100 ng/mL). After 18 hours, the number of tubes formed was counted in 3 fields. Mean data from 10 different experiments were shown as the mean $\pm$ standard error of the mean relative to an adjusted value of 1.0 for the mean value of the control. **P* < .01 (vs control). Representative pictures of control and BMP-7 (100 ng/mL) were shown.

particular, the role of VEGF has been studied extensively.¹⁶ Granulosa and theca cell layers of secondary follicles are one of the major sites of the VEGF expression.^{4,5} However, the regulatory mechanism of VEGF in these follicles remains to be elucidated. One potent inducer of VEGF, hypoxia-induced factor 1 α (HIF1 α), a transcription factor induced by hypoxia, is not present in preantral follicles.¹⁷ Gonadotrophins are also unlikely

Figure 3. Bone morphogenetic protein (BMP) 7 induced vascular endothelial growth factor (VEGF) receptor transcript relative abundance in human umbilical vein endothelial cells (HUVECs). Cultured HUVECs were stimulated with BMP-7 (100 ng/mL) for 24 hours. Total RNA was extracted from the cells and subjected to real-time polymerase chain reaction (PCR) to determine the VEGF receptor messenger RNA (mRNA) transcript relative abundance. Data were normalized by GAPDH mRNA levels. Data from 3 different experiments were combined and shown as the mean $\pm$ standard error of the mean relative to an adjusted value of 1.0 for the mean value of the control. * $P < .01$ (vs control).

candidates in the initial regulation of VEGF, because preantral follicles do not possess blood vessels, which are required to deliver FSH from the pituitary.²

The BMP cytokines are multifunctional growth factors belonging to the transforming growth factor β superfamily. In the ovary, BMP cytokines act as luteinization inhibitors by suppressing LH receptor expression in GC.⁷ Recent observations also emphasize the emerging role of BMP in angiogenesis: 2 genetic vascular diseases (hereditary hemorrhagic telangiectasia and pulmonary arterial hypertension) are caused by mutations in genes encoding components of the BMP signaling pathway.¹¹

In the present study, we hypothesized that BMP-7, a theca cell-derived cytokine that is expressed beginning at the secondary follicle stage,⁷ might act as an inducer of angiogenesis. We observed the effects of BMP-7 in the promotion of angiogenesis in both GC and endothelial cells. In human GC, BMP-7 induced VEGF expression, a critical angiogenic factor for endothelial cells, at both the mRNA and protein levels (Figure 1). During the process of follicular development, the vasculature of the follicle is limited to the theca layer outside the basement membrane.¹ Therefore, it is likely that follicles create a gradient of angiogenic factors to stimulate vascularization toward the basement membrane, thereby maximizing the supply of oxygen, nutrients, and hormones to GC. In the present study using HUVEC, we found that BMP-7 increased the number of endothelial cells, accelerated tube formation, and increased VEGF receptor expression (Figures 2 and 3). Thus, we propose that endothelial cells could be stimulated to form vasculature by BMP-7 via 2 distinct mechanisms: induction of VEGF expression in GC and increased sensitivity of endothelial cells to

VEGF. The BMP-7 has also been shown to elicit a prominent angiogenic response using the chick chorioallantoic membrane assay.¹² Other members of the BMP cytokines have also been shown to have angiogenic properties; in an in vitro assay, BMP-2 and BMP-4 induced significant proliferation of human endothelial cells,^{13,14} and Langenfeld et al also reported that BMP-2 induced tube formation of human endothelial cell in the Matrigel assays.¹³

In the present study, we found a new role of BMP-7 in folliculogenesis, where BMP-7 stimulated angiogenesis in endothelial cells via direct and indirect mechanisms. Thus, it is worthwhile to examine whether administration of BMP-7 to the ovary might promote folliculogenesis by increasing angiogenesis. Further study is necessary to test this hypothesis. Cultured GCs used in this study were obtained from IVF patients and may not represent the stages of growing follicles. However, our findings that VEGF-A were upregulated by BMP-7 in human GC are new and open new insights into our understanding of angiogenesis during folliculogenesis.

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Declaration of Conflicting Interests

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References

1. Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology—an overview. *Reprod Biol.* 2005;5(2):111-136.
2. Robinson RS, Woad KJ, Hammond AJ, Laird M, Hunter MG, Mann GE. Angiogenesis and vascular function in the ovary. *Reproduction.* 2009;138(6):869-881.
3. Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). *Endocrinology.* 2001;142(7):3244-3254.
4. Taylor PD, Hillier SG, Fraser HM. Effects of GnRH antagonist treatment on follicular development and angiogenesis in the primate ovary. *J Endocrinol.* 2004;183(1):1-17.
5. Yang MY, Fortune JE. Vascular endothelial growth factor stimulates the primary to secondary follicle transition in bovine follicles in vitro. *Mol Reprod Dev.* 2007;74(9):1095-1104.
6. Wulff C, Wilson H, Wiegand SJ, Rudge JS, Fraser HM. Prevention of thecal angiogenesis, antral follicular growth, and ovulation

- in the primate by treatment with vascular endothelial growth factor Trap R1R2. *Endocrinology*. 2002;143(7):2797-2807.
7. Shimasaki S, Moore RK, Otsuka F, Erickson GF. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev*. 2004;25(1):72-101.
 8. Shi J, Yoshino O, Osuga Y, Nishii O, Yano T, Taketani Y. Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells. *Fertil Steril*. 2010;93(4):1273-1279.
 9. Shi J, Yoshino O, Osuga Y, et al. Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin beta subunits, and anti-Mullerian hormone in human granulosa cells. *Fertil Steril*. 2009;92(5):1794-1798.
 10. Shi J, Yoshino O, Osuga Y, et al. Bone morphogenetic protein-2 (BMP-2) increases gene expression of FSH receptor and aromatase and decreases gene expression of LH receptor and StAR in human granulosa cells. *Am J Reprod Immunol*. 2011;65(4):421-427.
 11. David L, Feige JJ, Bailly S. Emerging role of bone morphogenetic proteins in angiogenesis. *Cytokine Growth Factor Rev*. 2009;20(3):203-212.
 12. Ramoshebi LN, Ripamonti U. Osteogenic protein-1, a bone morphogenetic protein, induces angiogenesis in the chick chorioallantoic membrane and synergizes with basic fibroblast growth factor and transforming growth factor-beta1. *Anat Rec*. 2000;259(1):97-107.
 13. Langenfeld EM, Langenfeld J. Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. *Mol Cancer Res*. 2004;2(3):141-149.
 14. Suzuki Y, Montagne K, Nishihara A, Watabe T, Miyazono K. BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and angiopoietin-1/Tie2 signaling. *J Biochem*. 2008;143(2):199-206.
 15. Aerts JM, Bols PE. Ovarian follicular dynamics: a review with emphasis on the bovine species. Part I: Folliculogenesis and pre-antral follicle development. *Reprod Domest Anim*. 2010;45(1):171-179.
 16. Fraser HM, Duncan WC. SRB Reproduction, Fertility and Development Award Lecture 2008. Regulation and manipulation of angiogenesis in the ovary and endometrium. *Reprod Fertil Dev*. 2009;21(3):377-392.
 17. Duncan WC, van den Driesche S, Fraser HM. Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and corpus luteum. *Endocrinology*. 2008;149(7):3313-3320.