

Organ-Specific Production Control of Vascular Endothelial Growth Factor in Ovarian Hyperstimulation Syndrome-Model Rats

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Abstract. Overproduction of vascular endothelial growth factor (VEGF) following human chorionic gonadotropin (hCG) stimulation has been implicated as one of the causative factors in the development of ovarian hyperstimulation syndrome (OHSS). The objective of this study was to clarify the action of hCG and progesterone, one of the possible factors for OHSS, on VEGF production and gene expression using OHSS-model rats. A total of 40 immature female Wistar rats were stimulated with 10 IU of equine chorionic gonadotropin for four consecutive days from the 22nd to 25th day of life followed by subcutaneous injection of 30 IU of hCG on the 26th day of life. RU486 and progesterone were injected 24 h after the hCG injection. Tissues and blood samples were collected on the 28th day. hCG elicited VEGF production in the OHSS-model rat ovaries. Ovarian weights of the OHSS model rats were significantly increased through day 26 by the ovarian stimulation with single dose of 30 IU hCG. Addition of anti-progesterone RU486, which reduced the ovarian enlargement, attenuated VEGF production dose dependently whereas the VEGF gene expression was stable. In the lung and liver, neither hCG nor RU486 affected VEGF production and gene expression. These results suggested that progesterone regulates VEGF production at the post-transcriptional level in a tissue specific manner in the hyperstimulated ovary.

Key words: Rat, Ovary, OHSS, hCG, VEGF, Progesterone

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OVARIAN hyperstimulation syndrome (OHSS) is the most serious complication of ovulation induction with human menopausal gonadotropin (hMG) and human chorionic gonadotropin (hCG). This iatrogenic condition is potentially lethal and occurs in 0.3–5% of stimulated ovarian cycles [1]. Clinical manifestations of OHSS are massive extravascular fluid accumulation and hemoconcentration similar to that in syndromes due to capillary leakage. Patients may be complicated by renal failure, hypovolemic shock, thromboembolic

episodes, and adult respiratory distress syndrome. Although the pathophysiology of this syndrome has not been elucidated completely, it seems likely that the increased capillary permeability triggered by the release of vasoactive substances secreted by the ovaries under hCG stimulation plays a key role in the onset of this syndrome.

Vascular endothelial growth factor (VEGF) has been implicated as a prime causative factor of OHSS progression [2, 3]. VEGF is a potent mitogen for vascular endothelial cells [4], also known as vascular permeability factor (VPF) based on its ability to induce vascular leakage [5]. High concentrations of VEGF were found in the follicular fluid from women with severe OHSS, which lead to increased endothelial cell permeability with attenuation by VEGF

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antibody [6]. VEGF was also detectable in the serum [7] and ascites [8] from OHSS patients. Serum levels of VEGF concentrations were proposed to be useful to predict the risk of OHSS [9, 10]. Several authors have shown that the source of VEGF in OHSS seems to be the hyperstimulated ovary, since the VEGF concentration in follicular fluid is 100-times higher compared to the serum. Therefore, ovarian VEGF is considered to play a major role in inducing OHSS. However, previous studies have not concluded that VEGF is a pivotal protein in the pathogenesis of OHSS. The course of severe OHSS cannot be predicted by the overall pattern of circulating free VEGF [11]. The rise in vascular permeability induced by ascites in OHSS patients could not be blocked by the administration of anti-VEGF antibodies in an *in vitro* assay and an *in vivo* animal experiment [12]. These reports suggest that other factors including kinin-kallikrein system [13], renin-angiotensin system, prostaglandin, cytokines and nitric oxide must also be involved in the pathogenesis of OHSS.

We have focused on the role of progesterone during ovulatory process since a potent synthetic progesterone antagonist RU486 inhibited the ovulation and progesterone synthesis in pregnant [14] and immature rats [15]. We have established an experimental model of OHSS in immature female rats [16], in which the extension of OHSS was attenuated by the addition of RU486, and the administration of progesterone concomitant with RU486 reversed the decline in capillary permeability. These results suggested that progesterone might be involved in the development of OHSS. Here we designed a study to elucidate whether progesterone is involved in the production and gene expression of VEGF using an experimental rat OHSS model.

Materials and Methods

Animal model

A total of 40 immature female Wistar rats aged 22 days weighing between 41 and 49 g, obtained from Kyudo Co. Ltd. (Kumamoto, Japan), were used throughout the studies. OHSS-model rats were prepared as previously described [16]. Briefly, the rats were stimulated with 10 IU of equine chorionic gonadotropin (eCG, Teikoku Zouki, Tokyo, Japan) in 0.1 ml of 0.9% saline for four consecutive days from the

22nd to 25th day of life followed by a subcutaneous injection of 30 IU of hCG (Mochida Pharmaceutical, Tokyo, Japan) on the 26th day of life. RU486 (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.2 ml of 70% ethanol and injected 24 h after the hCG injection. Rats were killed by cervical dislocation on the 28th day and the ovaries and other tissues were removed. Blood samples were collected and the serum was kept at -80°C until assayed. All procedures performed in this study were approved by the Animal Care and Use Committee of the Kumamoto University School of Medicine.

RNA isolation

Each tissue sample was weighed and total RNA was isolated according to the single step method using RNeasyTM (Tel-Test Inc., Friendswood, TX) reagent. Tissues were homogenized in RNeasyTM (2 ml per 100 mg-tissue) buffer using a glass/Teflon homogenizer. The homogenates were centrifuged, and the supernatants were collected. Chloroform (0.1 ml/1 ml-homogenate) was added and shaken vigorously for 15 sec and kept on ice for 5 min. These samples were centrifuged at $12,000 \times g$ for 15 min. The aqueous phase was transferred into fresh tubes, an equal volume of isopropanol was added and the samples were stored for 15 min at 4°C . The RNA was pelleted by centrifugation ($12,000 \times g$ for 15 min), washed once with 75% ethanol, and dissolved in diethylpyrocarbonate (DEPC)-treated water. The concentration was determined by absorbance at 260 nm.

RT-PCR

In all cases, 5 μg of DNase-treated total RNA was reverse transcribed. The RNA was incubated at 42°C for 50 min with 200 U of Superscript II reverse transcriptase (GIBCO) and the following reagents: 1) $10 \times$ PCR reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 μl ; 2) 50 mM MgCl_2 , 2 μl ; 3) random primer (0.5 mg/ml), 5 μl ; 4) dNTP mixture (10 mM each of dATP, dCTP, dGTP, and dTTP), 1 μl ; and 5) dithiothreitol (0.1 M), 1.0 μl . Then, 1 μl of RNase H was added and the incubation was carried out for 20 min.

Oligonucleotide primer pair for rat VEGF gene specific to #66-#86 and #628-#608 were synthesized based on the rat sequence (Table 1). This primer

Table 1. Primer sequences and PCR products used for RT-PCR analysis

Target	Sequence (5' → 3')	Product size (nt)	References
rat VEGF	up: CTGCTCTCTTGGGTGCACTGG dn: TCCTTCCTCGGAGGAGT	VEGF ₁₂₀ : 431 VEGF ₁₆₄ : 563 VEGF ₁₈₈ : 635	Koos, 1995 [24]
VEGFR-1 (flt-1)	up: CAGCACCTTGACCTTGAAC dn: TGTATTGAGGTCCGTGGTG	513	Finnerty <i>et al.</i> , 1993 [17]
VEGFR-2 (KDR/flk-1)	up: GAGAATACACCTGCACAGC dn: CCTTCACGTGTCTCCATTC	530	Finnerty <i>et al.</i> , 1993 [17]
DNA competitor for rat VEGF	up: CTGCTCTCTTGGGTGCACTGGGTACGGTCATCATCTGACAC dn: TCCTTCCTCGGAGGAGTCGCCATCCTGGGAAGACTCC	372	

pair encompasses a region containing the alternative splicing sites in the VEGF sequence, hence it generates products from any of the known forms of VEGF mRNA. VEGF receptor-1 (VEGFR-1/flt-1) and receptor-2 (VEGFR-2/ KDR/flk-1) specific primers [17] were also synthesized. The integrity of extracted mRNA was tested by amplification of 620 nt splice product of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using primer specific to #361-#380 and #961-#980 of the GAPDH DNA. The 30 cycle PCR reaction (denaturation at 95°C for 1 min, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec) using cloned *Thermus aquaticus* (*Taq*) DNA polymerase (Takara) was carried out according to GeneAmp DNA amplification Reagent Kit (Perkin-Elmer/Cetus, Norwalk, CT) instructions with minor modifications. For each PCR analysis, a blank was prepared using all reagents and 1.0 µl of the matching RT blank. In all experiments, this RT-PCR reagent blank yielded no detectable PCR products, indicating that all reagents were free from target sequence contamination.

The PCR samples were analyzed through agarose gels. Five microliters of each PCR mixture were added to 1 µl of 6 × loading buffer and fractionated by electrophoresis in a 1.5% (w/v) agarose gel using a constant 100-V field. Gels were stained with ethidium bromide and examined on a 312 nm UV transilluminator.

Competitive RT-PCR

A 372 nt competitive DNA template was designed by Competitive DNA Construction Kit (Takara). The heterologous DNA competitor has nucleotide sequences different from the rat VEGF DNA except for the se-

quences of the primer annealing sites (Table 1). The competitor copies were determined by absorbance at 260 nm.

The following reagents were added to 500 µl polypropylene microcentrifuge tube: 1) 10 × PCR reaction buffer 5 µl; 2) dNTP mixture (2.5 mM each) 4 µl; 3) sense primer 0.5 µl; 4) antisense primer 0.5 µl; 5) 5 U of cloned *Taq* DNA polymerase 0.25 µl; 6) 1 µl of undiluted RT solution; 7) DNA competitor (10⁴ to 10⁶ copies) 5 µl; and 8) water to give a final volume of 50 µl. Reaction mixtures were overlaid with 50 µl of light mineral oil (Sigma) and PCR reaction (30 cycles: 94°C, 30 sec; 60°C, 30 sec; and 72°C, 30 sec) was carried out. The PCR samples were analyzed through agarose gels. Five microliters of each PCR mixture were added to 1 µl of 6 × loading buffer and fractionated by electrophoresis in a 1.5% (w/v) agarose gel using a constant 100-V field. Gels were stained with ethidium bromide and examined on a 312 nm UV transilluminator. The T and C (T = amount of amplified VEGF₁₆₄, C = amount of amplified competitor) values for each set of samples were determined quantitatively after electrophoresis using NIH Image. For each set of samples, a graph was plotted of log (T : C) against the initial amount of competitor. The initial amount of target DNA template was estimated by determining the amount of competitor which gives the T : C ratio of 1.

RNA analysis

To determine the relative abundance of VEGF mRNA per unit of total RNA from different experimental animals, Northern blotting analysis was carried out. Ten µg of total RNA from ovarian tissue were fractionated on 1% formaldehyde-agarose gel and

transferred to GeneScreen Plus (DuPont) membrane. Rat VEGF specific probe (#66-#628 for VEGF₁₆₄, 563 nt) was labeled with Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech UK Ltd.). The blot was hybridized at 68°C for 3 h in QuikHyb solutions (Stratagene). After the hybridization, the blot was washed twice in $2 \times$ standard sodium citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) for 15 min at room temperature and then once in $0.1 \times$ SSC, 0.1% SDS for 30 min at 60°C. The blot was exposed at -80°C for 3 days. All signals were analyzed using a densitometer and lane-loading differences were normalized using S28. Experiments were repeated twice.

Protein extraction

Tissues were homogenized in 0.5 ml of modified RIPA buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.1 mM dithiothreitol, 1 mM phenylmethyl fluoride, and 2% of aprotinin with grass/Teflon homogenizer. Supernatant was obtained by centrifugation at $12,000 \times g$ for 15 min. Protein was determined by the Bradford method [18] with bovine serum albumin as standard.

SDS-PAGE, Western blotting and antibodies

Aliquots of the supernatants were fractionated by SDS-PAGE on 12% gels and electroblotted onto nitrocellulose membranes by standard procedures. All blots were blocked with 1% BSA in Tris-buffered saline. Immunoreactive VEGF was detected on blot by incubation at 4°C overnight with 1 : 200 diluted affinity-purified rabbit polyclonal antiserum against rat VEGF (IBL). After washing, the membrane was incubated with the anti-rabbit IgG (Amersham Pharmacia Biotech) antibody at 1 : 200 dilution for 2 h and labeled with [¹²⁵I] protein A at room temperature for 1 h.

VEGF and steroid hormone assays

VEGF concentrations in the ovarian tissue homogenates were determined by enzyme-linked immunosolvent assay (ELISA) (Quantikine™ M Mouse VEGF Kit; R&D Systems Inc., Minneapolis, MN). The homology between mouse and rat VEGF was 98%, therefore this assay system is valid for rat VEGF assay [19]. A house data showed that its %cross reactivity

with rat VEGF was approximately 70%. The lower limit of detectability was 7.8 pg/ml. Pooled samples of rat ovarian extract were used in a single assay to determine the intra-assay coefficients of variation (typically <5%). Results were expressed as the amount of VEGF per protein concentration. Serum progesterone and estradiol contents were determined by enzyme immunoassay (MBL Co., Nagoya, Japan). The minimum detectable amounts of progesterone and estradiol were 10 pg/ml and 10 ng/ml, respectively.

Statistical analysis

Data were presented as means \pm SEM and examined using Student's *t*-test. Significant differences were established as $P < 0.05$. The Bonferroni correction for multiple comparison was applied to determine the effects of RU486 on ovarian VEGF production.

Results

Preparation of OHSS model rat

The ovarian weights of the OHSS model rats were significantly increased through days 26 to 28 (26th day, 211.81 ± 21.03 mg vs 28th day, 300.92 ± 18.99 mg) by ovarian stimulation with a single dose of 30 IU hCG. Administration of 5 or 20 mg/kg of RU486 on day 27th reduced the increments of ovarian weight on day 28 (245.09 ± 20.71 mg and 256.68 ± 24.53 mg, respectively). Serum estradiol and progesterone concentrations on day 28, 48 h after the stimulation with hCG, were 32.7 ± 1.5 pg/ml and 217.6 ± 28.4 ng/ml, respectively. Five or 20 mg/kg of RU 486 did not affect the serum concentration of those sex steroid hormones (5 mg/kg, 37.3 ± 1.5 pg/ml and 243.5 ± 42.4 ng/ml; 20 mg/kg, 32.3 ± 3.5 pg/ml and 184.4 ± 38.7 ng/ml, respectively). The result is similar to that of a previous study in which we characterized the OHSS model rat [16], thus we employed the model rats in the following studies.

VEGF production in the OHSS rat

Cytosolic extracts of OHSS rat ovary were prepared for SDS-PAGE. As shown in Fig. 1, a 45 kDa protein was recognized against the affinity-purified anti rat VEGF serum. VEGF production was stimu-

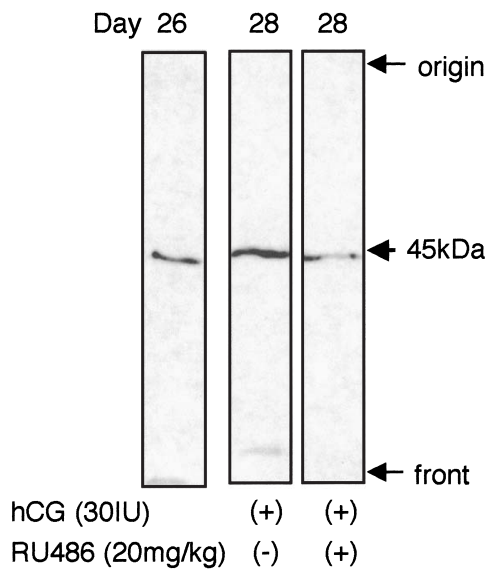


Fig. 1. Western blotting analysis of changes in VEGF protein production after administration of hCG (30 IU, on day 26) and RU486 (20 mg/kg, on day 27) in OHSS model rat ovary on the 28th day. Large arrow indicates the position of the single 45 kDa protein.

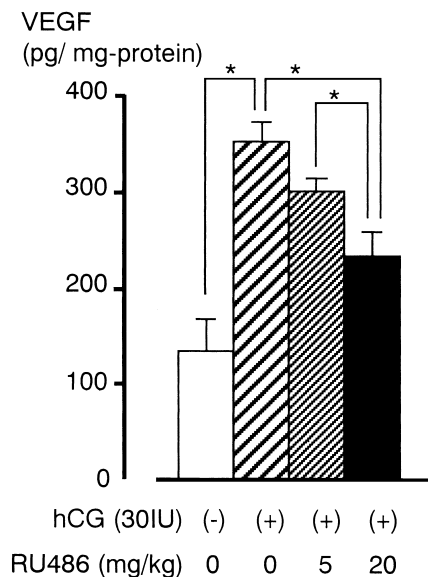


Fig. 2. Enzyme linked immunosorbent assay (ELISA) for changes in VEGF protein production after administration of hCG (0 or 30 IU on 26th day) and RU486 (0, 5 or 20 mg/kg on 27th day) in OHSS model rat ovary. VEGF concentration in supernatants of ovaries on 28th day was determined by ELISA, as described in Materials and Methods. Data show the relative amount to protein concentration. Values are represented as means \pm SE of determinations ($n = 5$). * = significantly different ($P < 0.05$).

lated by the addition of hCG. Production of VEGF was attenuated at 24 h after administration of RU486. We subsequently examined the amount of VEGF in the OHSS model rat ovaries by ELISA (Fig. 2). VEGF production was stimulated approximately 3-fold after administration of hCG. RU486 attenuated VEGF production elicited by hCG in a dose dependent manner. These results indicated that the decrease in VEGF production induced by RU486 injection was elicited by the anti-progesterone effect of RU486.

Because VEGF is produced in a wide range of tissues, we also examined the production and the effects of hCG and RU486 in the lungs and liver of the OHSS model rats (Fig. 3). VEGF protein was detected in both the lung and the liver. VEGF production in these organs was not affected at 24 h after the addition of hCG and RU486 in contrast to the ovary.

VEGF and its receptors gene expressions in OHSS rat ovary

RT-PCR amplification of ovarian RNA from OHSS model rats with VEGF primers generated two major products after 30 cycles of amplification (Fig. 4a). The sizes of these products corresponded to those expected from transcripts for VEGF₁₂₀, VEGF₁₆₄ (431 and 563 nt, respectively) based upon sequences previously reported [20]. A similar pattern of VEGF PCR products was seen in all groups. To confirm that this PCR product was produced from VEGF transcripts, the 563 nt product was extracted from the gel and subcloned into a pCRII vector (Invitrogen Corp., San Diego, CA). The 563 nt product was identical to the known rat VEGF₁₆₄ gene after sequencing (data not shown). hCG barely enhanced the yield of all three products on day 27, and RU486 did not affect the expression of VEGF mRNA. The yields of cytoplasmic GAPDH PCR product were similar at all time points. The mRNA expression for two known high affinity VEGF receptors, KDR/flk-1 and flt-1 were also tested by this condition. The RT-PCR revealed that neither hCG nor RU486 affected the expression of these VEGF receptor genes.

Using the competitive RT-PCR procedure, we quantitated VEGF gene expression in ovarian RNA from OHSS model rats (Fig. 5a). Comparison between the two amplifications, VEGF₁₆₄ gene and the competitor, showed that the amount of VEGF₁₆₄ cDNA on day 26 corresponded to $10 \times 10^4.5$ copies of competitor cDNA,

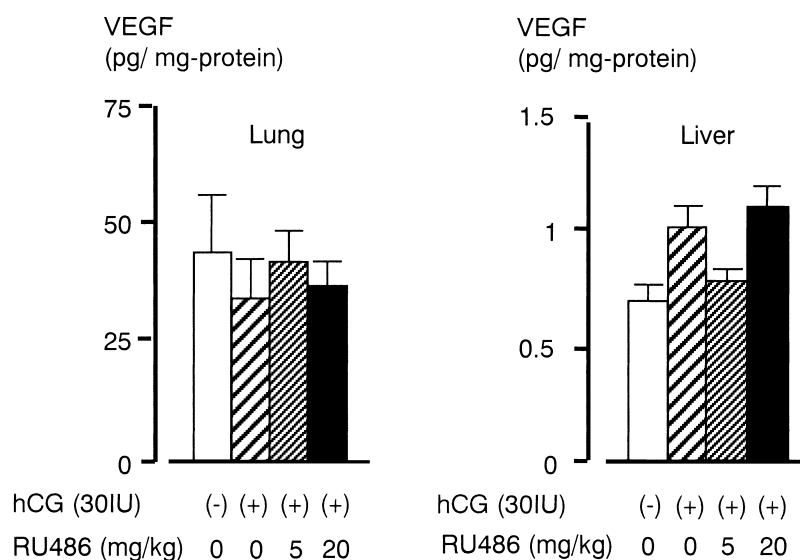


Fig. 3. Effects of hCG and RU486 on VEGF production in lung and liver of OHSS model rats. Rats were treated in the same way as shown in Fig. 2. Data show the relative amount to protein concentration. Values are represented as means \pm SE of determinations ($n = 3$). All changes of VEGF protein amounts were not significant.

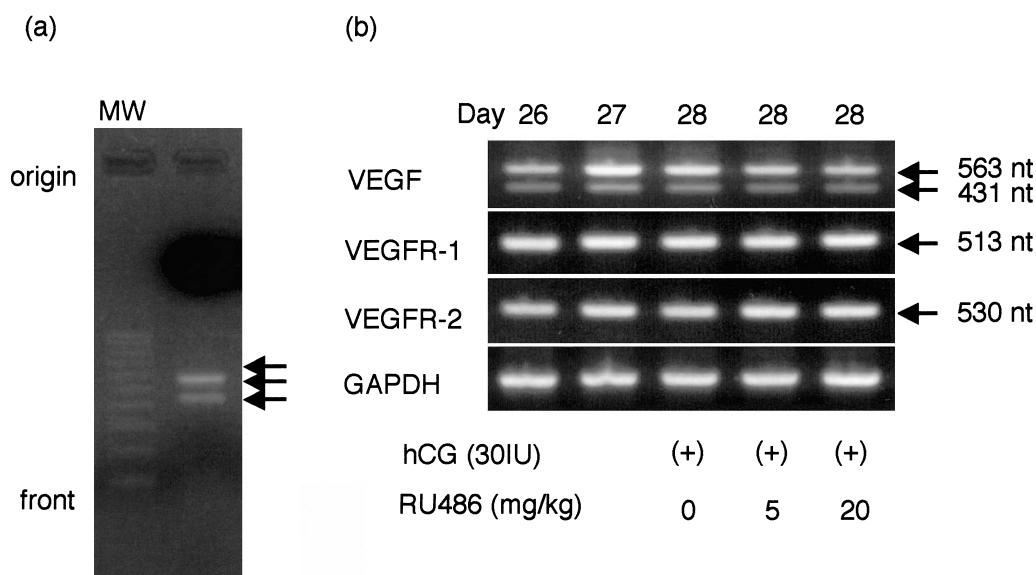


Fig. 4. VEGF and VEGF receptors expression in OHSS models rat ovary. (a) DNA size standard, negative control and RT-PCR products for VEGF mRNA on the 26th day of life. Arrows indicate the putative size of transcripts for rat VEGF gene. (b) RT-PCR amplification of ovarian RNA from OHSS model rats with the primers for VEGF and two known high affinity VEGF receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1). GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

and that VEGF gene on day 28 was increased to 10exp5.3 copies. RU486 did not decrease the copy number of VEGF (10exp5.1 copies).

Northern blot analysis was employed in a further study to quantify VEGF gene expression (Fig. 5b). VEGF gene transcript appeared as a single band located at the 3.7 kb site designated VEGF₁₆₄. hCG

stimulated VEGF gene expression and RU486 had negligible effect on the yield of VEGF mRNA.

Discussion

An experimental model of OHSS has been docu-

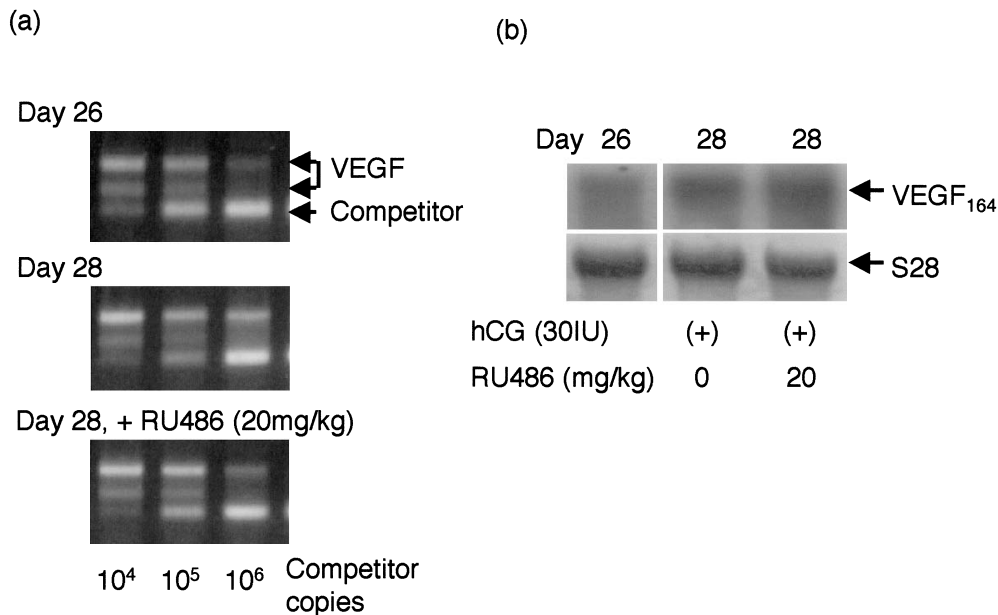


Fig. 5. (a) Competitive RT-PCR for VEGF mRNA from OHSS model rats. (b) Northern blot analysis of changes in VEGF mRNA expression after administration of hCG and RU486 in OHSS model rat ovary. Arrows indicate the position of VEGF₁₆₄ and S28 as internal control.

mented in adult rats [21]. We have established an OHSS model rats [16] with immature female rats since immature animals are not influenced by the corpus luteum from preceding cycles, and also because the ovarian physiology can be simplified. Our results demonstrated that the VEGF gene and protein are expressed in the ovaries of OHSS model rats, and that hCG stimulates the levels of VEGF mRNA and protein. These findings are in general agreement with recent studies for the pathophysiological character of OHSS in humans. It is thought that VEGF expression is temporally and spatially related to the proliferation of blood vessels in mammalian ovaries, suggesting that VEGF is a mediator of the cyclical growth of blood vessels that occurs in the ovarian tissue [22, 23]. VEGF mRNA is expressed in luteinized rat [24] and human [22] granulosa cells. The VEGF expression was dose- and time-dependently enhanced by hCG in human granulosa cells cultured *in vitro* [25]. The rise in the serum VEGF concentration that occurs after hCG administration is thought to be a marker of OHSS in *in vitro* fertilization cycles [10]. These results support the idea that our OHSS model rats mimic the human OHSS from the view of VEGF.

A single VEGF gene generates at least five different molecular isoforms (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆) in human [26], and at least

three (VEGF₁₂₀, VEGF₁₆₄, VEGF₁₈₈) in rat [27]. VEGF₁₆₅/VEGF₁₆₄ is the predominant molecular species produced by a variety of cells. Two major forms of the VEGF transcripts were confirmed by RT-PCR in the OHSS model rat ovary, and the levels of these transcripts were increased by the stimulation of hCG. Two high-affinity VEGF receptors, VEGFR-1 and VEGFR-2, were also identified in the ovarian tissues of the OHSS-model rat but their expression was not stimulated by hCG.

Analysis of the human VEGF promoter region reveals several binding sites for the transcription factors SP-1, AP-1, AP-2, hypoxia regulatory elements [28] and estrogen response elements (ERE) [29]. Several cytokines and growth factors are thought to up-regulate VEGF mRNA expression and/or induce release of VEGF protein. Estrogen has been also implicated as one of the regulatory factors of VEGF expression, as shown in the regulation of VEGF gene expression by estradiol in rat uterus [30]. Though high estradiol concentration is known to be closely related to OHSS, estradiol itself is not a causative factor of OHSS. Elevated serum estradiol concentrations predict the development of OHSS in only one-fourth of all cases, and severe OHSS has developed in patients with partial 17,20-lyase deficiency despite low serum estradiol concentration [31].

Progesterone is another major sex steroid hormone during the ovulatory process. Progesterone receptors (PR) begin to appear in the granulosa cells of the dominant follicle in the periovulatory period [32]. Serum progesterone concentration is also increased during the preovulatory and luteal phase. A positive correlation between VEGF and progesterone concentrations in human follicular fluid has been established in natural [33] and IVF-ET [7] cycles. The stimulatory effects of progesterone on the VEGF production have been reported in breast cancer cells [34] and bovine retinal pigment epithelial cells [35]. Our present study is the first attempt to clarify the control mechanism of ovarian VEGF production by progesterone in an OHSS model.

VEGF protein production was significantly decreased at 24 h after administration of RU486 accompanied with attenuation of progression of OHSS in a dose dependent manner. RU486 is a synthetic steroid hormone that binds to the progesterone receptor and acts as a progesterone antagonist. RU486 is reported to have a long life and high affinity to the progesterone receptor, and also acts as a glucocorticoid receptor [36]. Our previous study indicated that 5 mg/kg of RU486 was sufficient to act as an anti-progesterone in this model [16].

Unlike VEGF production, VEGF mRNA expression was not affected by RU486 in our experiments in agreement with a previous study on isolated macaque granulosa cells [37]. The probe employed for Northern blotting recognized the most abundant isoform VEGF₁₆₄. RT-PCR study showed that the relative expression of the three major PCR transcripts was not altered by the addition of hCG and RU486. As progesterone response elements (PRE) have not been found in the known VEGF 5'-sequence, progesterone may exert nongenomic actions on VEGF production in granulosa-luteal cells.

The rapid actions of steroids are likely to be transmitted by specific membrane receptors that have been characterized only recently. The cloning and functional expression of a putative progesterone membrane receptor have been achieved [38]. Despite the absence of classical PR in rodent luteal cells, luteal cells obtained from 19-day pregnant rats responded to the synthetic progestin promegestone (R5020) in a dose-dependent manner, with an increase in progesterone output [39]. Progesterone may also exert indirect influences on the VEGF production in granulosa-luteal

cells. Morphometric studies have confirmed that endothelial cells constitute approximately 50% of cells in the mature corpus luteum [40]. PR were present not in luteal but in endothelial cells in rodent corpus luteum, suggesting that progesterone serves as a paracrine signal which may be necessary for the induction of apoptosis in the rat corpus luteum [41].

These stimulatory effects of progesterone for ovarian VEGF production contrast with the data from humans. Lee *et al.* investigated that *in vitro* incubation of human luteinized granulosa cells with a 3 β -HSD inhibitor did not alter VEGF production [42]. There may be differences in the regulation of VEGF production among species.

VEGF expression has been also detected in organs where endothelial cells are normally quiescent, such as kidney, heart, lung and brain [43, 44]. Pleural effusion is one of the major symptoms in OHSS, thus the lungs are among the target organs involved in OHSS. We tested the expression of VEGF in the lungs of the OHSS model rat. The liver was employed as a negative control. VEGF mRNA expression was found in the lung and liver of the OHSS model rat. The lung produced 0.4 pg/mg protein of VEGF without hCG stimulation, which is approximately 50-fold higher than the VEGF production in the liver, and reached one-third of the amount in the ovary. In contrast to the marked effect on ovarian VEGF, hCG and RU486 had little effect on the yield of either VEGF mRNA or protein in lung and liver. These results suggest that organ specific pathways may regulate ovarian VEGF expression.

The female reproductive organ is unique in that it is the only site where angiogenesis occurs in a repetitive cyclic fashion. There is an analogy between the primate corpus luteum and the endometrium. These reproductive tissues are complex tissue consisting of different cell types, including endothelial, stromal and inflammatory cells. The hormonal receptivity and distribution of these cell populations change during the menstrual cycle. In addition to estradiol-induced VEGF expression in endometrial epithelial and stromal cells, infiltrating immune cells are another source of VEGF in normal endometrial cells [45]. This shows that cytokines can mediate neoangiogenesis in the endometrium by inducing VEGF gene transcription in inflammatory conditions. It has been well documented that ovulation is an inflammatory process [46, 47]. Ovarian angiogenesis may also be under the control of

both endocrine factors, including hCG and progesterone, and paracrine factors [48]. In our present experimental model, VEGF production still remained after the addition of 20 mg/kg of RU486. This indicated that progesterone is not a definite factor for VEGF control in the ovary. The production of VEGF protein appeared to be controlled by plural factors including progesterone. We did not test the other potent stimu-

lator for VEGF production, e.g. $\text{TNF}\alpha$. The cross talk of various factors involved in the progression of OHSS thus remains to be elucidated.

In conclusion, our results demonstrate that progesterone is implicated in the development of OHSS, in part, to enhance ovarian VEGF production by post-transcriptional and organ-specific control.

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