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Progestins suppress estrogen-induced expression of vascular endothelial growth factor (VEGF) subtypes in uterine endometrial cancer cells

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Abstract

Vascular endothelial growth factor (VEGF) contributes to the early advancement of uterine endometrial cancers that conserve hormone dependency via angiogenic activity. This process prompted us to study sex steroidal suppression of VEGF expression in Ishikawa cells (a line of well-differentiated uterine endometrial cancer cells). Estrogen transiently induced VEGF subtype ($VEGF_{165}$ and $VEGF_{121}$) secretion from Ishikawa cells. Progestins (progesterone, medroxyprogesterone acetate (MPA) and 17 α-hydroxyprogesterone) suppressed the estrogen-induced events. In conclusion, progestins could suppress VEGF-related angiogenic potential, which contributes to tumor growth in the early stage of uterine endometrial cancers that conserve estrogen dependency.

Keywords: Vascular endothelial growth factor; Endometrial cancer cell; Estrogen; Progestin; Angiogenesis

1. Introduction

Angiogenesis is essential for growth and nutrition of solid tumors larger than 2 mm in diameter [1]. An unorganized basement membrane in new capillary endothelial cells permits intravasation of tumor cells. A high density of microvessels in tumors is associated with this expansion and invasiveness [2–6]. Angiogenic factors stimulate various steps of angiogenesis [7].

Primary angiogenic factors induced from uterine endometrial cancers are basic fibroblast growth factor (FGF), platelet-derived endothelial cell growth factor (PD-ECGF) and vascular endothelial growth factor (VEGF) [8–10]. VEGF was initially recognized as a vascular permeability factor (34–42 kDa) which induced tumor ascites [11], and later VEGF was identified as a vascular permeability factor active in increasing blood vessel permeability, endothelial cell growth and angiogenesis [12]. It was also initially described as a secreted, direct-acting mitogen specific for vascular endothelial cells [13]. VEGF expresses in tumors [10,14,15], tissues and organs (namely reproductive organs, ovary, uterine endometrium and placenta [16–18]) with rapid endothelial turnover. The expression of VEGF in ovarian bovine granulosa cells was induced by estrogen, which demonstrated the estrogen-responsive element sequences in a transcription regulatory domain in the VEGF gene [16]. It is well known that some endometrial cancers are estrogen-dependent during growth [19].

To discover the advancement potential of uterine endometrial cancer associated with angiogenesis under the influence of sex steroids, we determined

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the secretion and expression, along with the mRNA expression, of VEGF and its subtypes in Ishikawa cells [20] by Western blot analyses with a sandwich enzyme immunoassay and RT–PCR.

2. Materials and methods

2.1. Chemicals

Estradiol-17β, tetrahydrocortisol, hydrocortisone, progesterone, medroxyprogesterone acetate (MPA) and 17α-hydroxyprogesterone were purchased from Sigma Chemical Co. (St. Louis, MO). They were solubilized in ethanol and added to the culture media to obtain a final concentration of ethanol below 0.1%.

2.2. Culture for uterine endometrial cancer cells

Ishikawa cells [20] were cultured in 90% Eagle's MEM and 10% fetal bovine serum (FBS). The culture was then placed in Eagle's MEM free of FBS and phenol red. After 48 h, various sex steroids were added to the culture dishes. The steroid concentration is indicated in each experiment.

2.3. Western blot analysis for human VEGF

Tissues (wet weight: 10–20 mg) were homogenized in WB-HB buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.2 mM phenylmethyl sulfonyl fluoride) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). The protein concentration of the samples was measured by the method of Bradford [20]. Equal amounts of protein (100 μg) were added to 4-fold amounts of sample buffer (62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol) and analyzed by 7.5% SDS–PAGE under non-reducing conditions. The gels were then transferred to nitrocellulose membranes (Hybond ECL Western, Amersham, Arlington Height, IL). The membranes were blocked with 5% milk (from dehydrate) in blocking buffer (20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20), incubated with rabbit anti-human VEGF antibody (1:1000) (Oncogene Research Products, Cambridge, MA), washed, and then incubated with anti-rabbit immunoglobulin, peroxidase-linked species-specific whole antibody from donkey (1:2000) (Amersham, Buckinghamshire, UK) prior to incubation with ECL chemiluminescence reagent (Amersham, Arlington Height, IL). Chemiluminescence was transferred to X-ray film at room temperature for 10 min before film development.

2.4. Enzyme immunoassay for determination of human VEGF antigen

All steps were carried out at 4°C. Tissues (wet weight: 10–20 mg) were homogenized in HG buffer (5 mM Tris–HCl (pH 7.4), 5 mM NaCl, 1 mM $CaCl_2$, 2 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 25 μg/ml aprotinin, 25 μg/ml leupeptin) with a Polytron homogenizer (Kinematics). This suspension was microcentrifuged at 12 000 rev./min for 3 min to remove the nuclear pellet. The protein concentration of samples was measured by the Bradford method to standardize VEGF antigen levels [21].

VEGF antigen levels in the samples were determined by a sandwich enzyme immunoassay using a Human VEGF Assay Kit-IBL (Immuno Biological Laboratories, Gunma, Japan). The levels of VEGF were standardized with the corresponding cellular protein concentrations.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR) to amplify VEGF mRNA

Total RNA was isolated from the cells using the acid guanidium thiocyanate–phenol–chloroform extraction method [22]. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MD) in a buffer of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM DTT and 0.5 mM deoxynucleotides to generate cDNAs using random hexamer (50 ng, Gibco BRL) at 37°C for 60 min. The RT reaction mixture was heated at 94°C for 5 min to inactivate MMLV-RTase.

Ten cycles of PCR for VEGF mRNA, consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, with reverse transcribed cDNA and 0.1 μM specific primers were carried out using an IWAKI thermal sequencer (TSR-300, Iwaki Glass, Tokyo, Japan) with Vent DNA

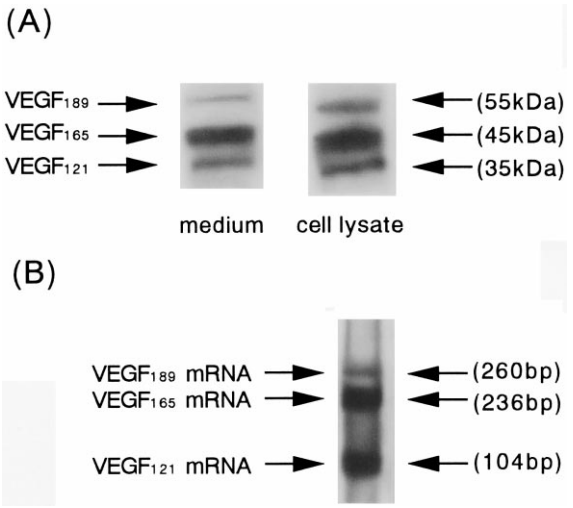

Fig. 1. Western blot analysis for the secretion and expression of VEGF and Southern blot analysis for the expression of VEGF mRNAs in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM without fetal bovine serum (FBS), phenol red or steroids. To analyze VEGF subtypes and their mRNAs, (A) SDS–PAGE with the culture medium and the cell lysate, and (B) RT–PCR–Southern blot with the isolated total RNA were carried out.

polymerase (New England Biolabs, Beverly, MA) in a buffer of 10 mM KCl, 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.15 mM deoxynucleotide phosphates. Additionally, 23 cycles of PCR for VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard were also carried out.

The oligodeoxynucleotides of specific primers in PCR (Fig. 1) were synthesized according to the published information (cDNA for VEGF [23] and GAPDH [24]) as follows: sense primer for VEGF mRNA: 5′-AGGCCAGCACATAGGAGAGA-3′ (in exon 4); antisense primer for VEGF mRNA: 5′-ACCGCCTCGGCTTGTCACAT-3′ (in exon 8); sense primer for GAPDH mRNA: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (in exon 2); antisense primer for GAPDH mRNA: 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (in exon 8).

2.6. Southern blot analysis for quantities of VEGF mRNA expression

PCR products were applied to 1.2% agarose gel, and electrophoresis was performed at 50–100 V. PCR products were capillary transferred to an Immobilon transfer membrane (Millipore Corp., Bedford, MA) for 16 h. The membrane was dried at 80°C for 30 min, and was UV-irradiated to tightly secure the PCR products. PCR products on the membrane were prehybridized in a buffer of 1 M NaCl, 50 mM Tris–HCl (pH 7.6) and 1% SDS at 42°C for 1 h, and hybridized in the same solution with the biotinylated oligodeoxynucleotide probes synthesized from the sequences of VEGF and GAPDH cDNAs between the specific primers at 65°C overnight. Specific bands hybridized with the biotinylated probes were detected with Plex Luminescent Kits (Millipore Corp.), and X-ray film was exposed on the membrane at room temperature for 10 min. The quantification of Southern blot was carried out with Bio Image (Millipore, Ann Arbor, MI). The intensity of specific bands was normalized with that of GAPDH.

2.7. Statistics

Statistical analyses were performed with one-way ANOVA and Student's *t*-test. Differences were considered significant when P was less than 0.05.

3. Results

In Ishikawa cells, mainly $VEGF_{165}$ and $VEGF_{121}$ and their mRNAs were detected by Western blot and RT–PCR–Southern blot. $VEGF_{165}$ and its mRNA were only faintly detected, and $VEGF_{206}$ and its mRNA were not detected. Among the four subtypes of VEGF, the populations of $VEGF_{165}$ and $VEGF_{121}$ were dominantly expressed. The two main subtypes and their mRNAs expressed are shown in parallel in Fig. 1. The simultaneous determinations of total VEGF level by the sandwich enzyme immunoassay and subtype mRNAs with RT–PCR–Southern blot provided enough information to reliably identify the subtype protein expression populations.

The signal intensity curve for mRNA expression is necessary for an accurate measurement of mRNA by RT–PCR. The following PCR templates were prepared from reverse transcribed total RNA (100 μg): 1.5, 3, 6, 12, 24 and 48 μg total RNA reverse transcribed (RNA–RT). PCR–Southern blots were carried out as described in Section 2. The signal inten-

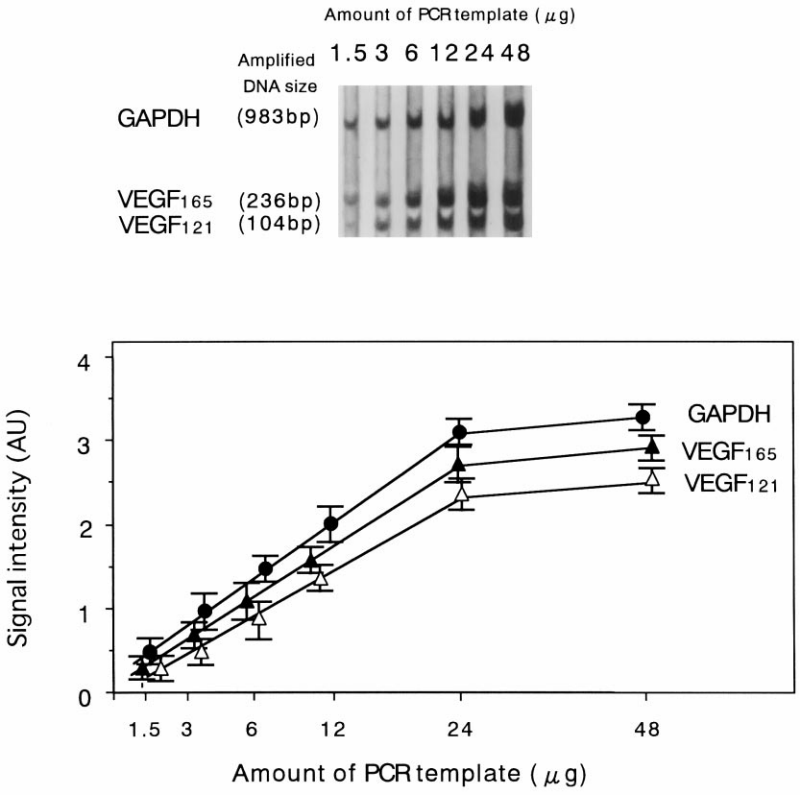

Fig. 2. Signal intensity curve for VEGF mRNA levels in a series of reverse transcribed-total RNA of Ishikawa cells by RT–PCR–Southern blot analysis. Ishikawa cells in a plate were incubated under the same conditions as for Fig. 1. The following PCR templates were prepared from reverse transcribed total RNA (100 μg) in Ishikawa cells: 1.5, 3, 6, 12, 24 and 48 μg RNA-RT. PCR–Southern blots were carried out as described in Section 2. The mRNA levels in Ishikawa cells were assigned as arbitrary units (AU)/GAPDH mRNA (AU/GAPDH mRNA). Data are the mean ± SD of six determinations.

sity curve for VEGF mRNA levels ranging from 1.5 to 24 μg reverse transcribed-total RNA of Ishikawa cells by RT–PCR–Southern blot was linear (Fig. 2). Therefore, semi-quantitative alternation of the mRNA levels proved to be reliable.

Estradiol dose-dependence up to 10^{-8} M significantly ($P < 0.05$) increased the levels of VEGF subtypes in the culture medium (in 24 h), in the cell lysate (in 3 h), and of mRNAs (in 2 h) (Fig. 3). Progesterone dose-dependence up to 10^{-6} M significantly ($P < 0.05$) diminished the levels of VEGF subtypes induced by estradiol in the culture medium, in the cell lysate, and in the mRNAs (Fig. 4). Therefore, the experimental concentrations of estradiol (10^{-8} M) and progesterone (10^{-6} M) for the appropriate effects were set.

Within several hours estradiol markedly ($P < 0.05$, significant) increased the levels of VEGF subtypes in the culture medium and maintained these elevated levels up to 36 h. It temporarily but significantly ($P < 0.05$) increased levels in the cell lysate with a peak at 3 h. Subtype mRNA levels, also increased temporarily ($P < 0.05$) with a peak at 2 h (Fig. 5). Progesterone significantly ($P < 0.05$) decreased the levels of VEGF subtypes induced by estradiol in the culture media and cell lysate and decreased the subtype mRNA levels (Fig. 5).

Tetrahydrocortisol and hydrocortisone (10^{-6} M) failed to significantly alter the levels of VEGF subtypes induced by estradiol in the culture media (in 24 h), in the cell lysate (in 3 h) or of mRNA levels (in 2 h) (Fig. 6). Progesterone, MPA, 17 α-hydroxyprogesterone and tamoxifen decreased all levels significantly ($P < 0.05$) (Fig. 6).

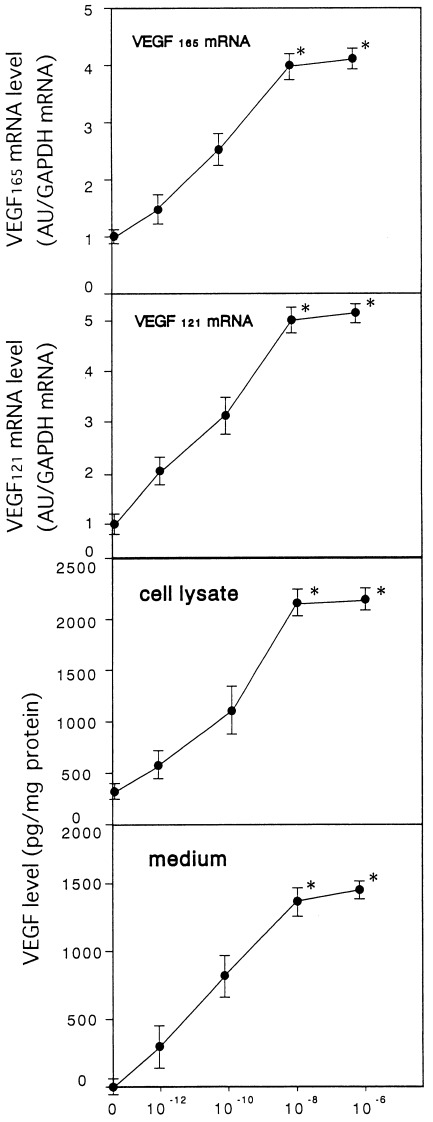

Fig. 3. Dose response curve showing the effects of estradiol on secretion and expression of VEGF subtypes and their mRNA expressions in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-12}–10^{-6} M estradiol. Levels of VEGF in the culture medium (incubated for 24 h) and in the cell lysate (incubated for 3 h) were measured by a sandwich enzyme immunoassay. The levels of $VEGF_{165}$ and $VEGF_{121}$ mRNAs in the cells (incubated for 2 h) were measured by RT–PCR–Southern blot analysis. Data are the mean ± SD of six determinations. *$P < 0.05$ versus controls.

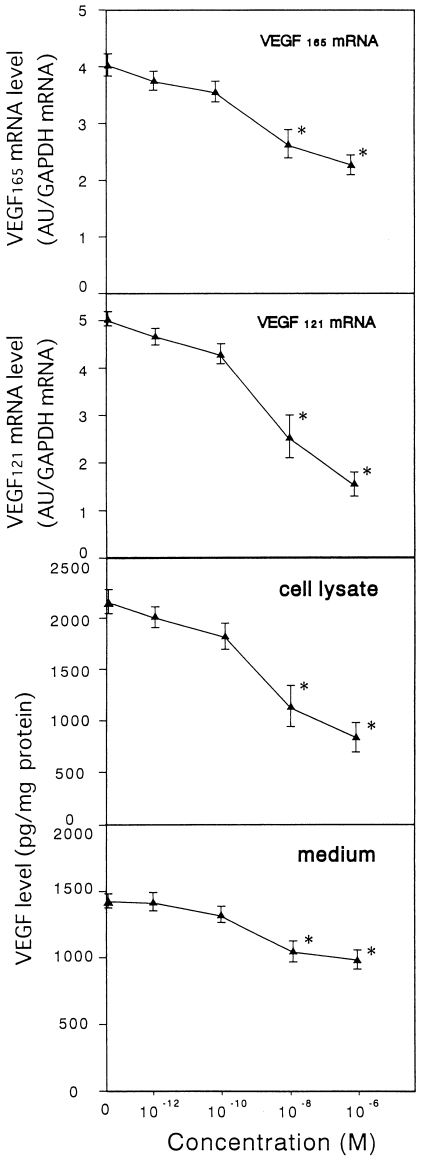

Fig. 4. Dose response curve showing the effects of progesterone on estradiol-induced secretion and expression of VEGF subtypes and their mRNA expressions in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-8} M estradiol and 10^{-10}–10^{-6} M progesterone. The levels of VEGF and its mRNA were then measured as described for Fig. 3. Data are the mean ± SD of six determinations. *$P < 0.05$ versus E_2.

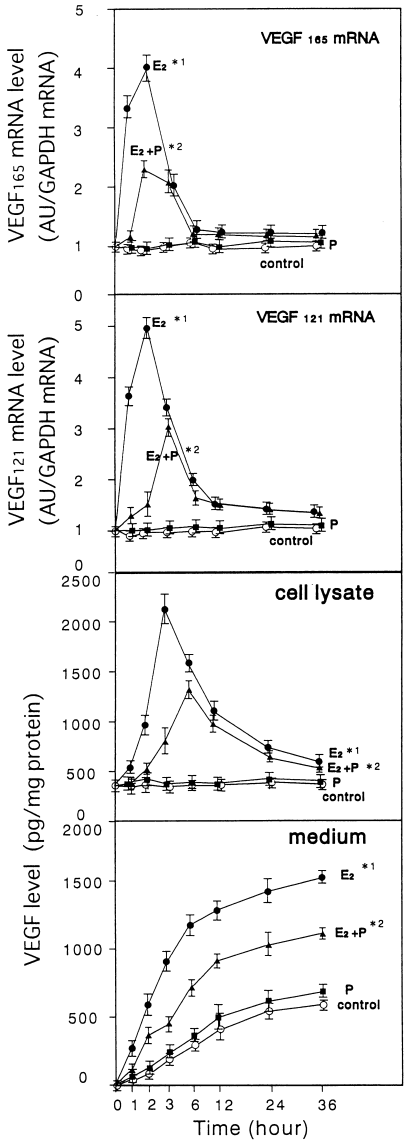

Fig. 5. Time course for effects of estradiol, estradiol plus progesterone, or progesterone on secretion and expression of VEGF subtypes and their mRNA expressions in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-8} M estradiol, 10^{-8} M estradiol plus 10^{-6} M progesterone, or 10^{-6} M progesterone. Levels of VEGF in the culture medium and cell lysate (incubated for 36 h), and levels of $VEGF_{165}$ and $VEGF_{121}$ mRNAs in the cells (incubated for 24 h) were measured as described for Fig. 3. Data are the mean ± SD of six determinations. *1 $P < 0.05$ versus controls; *2 $P < 0.05$ versus E_2.

4. Discussion

The tumor cell-derived angiogenic factor VEGF contributes to tumor growth in a paracrine fashion to surrounding microvessels [25]. Furthermore, VEGF facilitates metastasis of tumor cells in the steps of angiogenesis [26]. The expression and secretion of VEGF was induced by platelet-derived growth factor, transforming growth factor-β, interleukin-1, hypoglycemia, hypoxia [27–31], estrogen [16] and androgen [32,33]. VEGF content decreased to less than 20% within the ventral prostate of castrated rat [32]. Furthermore, androgen withdrawal induced apoptosis in Shionogi tumor cells, androgen-dependent in growth [33]. This might lead to an interesting androgen ablation therapy as an anti-angiogenic strategy.

In the case of uterine endometrial cancers, strong expression was observed of VEGF and its mRNA in the cancer cells, and of flt-1 and KDR mRNA in the endothelial cells of the surrounding microvessels [34]. From the aspect of VEGF subtypes, it was reported that four species of mRNA encoding VEGFs were identified in human uterine endometrium, and $VEGF_{165}$ and $VEGF_{121}$ were present in peripheral monocytes, which indicated tissue-specific splicing of the two other transcripts [35]. In ovarian and uterine cervical cancers, two VEGF subtypes, $VEGF_{165}$ and $VEGF_{121}$, are dominantly expressed [14,15]. The populations of $VEGF_{165}$ and $VEGF_{121}$ among the four VEGF subtypes in uterine endometrial cancers are dominant and essential to total angiogenic activity supplied from VEGF [10]. VEGF also contributes to tumor growth in the early stage of uterine endometrial cancers that conserve some estrogen dependency [10].

Among the main angiogenic factors, basic FGF in Ishikawa cells is persistently induced by estrogen. Progestins suppress the estrogen-induced basic FGF [36]. Basic FGF expresses in cancer and stroma cells of uterine endometrial cancers [36,37]. Also, basic FGF dominantly contributes to tumor spreading in the advanced stage of endometrial cancers [8]. However, PD-ECGF in the Ishikawa cell line is transiently induced by estrogen. Progesterone enhances the estrogen-induced PD-ECGF (data not shown). PD-ECGF expression has been demonstrated in the stroma cells of the normal uterine endometrium, and

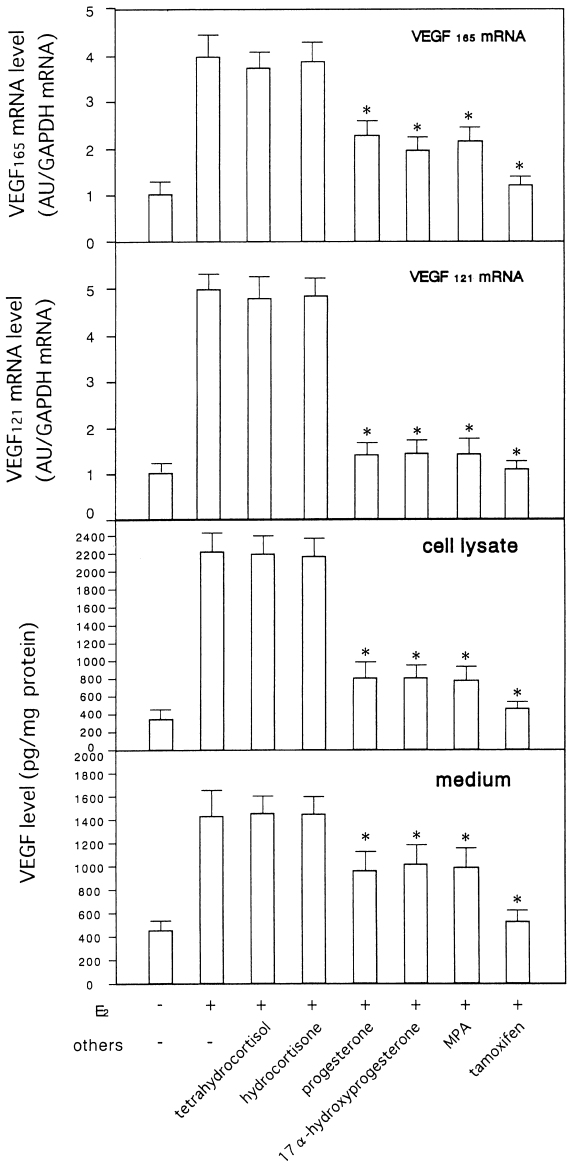

Fig. 6. Effect of various progestins on VEGF subtypes and the mRNA expressions in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-8} M estradiol and with 10^{-8} M estradiol plus various 10^{-6} M steroids. The levels of VEGF and its mRNA were then measured as described in Fig. 3. Data are the mean ± SD of six determinations. *$P < 0.05$ versus E_2.

in the stroma cells of endometrial cancer [38,39]. Furthermore, PD-ECGF contributes to tumor growth in the early stage of uterine endometrial cancers, especially myometrial invasion, via angiogenesis [9]. Distinct from basic FGF and PD-ECGF, VEGF expression has been demonstrated in glandular cells of normal uterine endometrium, and in the cancer cells of uterine endometrial cancer [34]. In rat uterus, treatment with estradiol and estriol rapidly induces VEGF subtypes $VEGF_{165}$ and $VEGF_{121}$ [40]. In this study, estrogen transiently induced VEGF subtypes ($VEGF_{165}$ and $VEGF_{121}$) in the culture medium and in the cell lysate of Ishikawa cells. Progesterone also stimulates the expression of $VEGF_{165}$ and $VEGF_{121}$ in rat uterus [40], but alteration of VEGF by progestin alone does not occur in Ishikawa cells [41], results consistent with those of the present study. This discrepancy of the progestin effect on VEGF expression between rat uterus, including interstitial cells, and Ishikawa cells may arise from cell specificity in different species. Actually, a low level of estrogen is always synthesized, even in menopausal women. Therefore, progestin treatment is essential in cases in which the estrogen-induced VEGF expression can be suppressed. We demonstrated that progestins sufficiently suppressed the induction by estrogen. In conclusion, progestins could suppress the VEGF-related angiogenic potential, which contributes to tumor growth in the early stage of uterine endometrial cancers which conserve estrogen dependency.

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