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Effects of various steroids on platelet-derived endothelial cell growth factor (PD-ECGF) and its mRNA expression in uterine endometrial cancer cells☆

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Abstract

Progestins diminish the estrogen-induced angiogenic potential related to basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) in uterine endometrial cancer cells. This led us to study the effect of various steroids on the expression of platelet-derived endothelial cell growth factor (PD-ECGF) as the other pertinent angiogenic factor in well-differentiated uterine endometrial cancer cell line Ishikawa.

In Ishikawa cells, estradiol induced the expression of PD-ECGF and its mRNA. The estrogen-induced expression was increased approximately two-fold by progesterone and by its metabolite, 17α-hydroxyprogesterone, but not by medroxyprogesterone acetate (MPA). Therefore, progesterone and 17α-hydroxyprogesterone as endogenous steroids might induce PD-ECGF-related angiogenic potential in uterine endometrial cancer cells, but not MPA as a synthetic steroid. In conclusion, the failure of PD-ECGF induction by MPA might be the great merit of anti-angiogenic treatment with MPA for uterine endometrial cancers.

Keywords: PD-ECGF; Angiogenesis; Endometrial cancer cell; Steroids

1. Introduction

Solid tumors beyond 2 mm in diameter require angiogenesis for growth and nutrition [1]. Unorganized basement membrane of new capillary endothelial cells permits intravasation of tumor cells, and a high density of microvessels in tumors is associated with their expansion and invasiveness [2–6]. Angiogenic factors stimulate various steps of angiogenesis [7].

Main angiogenic factors induced from uterine endometrial cancers are basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) [8–10]. PD-ECGF was originally cloned as a novel angiogenic factor (45 kDa polypeptide) from human platelet [11]. Thereafter, PD-ECGF was completely identifical with thymidine phosphorylase (TP) [12,13]. PD-ECGF/TP does not stimulate the growth of endothelial cells, but chemotaxis of them, and induces angiogenesis in vivo with the activation of thymidine phosphorylase as an enzyme [14,15]. Among normal tissues and organs, PD-ECGF is expressed in lymph nodes, peripheral lymphocytes, spleen, lung, liver, placenta [16] and uterine endometrium [17,18]. Among solid tumors, PD-ECGF is expressed in malignant gliomas, thyroid tumors, cancers of the breast, esophagus, stomach, colon, pancreas, gall bladder, kidney, lung [16], uterine cervix [19] and endometrium [20], and ovary [21].

Progestins diminish the basic FGF- and VEGF-related angiogenic potential induced by estrogen in well-differentiated uterine endometrial cancer cells [22]. The purpose of the present study was to determine the effects of various steroids on the expression of PD-ECGF as the other pertinent angiogenic factors in well-differentiated uterine endometrial cancer cell line Ishikawa.

2. Materials and methods

2.1. Chemicals

Estradiol-17β, tetrahydrocortisol, hydrocortisone, progesterone, medroxy-progesterone acetate (MPA) and 17α-hydroxyprogesterone were purchased from Sigma (St. Louis, MO, USA). They were solubilized in ethanol and

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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, derived from well-differentiated adenocarcinoma of the endometrium [23], were cultured in 90% Eagle's MEM and 10% fetal bovine serum (FBS). Afterwards, the culture was proceeded in Eagle's MEM free of FBS and phenol red. Forty-eight hours later, various steroids were settled in culture dishes. The steroid concentration is indicated in each experiment.

2.3. Enzyme immunoassay for determination of human PD-ECGF 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, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12,000 rpm for 3 min to obtain the supernatant. The protein concentration of samples was measured by the method of Bradford [24] to standardize PD-ECGF antigen levels.

PD-ECGF antigen levels in the sample were determined by the sandwich enzyme immunoassay described by Nishida et al. [25]. The levels of PD-ECGF were standardized with corresponding cellular protein concentrations.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) to amplify PD-ECGF mRNA

Total RNA was isolated from the cells by the acid guanidium thiocyanate-phenol-chloroform extraction method [26]. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MD, USA) 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.

Five cycles of PCR for PD-ECGF 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, were carried out with reverse transcribed cDNA, 0.1 μM specific primers and Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) 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 using an IWAKI thermal sequencer TSR-300 (Iwaki Glass, Tokyo, Japan). Additionally, 23 cycles of PCR for PD-ECGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard were done in the same manner.

The oligodeoxynucleotides of specific primers in PCR were synthesized according to the published information on cDNA for PD-ECGF [27] and GAPDH [28] as follows: sense primer for PD-ECGF mRNA: 5′-AGTCGGATGGCCATCAGCAT-3′ (in exon 2); antisense primer for PD-ECGF mRNA: 5′-TGGAATGCTTGTCCACAAGC-3′ (in exon 3); sense primer for GAPDH mRNA: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ [in exon 2]; antisense primer for GAPDH mRNA: 5′-CATGTGGGCCATGA GGTCCA CCAC-3′ (in exon 8).

Southern blot analysis for quantities of PD-ECGF 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, USA) for 16 h. The membrane was dried at 80 °C for 30 min, and was UV-irradiated to tightly fix PCR products. PCR products on the membrane were prehybridized in 1 M NaCl, 50 mM Tris–HCl, pH 7.6, and 1% sodium dodecyl sulfate at 42 °C for 1 h, and then hybridized in the same solution with the biotinylated oligodeoxynucleotide probes synthesized from the sequences of PD-ECGF 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, USA). The intensity of specific bands was standardized with that of GAPDH mRNA.

2.5. Statistics

The levels of PD-ECGF and its mRNA were measured from three parts of the same tissue in triplicate. Statistical analysis was performed with Student's *t*-test and one-way ANOVA. Differences were considered significant when P was less than 0.05.

3. Results

The signal–intensity curve for the mRNA expression is necessary for an accurate measurement of the mRNA by RT-PCR. PCR templates were prepared from reverse transcribed total RNA (100 μg) in uterine normal endometrium as follows: 1/2X, 1.5 μg total RNA reverse transcribed (RNA-RT); 1X, 3 μg RNA-RT; 2X, 6 μg RNA-RT; 4X, 12 μg RNA-RT; 8X, 24 μg RNA-RT; and 16X, 48 μg RNA-RT. PCR–Southern blots were carried out as described in Section 2. The signal–intensity curve for PD-ECGF mRNA levels ranging from 1/2X–8X of reverse transcribed-total RNA of normal endometrium by RT-PCR–Southern blot was linear (Fig. 1), so semiquantitative

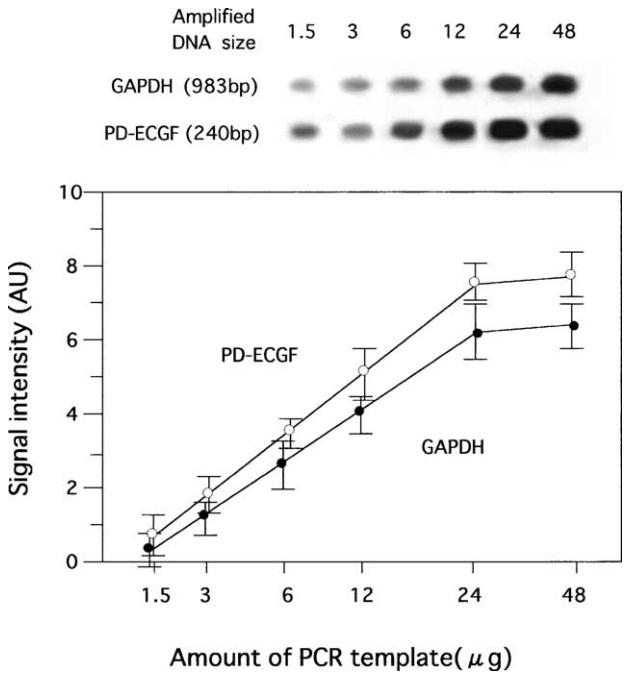

Fig. 1. Signal–intensity curve for PD-ECGF mRNA levels in a series of reverse transcribed-total RNA of Ishikawa cells by RT-PCR–Southern blot analysis. Ishikawa cells in a plate were incubated in eagle MEM medium without FBS, phenol red or steroids. RT was carried out on total RNA isolated. Then PCR-SBA was performed on a series of revere transcribed-total RNA (3 μg) ranging from 1/2X to 16X. The levels of mRNA expressions in Ishikawa cells were assigned as arbitrary units/GAPDH mRNA (AU/GAPDH mRNA). Data are the mean ± S.D. of nine determinations.

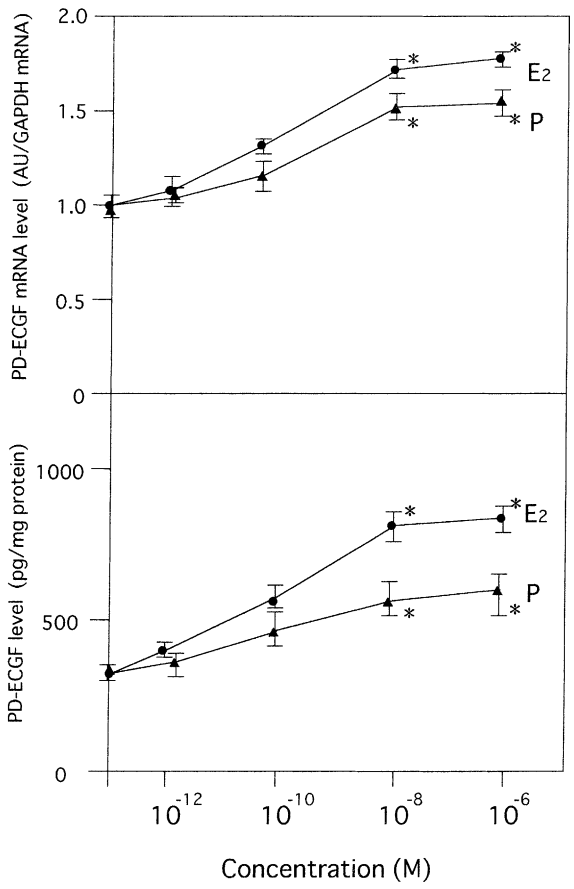

Fig. 2. Dose–response curve showing the effects of estradiol alone and progesterone alone on PD-ECGF and its mRNA levels in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-12}–10^{-6} M estradiol alone and progesterone alone. Then the levels of PD-ECGF and its mRNA were measured by the sandwich enzyme immunoassay and RT-PCR–Southern blot analysis, respectively. Data are the mean ± S.D. of nine determinations. $^{*}P < 0.05$ vs. controls.

alternation of the mRNA levels was shown to be reliable.

In the dose–response curve showing the effects of estradiol and of progesterone on PD-ECGF and its mRNA expression in Ishikawa cells, estradiol alone and progesterone alone dose-dependently up to 10^{-8} M, significantly increased the levels of PD-ECGF (in 9 h) and its mRNA (in 6 h) (Fig. 2). Therefore, the concentration of 10^{-8} M estradiol and 10^{-8} M progesterone were set for the following time course study.

In the next experiment, the effects of estradiol alone and progesterone alone, and estradiol plus progesterone together on the expression of PD-ECGF and its mRNA were measured over a 48 h time period (Fig. 3). The administration of estradiol alone and progesterone alone led to a significant increase in PD-ECGF (in 9 h) and its mRNA level (in 6 h) in Ishikawa cells. With estradiol and progesterone together, a maximum two-fold induction in PD-ECGF and its mRNA level was obtained after 9 h and 6 h, respectively. The combined effect of estradiol and progesterone is thus additive and not synergistic.

Furthermore, with estradiol and 17α-hydroxyprogesterone as a progesterone metabolite, the induction was also significantly higher compared to the induction with estradiol alone. However, with hydrocortisone, tetrahydrocortisol and MPA, neither additive nor synergistic induction was shown. Additionally, tamoxifen greatly diminished the induction by estrogen (Fig. 4).

4. Discussion

Among main tumor-derived angiogenic factors, basic FGF, VEGF and PD-ECGF might be specific for the process of tumor growth and spreading in uterine endometrial cancers. The expression of basic FGF is significantly higher in uterine endometrial cancers than in normal uterine endometria, and is increased with dedifferentiation and advancing [8]. On the other hand, the expression of VEGF and PD-ECGF is the reverse, significantly lower in uterine endometrial cancers than in normal uterine endometria, and decreased with dedifferentiation and advancing [9,10]. Especially the active availability of PD-ECGF contributes to the acceleration of angiogenic activity in the early process of invasion of well-differentiated uterine endometrial cancers [20].

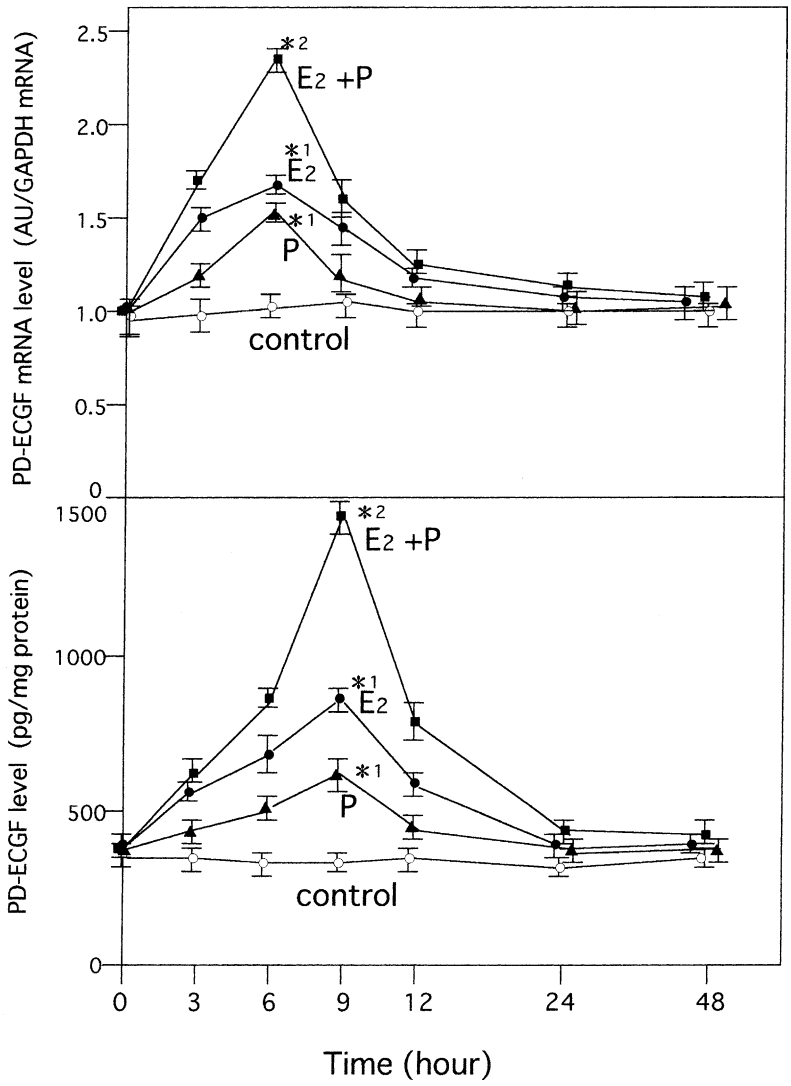

Fig. 3. Time course for effects of estradiol alone, estradiol plus progesterone, and progesterone alone on PD-ECGF and its mRNA levels in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-8} M estradiol alone, 10^{-8} M estradiol plus 10^{-8} M progesterone, and 10^{-8} M progesterone alone for 48 h. Then the levels of PD-ECGF and its mRNA were measured as described for Fig. 2. Data are the mean ± S.D. of six determinations. The symbols '*1' and '*2' indicate $P < 0.05$ vs. controls and $P < 0.05$ vs. E_2, respectively.

It is easy to consider that angiogenic factors derived from breast cancers, uterine endometrial cancers and ovarian cancers, which are often estrogen-dependent in growth, might be regulated by sex steroids. In fact, the expression of VEGF is increased by estrogen in granulosa cells [29]. The expression of basic FGF in uterine endometria and endometrial cancers was increased by estradiol, and progesterone canceled the estradiol-induced basic FGF expression [22,30,31].

In the present study, estradiol induced the expression of PD-ECGF and its mRNA, and tamoxifen greatly diminished the estrogen-induced PD-ECGF. Therefore, the estrogen-induced PD-ECGF might be driven via estrogen receptor cascades. The estrogen-induced PD-ECGF was increased approximately two-fold by progesterone and by 17α-hydroxyprogesterone in well-differentiated endometrial cancer cells, but not by hydrocortisone, tetrahydrocortisol or MPA. Progesterone and 17α-hydroxyprogestone might enhance the estrogen-induced PD-ECGF via progesterone receptors induced by estradiol. In general, the angiostatic steroids hydrocortisone, tetrahydrocortisol and MPA act directly on vascular endothelial cells [32]. Especially the former two do not modify the expression of angiogenic factors in cancer tissues. On the other hand, MPA diminishes the estrogen-induced basic FGF [22] and VEGF (data not shown) in well-differentiated uterine endometrial cancer cells, but not that of PD-ECGF. MPA probably uses a co-factor system in transcription of target genes different from other progestins, although the difference in molecular mechanisms of MPA and the other two progestins is still unknown.

In conclusion, the failure of PD-ECGF induction by MPA might be the great merit of anti-angiogenic treatment with

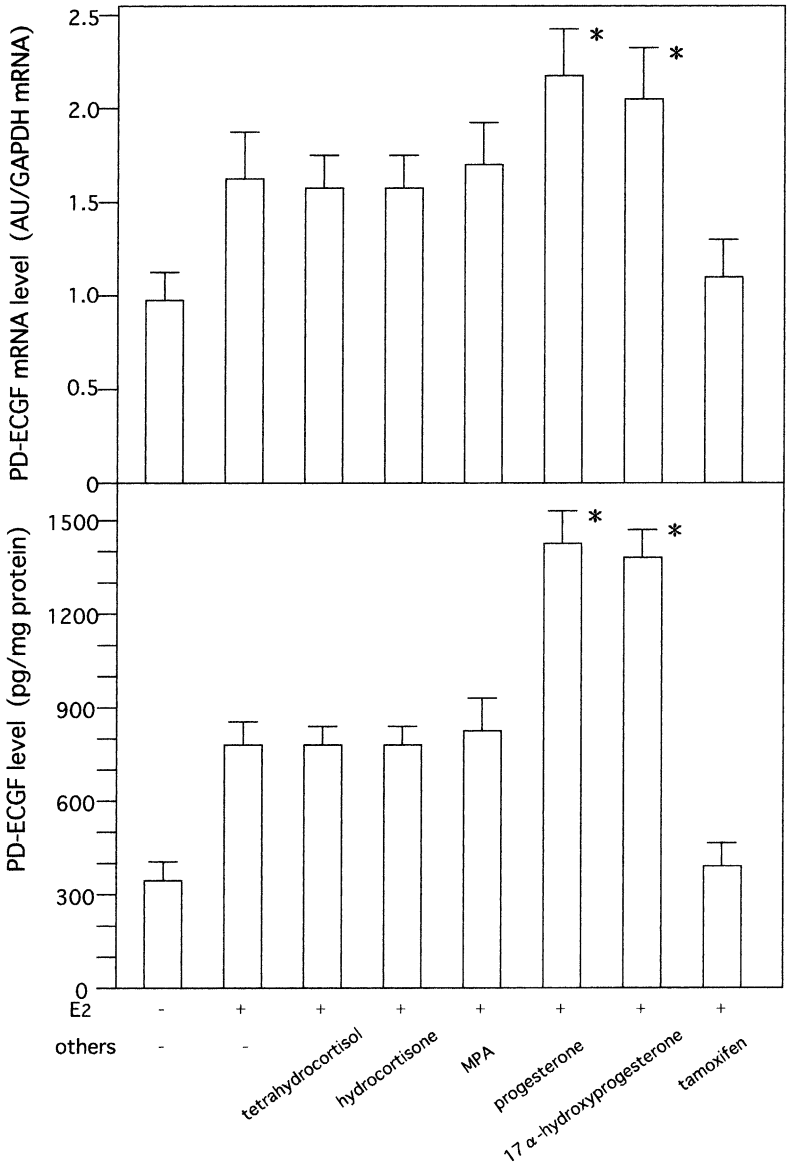

Fig. 4. Effects of various steroids on PD-ECGF and its mRNA levels in Ishikawa cells. Ishikawa cells in each plate were incubated in Eagle's MEM free of phenol red and FBS, with 10^{-8} M estradiol alone, 10^{-8} M estradiol plus 10^{-8} M various steroids, and 10^{-8} M estradiol plus 10^{-6} M tamoxifen. Then the levels of PD-ECGF and its mRNA were measured as described for Fig. 2. Data are the mean ± S.D. of six determinations. MPA, medroxyprogesterone acetate $^{*}P < 0.05$ vs. E_2.

MPA for uterine endometrial cancers. This indicates that angiogenic inhibitors, especially angiostatic steroids [32,33] on new capillary formation, might be effective for uterine endometrial cancer therapy regardless of direct anti-tumoral effects on cancer cells.

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