The effect of exogenous estradiol treatment on the mRNA expression of vascular endothelial growth factor and its receptors in cultured human oviduct mucosal cells

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Purpose: To evaluate the responses of cultured oviduct mucosal cells to exogenous estradiol treatment in regulating the mRNA expression of vascular endothelial growth factor (VEGF) and its receptors.

Methods: The mucosal layer of the ampullary regions of the human oviduct was isolated and cultured with (study groups) or without (control group) the addition of exogenous estradiol in five different concentrations. Semiquantitative reverse-transcriptase-polymerase chain reaction was performed on the oviduct mucosal cells before and after the 6-day culture.

Results: There were no significant differences in the mRNA expression of VEGF and its receptors, both KDR and flt-1, between the five study groups and the control group.

Conclusions: The mRNA expression of VEGF and its receptors is not altered by exogenous estradiol treatment in cultured oviduct. This helps to explain the mechanism of temporal regulation of VEGF in human oviduct, which reaches the peak level in the peri-ovulatory stage when both the serum estradiol and gonadotropins concentrations are high.

KEY WORDS: Estradiol; mRNA; oviduct; RT-PCR; VEGF.

INTRODUCTION

Vascular endothelial growth factor (VEGF), a known permeability-enhancing factor, has been postulated as a regulator of oviductal secretion by promoting vascular permeability and so serum transudation (1,2). This notion was supported by our previous observation of prominent expression of VEGF and its receptors in the ampullary regions of the human oviduct at the peri-ovulatory time (2,3), the same modulation pattern as the oviductal secretion which supports the early reproductive events

occurring there. Although both gonadotropins and estradiol (E₂) reach their peak serum levels in the peri-ovulatory stage, we have observed significant positive correlations between the mRNA expression of VEGF or its receptor flt-1 and the serum gonadotropins concentrations, but not the E₂ concentration (2,3). A direct stimulatory effect of gonadotropins on the oviductal VEGF expression has been recently confirmed by our in-vitro study of oviduct mucosal cell culture with human chorionic gonadotropin (4).

We postulated that, in contrast to the endometrium, the expression of VEGF and its receptors in the human oviduct was steroid-independent. In the endometrium, where the main role of VEGF is to induce angiogenesis to form a receptive endometrium for embryo implantation, the expression of VEGF is promoted by E₂ as well as progesterone (P) (5,6). However, the information on the roles of VEGF in the oviduct and its hormonal

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regulation is limited. We now perform this study to evaluate the in-vitro responses of the cultured oviduct mucosal cells to exogenous E₂ treatment in regulating the mRNA expression of VEGF and its receptors.

Since E₂ responsiveness of oviductal cells relies on the in-vivo cell-to-cell and cell-to-basement membrane contacts and may be lost in the standard cell culture system (7), an organ culture system that retains the in-vivo architecture of the oviduct mucosal cells was used (8). A better understanding of the causal relationship between E₂ and oviductal VEGF expression may help to explain the temporal regulation of VEGF expression in the human oviduct, which reaches the peak level in the peri-ovulatory stage when both the serum E₂ and gonadotropins concentrations are high.

MATERIALS AND METHODS

The study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

Subjects

Twenty parous women who underwent laparoscopic tubal sterilization or hysterectomy for benign gynecological conditions were recruited into the study. All subjects had regular monthly menstrual cycle and were not on any exogenous hormone in the previous 3 months. The cycle was divided into five stages that were determined by the date of commencement of last menstrual period (LMP) and were confirmed by appropriate serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), E₂ and P. There were five women in the early follicular stage (day 1–5) and the mid-follicular stage (day 6–12), three women in the peri-ovulatory stage (day 13–17) and early or mid-luteal stage (day 18–23), and four women in the late luteal stage (day 24–28). Informed consent was obtained from each participant. No subjects were included in cases in which the LMP did not correspond with serum LH, FSH, E₂ and P concentrations.

Oviduct Organ Culture

The procedures of oviduct tissue dissection were described in our previous publication (2). In brief, oviduct tissue excised during the operation was

immediately placed in 20 mL of Hepes buffered Quinn's Human Tubal Fluid (Irvine Scientific, Santa Ana, California, USA). The ampullary region of intact oviduct was collected after removing its surrounding connective tissue. The mucosal layer was isolated and sliced into 2-mm-wide pieces.

From our previous studies on the expression of oviductin and progesterone receptors in the human oviduct, we realized that the E₂ responsiveness of oviduct mucosal cells was lost in standard cell culture system (9). It was because the E₂ responsiveness relied on the in-vivo cell to cell and cell to basement membrane contacts in the oviduct mucosal layers. An organ culture system using intact oviduct mucosal tissues can retain the in vivo architecture of the mucosal cells and so their E₂ responsiveness. In order to evaluate the in-vitro oviductal responses to exogenous estradiol treatment, mucosal pieces of the ampullary regions of oviducts were processed by oviduct organ culture with or without exogenous estradiol-17 β (E₂, Sigma, Missouri, USA) treatment as follows:

The 2-mm-wide mucosal tissue pieces were cultured in the cell attachment medium for 3 days. The cell attachment medium was modified Eagle's medium (MEM, GIBCO BRL, Carlsbad, California, USA) with 40% fetal bovine serum (FBS) supplemented with 100 IU/mL penicillin and 20 μ g/mL streptomycin. The culture dishes were uncoated so as to minimize the cell adhesion to dish surface. Three days later, the attachment medium was replaced by the growth medium. The growth medium was MEM with 15% FBS supplemented with 100 IU/mL penicillin and 20 μ g/mL streptomycin, and the low percentage of FBS in the medium promoted cell proliferation. Exogenous estradiol-17 β (E₂, Sigma, Missouri, USA.) was added immediately to the cultures in five different concentrations (0.5 nmol/L, 50 nmol/L, 100 nmol/L, 200, nmol/L, and 500 nmol/L in Group 1–Group 5, respectively) while only solvent (100% ethanol) was added in the control group. The culture was incubated at 37°C with 5% CO₂ for a further 3 days. Then, the growth medium was removed and the cultured oviduct pieces were minced and stored at –70° subsequent mRNA isolation.

The range of E₂ concentrations and the duration of cell culture were derived from the previous work on the E₂ effect on oviductin expression in the human oviduct mucosal cells (8). The procedures of the organ culture were performed under room

temperature by the same investigator. All the samples were exposed to the same conditions except the E₂ concentrations in the growth medium. The origin of the cultured cells was examined by the contribution of epithelial cells relative to stromal or fibroblast cells as revealed in immunocytochemistry.

Semi-Quantitative Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

The procedures of mRNA isolation and RT-PCR were described in details in our previous study (2,3). In short, mRNA was extracted from the cultured oviduct pieces using the Oligotex direct mRNA kit (Qiagen, Hilden, Germany), and 100 ng of mRNA was used for cDNA synthesis with Multiscribe reverse transcriptase (PE Biosystems, Foster City, California, USA). All the resultant cDNA was then used for PCR with Amplitaq Gold DNA polymerase (PE Biosystems, Foster City, California, USA). Each PCR cycle consisted of denaturation at 95°C for 45s, annealing at 55°C for 45s, and extension at 72°C for 1 min. Thirty cycles were performed, followed by a final extension at 72°C for 5 min. Beta-actin was co-amplified with VEGF or its receptors to provide a semi-quantitative internal control for RNA quantity. The RT-PCR assay has been validated (2,3). Forward and reverse primers specific to beta-actin, VEGF and its receptors (KDR and flt-1) were previously described (2,3).

Apart from VEGF and its receptors, the effect of E₂ on an unrelated estrogen-regulated gene, progesterone receptor AB isoform (PRAB), in the cultured oviduct mucosal cells was also examined as a measure of E₂ responsiveness of the culture system. Forward and reverse primers specific to the PRAB were designed from the published cDNA sequences (10). The sequences of the primers were: forward—ACA GAA TTC ATG ACT GAG CTG AAG GCA AAG GGT and reverse—ACA AGA TCT CAA ACA GGC ACC AAG AGC TGC TGA. In addition, the expression of estrogen receptor (ER) in the cultured oviduct mucosal cells was checked. Forward and reverse primers specific to the ER- α and ER- β were designed from the published cDNA sequences (11). The sequences of the ER- α oligonucleotide primers were: forward—TGT GCA ATG ACT ATG CTT CA and reverse—GCT CTT CCT CCT GTT TTT A. The sequences of the ER- β oligonucleotide primers were: forward—GTC CAT CGC CAG TTA TCA CAT and reverse—GCC TTA CAT CCT TCA CAC GA.

Twenty percent of each PCR reaction was separated by gel electrophoresis on 2% agarose gel with 0.5 μ g/mL ethidium bromide in tris-borate ethelenediaminetetra acetic acid buffer. The separated PCR products were visualized under ultraviolet light. A video camera sent the UV-illuminated gel image to a computer, where the software package Gel Doc (Model GS-1000; Bio-Rad Laboratories, Hercules, California, USA.) enabled an image of the gel to be recorded. The integrated optical density (IOD) was determined for each PCR product by the Molecular Analyst Software (Bio-Rad Laboratories, Hercules, California, USA.). The IOD ratio between the PCR-amplified experimental gene and the co-amplified beta-actin control was obtained for each sample and then normalized to the lowest value for the subsequent analysis.

Statistics

The results were analyzed using the Statistical Package for Social Sciences. The paired-samples *t*-test was used to examine the differences in the mRNA expression of VEGF or its receptors between the cultured oviduct cells with and without the addition of exogenous E₂. Statistical significance was accepted at *p*-value < 0.05.

RESULTS

E₂ Responsiveness of the Culture System

After 6 days of culture, the pieces of oviduct mucosa maintained their in-vivo morphological characteristics including actively beating cilia. The purity of the cell culture was confirmed by immunocytochemistry. It showed that >95% of the cells stained positively for the specific epithelial cell marker cytokeratin and <5% stained positively for the fibroblast cell marker vimentin. This finding confirmed the epithelial cell origin of the cultured cells. Also, mRNA expression of both ER- α and ER- β was observed in the post-cultured oviduct mucosal cells in both study groups and control group. Most importantly, the PRAB mRNA expression in the study groups was significantly higher than the control group (2.2 ± 0.1 , 2.5 ± 0.1 , 3.0 ± 0.1 , 3.5 ± 0.2 and 3.5 ± 0.2 in Groups 1–5, respectively vs. 1.9 ± 0.1 in the control, *p* < 0.05). This is shown in Fig 1. This stimulatory effect of E₂ on the PRAB mRNA expression in cultured oviduct mucosal cells confirmed the E₂ responsiveness of the culture system.

Fig. 1. The relative mRNA expression of progesterone receptor AB isoform (PRAB), vascular endothelial growth factor (VEGF), and VEGF receptors (KDR and flt-1) in the oviduct mucosal cells before and after 6 day culture with or without exogenous estradiol added in various concentrations.

The effect of Exogenous E₂ on the mRNA Expression of VEGF and Its Receptors

There were no significant differences in the VEGF mRNA expression between the five study groups and the control group (1.4 ± 0.1 , 1.4 ± 0.1 , 1.6 ± 0.1 , 1.5 ± 0.1 and 1.5 ± 0.1 in Group 1–5, respectively vs. 1.5 ± 0.1 in the control, $p > 0.05$). Also, there were no significant differences in the mRNA expression of KDR or flt-1 between the study groups and the control group (For KDR: 1.6 ± 0.1 , 1.6 ± 0.1 , 1.5 ± 0.1 , 1.6 ± 0.1 and 1.6 ± 0.1 in Group 1–5, respectively vs. 1.6 ± 0.1 in the control, $p > 0.05$; for flt-1: 1.4 ± 0.1 , 1.6 ± 0.1 , 1.4 ± 0.1 , 1.6 ± 0.1 and 1.7 ± 0.1 in Group 1–5, respectively vs. 1.6 ± 0.1 in the control, $p > 0.05$). This is shown in Fig. 1.

DISCUSSION

The amount of oviductal fluid produced is under the influence of steroid hormones and is greatest in the peri-ovulatory period (12). Estrogen increases fluid secretion whereas progesterone decreases fluid secretion (13). This modulation of oviductal fluid secretion can be at least partly accountable by the hormonal regulation of the biosynthetic activity of oviductal epithelium. It has been observed that the expression of oviductin, which is specific to the oviduct may reflect the biosynthetic activity of oviductal epithelium, which is increased by estrogen and reduced by progesterone (14). This is probably

mediated by the changes in the morphology of the non-ciliated cells and indirectly by the modulation of blood flow to the oviduct. Estrogen not only promotes hypertrophy and secretory activity of the non-ciliated cells (15) but also increases the blood flow to the oviduct by decreasing the sympathetic discharge (16).

Serum transudation is another important mechanism of oviductal fluid formation, and it relies on both driving force for water movement as well as vascular permeability especially for the large-sized molecules like albumin. VEGF, a known vascular permeability promoter, has been postulated as a regulator of oviductal secretion (1). While we have demonstrated that the temporal and spatial modulations of VEGF in the oviduct follow that of the oviductal section, the mechanisms for their regulations remain largely unknown.

VEGF has been localized in various tissues of the female reproductive system, where the cyclical processes in the course of an ovulatory cycle suggests a hormonal regulation of its expression. In the endometrium, where the main role of VEGF is to induce angiogenesis to form a receptive endometrium for embryo implantation (17), the expression of VEGF is promoted by E₂ and P (5,6). However, in the ovary where VEGF plays more than a simple angiogenic role, its expression is not affected by E₂ or P (18). Instead, it is stimulated by gonadotropins and this relationship holds even in steroid-ablated conditions (18). Although the regulatory mechanisms

of VEGF expression in the endometrium and the ovary have been well studied, the information on the oviduct is scarce.

In our previous in-vivo studies, the lack of correlations between the serum E₂ concentration and the mRNA expression of VEGF or its receptor flt-1 in the oviduct suggests a steroid-independent regulation (2,3). This is confirmed by the results of this in vitro experiment. Together with our previous culture experiments on oviduct mucosal cells with hCG (4), which revealed increased mRNA expression of VEGF and its receptor flt-1 in a dose-dependent manner, it is likely that the VEGF expression in the oviduct is directly stimulated by gonadotropins without the influence of estradiol.

In summary, the mRNA expression of VEGF and its receptors in the cultured oviduct mucosal cells is not altered by exogenous E₂ treatment. This finding helps to explain the regulatory mechanism of the peak oviductal expression of VEGF around the time of ovulation when both the serum gonadotropins and estradiol concentrations are high.

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