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EFFECT OF HUMAN CHORIONIC GONADOTROPIN (HCG) ON EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR A (VEGF-A) IN HUMAN MID-SECRETORY ENDOMETRIAL CELLS IN THREE-DIMENSIONAL PRIMARY CULTURE

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Abstract : Several lines of evidence suggest that human uterine endometrial cells can bind human chorionic gonadotropin (hCG) which, in turn, influences the physiology of implantation stage endometrium. Vascular endothelial growth factor (VEGF) appears to be a candidate mediator in this process. However, our knowledge about hCG action on VEGF in human endometrial cells is very thin. In the present study, we have examined microscopically hCG binding to dissociated human endometrial cells collected from mid-luteal phase and maintained in three-dimensional primary co-culture on rat-tail collagen type I biomatrix and examined the effect of different concentrations (0, 1, 10, 100 and 1000 IU/ml) of hCG on VEGF expression and secretion by endometrial cells maintained in the above system. We report that both cytokeratin positive epithelial cells as well as vimetin positive stromal cells from human mid luteal phase endometrium could bind hCG and that their number increased ($P<0.01$) steadily with time. Administration of hCG enhanced ($P<0.05$) immunoreactive VEGF protein expression in dose dependent manner in endometrial cells retrieved from mid-luteal phase of cycle, and co-cultured in a three-dimensional cell culture system, but with no marked change in VEGF secretion. Collectively, it appears that hCG influences VEGF protein synthesis in human mid-luteal phase endometrial cells, but has little effect on post-translational regulation and secretion. From physiological homeostasis point of view, it is likely that synthesis and secretion of VEGF exhibits a modular and factorial regulation to achieve a fine tuning of this potent vasotropic agent in receptive stage endometrium.

Key words : endometrium, epithelial cells, hCG, stromal cells, VEGF

INTRODUCTION

Blastocyst implantation in the human is a finely regulated process. Luteal phase progesterone provides the hormonal drive for inducing endometrial secretory maturation

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that is essential for blastocyst implantation (1–3). The progesterone driven endometrial secretory response presumably acts on the preimplantation stage embryo, which subsequently undergoes functional differentiation and secretes substances to further act on the progesterone-dominated endometrium (4). Such putative mutual interaction between maternal endometrium and implantation stage embryo thus appears significant towards endometrial receptivity (5, 6). Of various embryo-derived factors, chorionic gonadotropin (CG) is one of the earliest identified embryo-derived factor (7, 8) that may act on receptive stage endometrium and influence morphological and biochemical parameters concerning implantation (9–12). Intrauterine infusion of hCG in women during their luteal phase of menstrual cycle resulted in distinctive modulation within the uterine luminal fluid of several cytokines including vascular endothelial growth factor (VEGF) (10). The major isoform VEGF-A is a dimeric glycoprotein that influences vascular permeability and endothelial cell proliferation (13, 14). VEGF-A is expressed in a stage specific manner in human endometrium throughout the menstrual cycle (15, 16) with a higher expression of VEGF mRNA during the secretory phase (17). It is also expressed in implantation stage endometrium (18) and exhibits a vectorial secretion towards luminal cavity with a putative embryotropic function (10, 19). Systemic administration of VEGF monoclonal antibody during the peri-implantation period led to failure of pregnancy in rhesus monkeys (20).

Taken together, it appears that chorionic gonadotropin (CG) secreted by preimplantation stage embryo may influence VEGF synthesis and secretion by synchronous maternal endometrium, and that endometrial VEGF in turn may support embryo implantation. However, we have very little knowledge about the underlying paracrinology of CG induced VEGF synthesis and secretion by endometrial cells in the human. The aim of the present study therefore was to examine the potential role of human chorionic gonadotropin (hCG) in modulating expression and secretion of VEGF-A in mid-secretory endometrial cells using an *in vitro* three-dimensional cell culture model.

MATERIALS AND METHODS

Samples

Human mid-secretory stage endometrial samples were obtained from healthy fertile women patients (n=14; age: 20–38 years) admitted to the Department of Obstetrics and Gynecology of the All India Institute of Medical Sciences for undergoing treatment due to benign uterine fibroid and vaginal prolapse. Human endometrial samples from operation theatre were collected on ice in sterile DMEM-F12 (1:1) medium containing 5% (v/v) FCS, gentamicin (10 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2 μg/ml) solutions and immediately transported on ice to the cell culture laboratory. The patients who had a past history of immune diseases, malignancy of uterus, and any chronic diseases or who were on oral contraceptive pills were excluded from the study. Ethical approval for the study was obtained from the Ethics Committee, All India Institute of Medical Sciences and written consent was taken from individual patients after explaining the nature of the study.

Processing of tissue samples

All samples were divided into two parts: one part was fixed in phosphate-buffered neutral formaldehyde (4% w/v), pH 7.4, while the second part was processed for endometrial cell isolation and culture. Fixed tissue samples were washed with phosphate buffered saline (PBS) and then dehydrated through graded series of ethanol. After quick washing with pre-chilled acetone, tissue was put in cedar wood oil till clearing. After processing through graded xylene and paraffin, tissue samples were embedded in paraffin wax. Blocks were cut into 5 µm thick sections. Sections were de-waxed in xylene, rehydrated in graded concentrations of ethanol and finally distilled water. Hematoxylin staining was done for endometrial dating using Noyes' criteria. The major part of the tissue sample was further processed for endometrial cell isolation and culture on rat-tail collagen type I as described previously (21). Two samples that failed to show features typical of mid-secretory phase endometrium were discarded for this study.

Cell isolation and culture

The details of endometrial cell isolation and culture on rat-tail collagen I matrix have been given elsewhere (21–23). Briefly, tissue sample was washed three times in sterile, cold Ca^{2+}–Mg^{2+} free Hank's balanced salt solution, minced on ice and incubated at 37°C with 0.2% (w/v) collagenase in Hank's balanced salt solution (HBSS) supplemented with 2% (v/v) fetal calf serum, 15 mM HEPES for 30 min in shaking water bath. The resulted cell suspension was then washed twice with HBSS at 300x g for 10 min and finally resuspended in 1 ml of HBSS. This was then placed on 40% percoll gradient and centrifuged at 600x g for 20 min, cells at the interface collected and washed with HBSS. Final washings were done in DMEM-F12 (1:1) medium and the pellet was resuspended in 1 ml of medium to determine cell number and cell viability by the trypan blue method. Cell viability was found to be 90±3%. The isolated cells from individual subjects (n=12) were cultured at 37°C in humidified chamber with 5% CO_2 in air at plating density of $3–5 \times 10^5$ cells per square centimeter in rat-tail collagen type I gel matrix (1.2 mg/ml) as described elsewhere (24). Medium was changed after 24 h of plating. After 48 h of initiation of culture the cells were subjected to serum-free, DMEM-F12 (1:1) medium supplemented with insulin (10 µg/ml), transferrin (5.5 µg/ml), selenium (6.7 ng/ml) and hydrocortisone (5 µg/ml)). After 3 h of serum free medium cells were divided into five groups in triplicates containing different doses of hCG (0, 1, 10, 100, 1000 IU/ml) as shown in Table 1. Spent media were collected after 24 h. The medium was centrifuged at 10,000x g for 5 min and the supernatant was collected and stored at -20°C for subsequent immunoassay of VEGF and Western blot analysis. The endometrial cells grown in culture were further processed for immunocytochemistry as described below. Supplies were obtained from Sigma Chemical Co. (St. Louis, MI, USA).

Viability assay

In order to ascertain whether addition of hCG influenced cell viability under the culture conditions, MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide) based viability assay was performed as described elsewhere (25, 26). $2\text{-}3 \times 10^5/cm^2$

cells were cultured in triplicate in 96-well flat bottom tissue culture plates coated with 100 ml rat-tail collagen (1.2 mg/ml) matrix. After 48 h of cell seeding as describe above, the cells were incubated with either 1000 IU/ml of hCG or control medium without hCG for 24 h. At the end of culture, MTT based viability assay was performed as described elsewhere (25, 26). Briefly, 5 mg/ml MTT dissolved in pre-warmed PBS and filtered by 0.2 µm filter was added to culture well and incubated for 2 h, and the completion of reaction was followed under the microscope. At the end of the incubation period the medium was removed, the cells were lysed and converted dye solubilized with acidic isopropanol. All fine chemicals were obtained from Sigma Chemical Co. Absorbance of the product was measured at a wavelength of 570 nm with background subtraction at 630 nm on microplate reader (Model 550, Microplate reader, Bio-Rad laboratories Inc, Hercules, CA, USA). All fine chemicals were obtained from Sigma Chemical Co.

Binding of biotinylated hCG to cultured endometrial cells

Biotin labeling of intact rhbCG (Sigma Chemical Co.) with or without heat inactivation was carried out using a commercially available Biotin labelling kit from Roche (Penzberg, Germany) and using a procedure provided by the manufacturer. Free biotin was separated using G25 Sephadex column obtained from GE Healthcare (Tiong Bahru, Singapore) and the biotinylated product was eluted using PBS. Dissociated endometrial cells from individual patients (n=12) were grown on collagen coated cover slips for 24 h as described above and following washings in serum free medium, naïve or heat inactivated biotinylated rhbCG (1000 IU) was added. The culture was terminated at different time points as shown in Table 2, and fixed with 4% (w/v) paraformaldehyde after washing with PBS. Intracellular peroxidase activity was quenched and cell bound biotinylated hCG was detected using Vectastain kit obtained from Vector Laboratories (Burlingame, CA, USA) and visualized using 3,3'-diaminobenzidine hydrochloride and freshly added H_2O_2. The slides were viewed under light microscope (Leitz DIALUX 20) and photographic images were grabbed using a photographic system (Leica WILD MPS 28). In another set of study with parallel cultures of endometrial cells were treated with naïve or heat inactivated biotinylated rhbCG as above for different time points as given in Table II, and hCG binding was detected using fluorescence labeled antibody against biotin with DAPI nuclear stain. Fluorescence labeled antibodies and DAPI were obtained from Molecular Probes (Eugene, OR, USA). The stained coverslips were mounted with antifade mounting medium and examined under a Confocal Laser Scanning Microscope (DMIRE2, Leica Microsystems, Wetzlar GmbH, Germany). For each of the time point, numbers of positive cells per 200 cells in triplicate were counted.

Immunoassay of VEGF

ELISA based immunoassay for VEGF-A was carried out using a commercially available kit (Duo set ELISA development system) from R&D Systems (Minneapolis, MN, USA) following a procedure protocol described elsewhere (20). Briefly, 96 well microplate was coated with goat antihuman VEGF (dilution 1:700) capturing antibody and was incubated with samples/standards at room temperature. Following washing the

wells were incubated with biotinylated goat antihuman VEGF antibody (1:180), and then with streptavidin – horseradish peroxidase at room temperature. Final washing was followed by incubation with substrate solution containing H_2O_2 and 3,3',5,5'-tetramethylbenzidine and observed for colour development. Reaction was stopped using stop buffer (2N H_2SO_4). The optical density of each well was determined using ELISA microplate reader (Microplate manager 4) at 450 nm. A seven point standard curve using 2-fold serial dilution of a standard of 4000 pg/ml as supplied by the manufacturer was performed and the concentrations of unknown samples were determined.

Western immunoblot

The endometrial secretory proteins in spent media were subjected to separation by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot as described earlier (27, 28). The antibody against rhVEHF-A (R&D Systems, Minneapolis, MN, USA) was raised in goat and was used at a dilution of 1:500. 15 µg of the endometrial secretory protein from each sample was separated on 12% SDS-PAGE and transferred on to nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA). After washing the membrane in Tris buffer saline (TBS) it was incubated in 1% (w/v) bovine serum albumin in TBS for 1 h in room temperature to block non specific binding of the antibody. Following incubation with the primary antibody for 1 h at room temperature, membrane was incubated with biotinylated-secondary antibody and the bands were visualized using Vectastain ABC immunoperoxidase kit (Vector Laboratory, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) as per manufacturers instruction. The molecular weights of bands were determined using the calibrated molecular weight standards obtained from GE Healthcare (Tiong Bahru, Singapore).

Immunocytochemical staining of cultured cells

After termination of culture endometrial cells were washed thrice with PBS and were fixed in freshly prepared 4% (w/v) paraformaldehyde, pH 7.4 for 15 min at room temperature. Permeabilization of cells was done with 0.1% (v/v) Triton X-100 for 10 min and nonspecific immunoglobulin binding sites were blocked by pre-incubation with blocking serum obtained from Vector Laboratories (Burlingame, CA, USA). This was followed by the incubation with anti-human VEGF-A (dilution 1:20) antibody (R&D Systems, Minneapolis, MN, USA) at 4^0C overnight in a humidified chamber. After washing the excess primary antibody, cells were incubated with anti-goat IgG antibody conjugated with Alexa Fluor 633 at room temperature in a humidified chamber for 1 h. The excess of secondary antibody was washed and cells were counterstained with the nuclear dye, diamidino-6-phenylindole (DAPI). Appropriate primary antibody and secondary antibody controls were run simultaneously. Co-localization for immunoreactive cytokeratin as epithelial cell marker or vimentin as fibroblast marker along with VEGF immunostaining was performed following a procedure described elsewhere (29). Antibodies labeled with fluorescence dyes (Alexa Fluor 488, Alexa Fluor 633, and Texas red) and DAPI were obtained from Molecular Probes (Eugene, OR, USA). The stained coverslips were mounted

with antifade mounting medium (Molecular Probes, Eugene, OR, USA) and examined under a Confocal Laser Scanning Microscope (DMIRE2, Leica Microsystems, Wetzlar GmbH, Germany). Numbers of immunopositive cells per 200 cells in triplicate were counted.

Statistics

Kruskal-Wallis test and Wilcoxon rank test were performed depending on the requirement of one-way and between groups analysis, respectively (30). Statistical analysis was done using SPSS software (Chicago, IL, USA).

RESULTS

As shown in Figures 1A and 1B, mid-luteal phase endometrial cells in three-dimensional primary mixed cell culture expressed immunoreactive VEGF-A, and application of hCG resulted in an increase in the number of cells showing VEGF immunoprecipitation compared with control treatment, significant (P<0.01) changes being observed following application of 100 IU (P<0.05) and 1000 IU (P<0.01) hCG per ml medium (Table I). VEGF was also detected in gland-like structures that were occasionally observed in the present study (Fig. 1C). Co-localization with either cytokeratin or vimentin revealed that VEGF protein was expressed in both cytokeratin positive epithelial and vimentin positive stromal cells (Fig. 1F-M). There was however no significant difference in concentrations of immunoreactive VEGF in spent medium from cells subjected to hCG treatment ranging from 0 to 1000 IU/ml (Table I). Immunoreactive secretory VEGF–A was detected in three bands, 46 K, 30 K and 14.3 K in spent media with marginally (P<0.05) higher optical density for 14.3 K band following hCG treatment (Fig. 2). The possibility that the observed increase in cellular expression of VEGF was not associated with any drift in cell viability was ruled out as treatment with 1000 IU/ml hCG did not result in any significant change in cell viability ($95 \pm 12\%$) compared with control (assuming 100%) treated cells. Also, a small population of cells showed nuclear localization of VEGF protein (Fig. 1D). As shown in Table II, the cultured endometrial

TABLE I : VEGF protein expression and secretion by endometrial cells following hCG treatment.

Group (n)	*Treatment (hCG, IU/ml)*	*Median value (ranges)*	
		Spent medium (pg/ml)	*Intracellular expression (% cells with immuno-precipitation)**
1 (12)	0	380 (135-2005)	11 (0-17)
2 (12)	1	478 (241-1490)	17 (0-22)
3 (12)	10	422 (164-2080)	25 (2-29)
4 (12)	100	386 (105-1580)	37 (9-43)[a]
5 (12)	1000	481 (127-1610)	56 (11-81)[b]

*P<0.01 in Kruskal-Wallis test. [a]P<0.05, [b]P<0.01 compared with groups 1-3.

TABLE II : Time course of biotinylated rhβCG binding to endometrial cells *in vitro*.

Time	*Number of cells in per cent Median value (range)*	
	*IHC**	*IF**
1 min	N D	N D
10 min	8 (5-12)	11 (8-14)
30 min	10 (8-12)	17 (14-20)
60 min	16 (10-19)	28 (19-38)
6 h	39 (18-52)[a]	51 (35-55)[a]
16 h	77 (71-87)[b,c]	82 (70-92)[b,c]

*P<0.01 in Kruskal-Wallis test. [a]P<0.05, [b]P<0.01 compared with 10 min, 30 min and 60 min. [c]P<0.01 compared with 6 h.

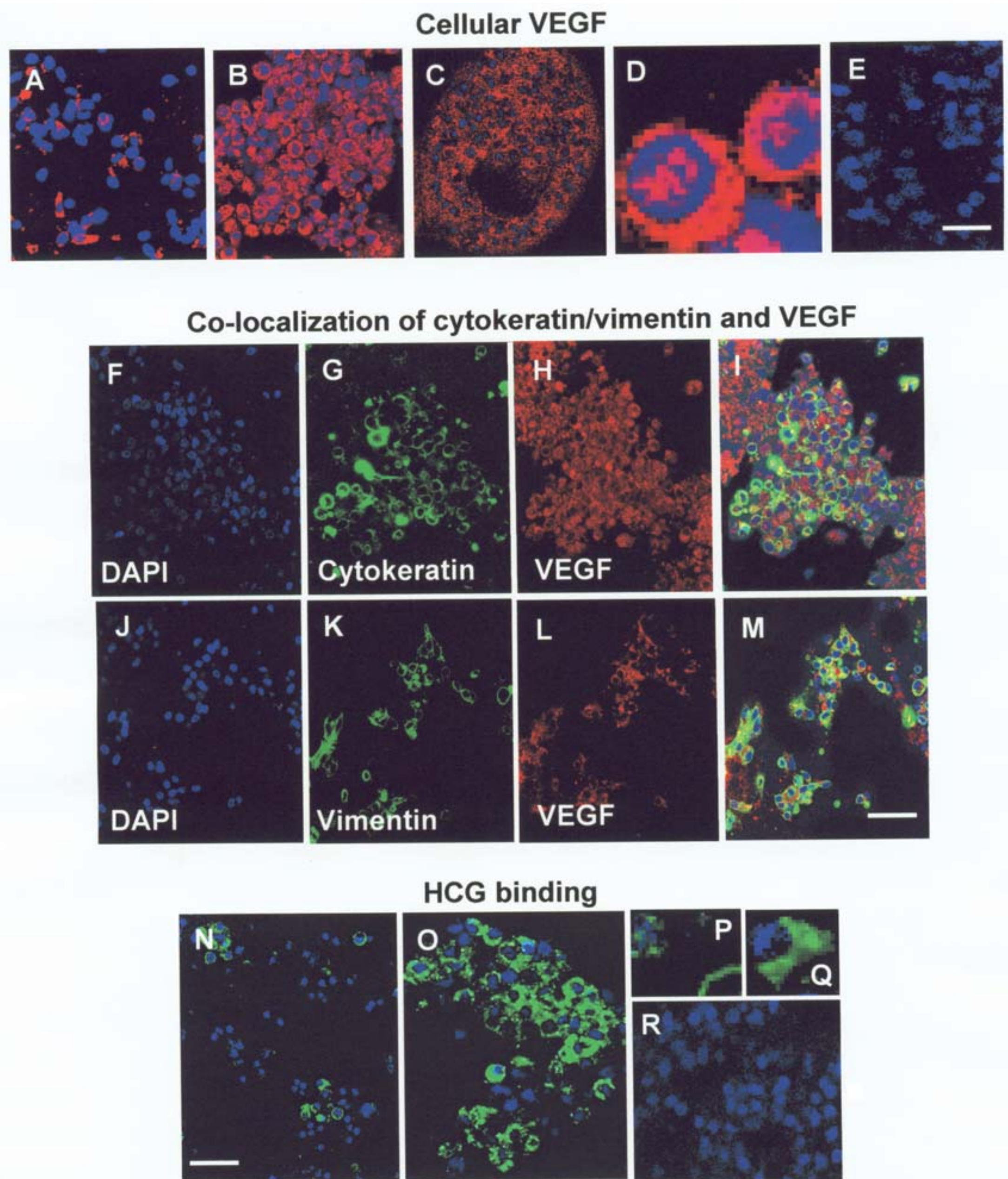

Fig. 1 : Confocal laser scanning fluorescent images of human endometrial cells showing immunocytochemical localization of VEGF-A protein detected with anti-VEGF polyclonal antibody (A-D, Alexa Fluor 633) and binding of biotinylated hCG (N-R, Alexa Fluor 488). Cells in parallel cultures were exposed to medium without hCG (A) or with 1000 IU/ml hCG (B). Immunoreactive VEGF is detected in cells of gland-like structure (C) and cell nucleus (D). E, control immunohistochemistry of cultured endometrial cells following incubation in medium lacking primary antibody for VEGF. Both epithelial (F-I) and stromal (J-M) cells cultured on collagen matrix and counterstained with nuclear dye DAPI (F, J) showing immunopositivity for specific markers cytokeratin (G) and vimentin (K), respectively and for VEGF (H, L) as well. Overlaid images (I, M) depict colocalization of these proteins. Endometrial cells show binding with biotinylated rhbCG (N-R) after 10 min (N) and 16 h (O) along with detectable events of ligand patching, capping and internalization (P, Q). Heat inactivated rhbCG fails to bind to the cells (R). Bars = 15 μm (D, P and Q), 40 μm (A-M and N, O, R).

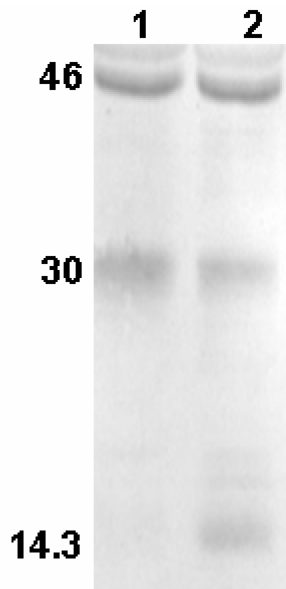

Fig. 2 : Western immunoblot profiles for VEGF-A in cell culture supernatant conditioned by endometrial cells treated with medium containing no hCG (lane 1) and 1000 IU/ml (lane 2). Three distinct bands corresponding to 46 kDa, 30 kDa and 14.3 kDa are seen.

cells showed detectable binding of biotinylated hCG as early as 10 min (Fig. 1N) and it increased (P<0.01) progressively with time resulting in about 80% cells binding rhbCG after 16 h (Fig. 1O). While such binding was not seen in binding experiment with heat-inactivated rhbCG (Fig. 1R), binding of pure hCG molecule showed typical patching, capping and internalization events in the mid-luteal phase endometrial cells in culture (Fig. 1P,Q).

DISCUSSION

There has been substantial evidence to support the idea that uterine endometrial cells of various mammalian origin including human can bind human chorionic gonadotropin (hCG) (31–34). It has been suggested that hCG may influence the physiology of implantation stage endometrium (6, 35). For example, intrauterine infusion of hCG can enhance VEGF secretion during luteal phase (10). Furthermore, a large number (~60) of endometrial genes have been shown to be differentially expressed in implantation stage endometrium of the baboon in presence of hCG (35). Collectively, it appears that CG may then influence both endometrial vasotropic and vectorial embryotopic functions towards implantation (6, 35). It has also been reported that VEGF expression in human endometrial stromal cells was influenced by hCG in primary cell culture (36). Yet, our knowledge about hCG action on VEGF in endometrial cells is very thin, and physiological significance of above-mentioned observations in reference to blastocyst implantation is unclear for several reasons. Typically, physiology of endometrial stromal cells in absence of epithelial cells is discernibly different in implantation stage endometrium (37, 38). Furthermore, fibroblasts cultured on plastic surface tend to loose physiological polarity and differentiation (39). Especially, endometrial stromal cells in such condition tend to become epitheloid in morphology (40, 41). Results from the previous study (36) wherein human endometrial samples were collected from all phases of menstrual cycle and data related to VEGF synthesis and secretion in response to hCG were pooled cannot give any meaningful idea about the behaviour of implantation stage endometrium in response to hCG. Also, there is an uncertainity about the relevance of the amount of hCG used in previous studies, vis-à-vis, embryo derived hCG in the process of implantation (35, 42). Taken together, we proposed to examine the effect of different concentrations (0, 1, 10,

100 and 1000 IU per ml medium) of hCG on VEGF expression and secretion by endometrial cells retrieved from mid-luteal phase and maintained in three-dimensional primary co-culture on rat-tail collagen type I biomatrix. We also microscopically examined hCG binding to endometrial cells in the above system.

In the present study, we observed that both epithelial cells and stromal fibroblasts from mid-luteal phase endometrium could bind hCG and number of cells binding hCG increased steadily with time. While the fact that human endometrial cells can bind hCG, as discussed above, is not novel, we observed that hCG enhanced VEGF immunoreactive protein expression in both epithelial and stromal cells retrieved from mid-luteal phase and co-cultured in a three-dimensional cell culture system. In a previous study (36), human endometrial stromal cells recovered irrespective of any specific phase of cycle and cultured on poly-lysine coated plastic surface showed biphasic effect on VEGF expression in response to hCG: number of cells showing cellular expression of VEGF increased following treatment with a lower dose (2,500 ng/ml) of hCG, and no change with higher dose (20,000 ng/ml) of hCG compared with control treatment. On the other hand, VEGF secretion at both doses of hCG did not show any change compared with control treatment (36). In the present study, however, number of mid-luteal phase endometrial cells showing immunoreactive VEGF protein increased in a dose dependent manner with no marked change in VEGF secretion with different concentrations of hCG in the three-dimensional primary co-culture system. It appears that hCG indeed enhances VEGF synthesis in mid-luteal phase endometrial cells, final secretion of VEGF from mid-luteal phase endometrial cells is possibly dependent upon other factors. It is interesting to note that 121 amino acid isoform of VEGF-A (14.3 K) was marginally higher in secretion following hCG treatment compared with control despite no marked change in overall secretion. It is generally known that VEGF-A_{121} lacks products of exons 6 and 7 and therefore is readily secreted, while it possesses product of exon 8 that is involved in angiogenesis (43). The physiological significance of this observation in reference to the implantation stage endometrium is only speculative. Collectively, it appears that hCG may influence VEGF protein synthesis in human mid-luteal phase endometrium, but has little effect on its secretion. Since VEGF is an extremely potent vasotropic agent in endometrium, it requires fine regulation for physiological homeostasis around the time of blastocyst implantation (44). In this connection, the observation that mid-luteal phase endometrial cells are VEGF-ready under the influence of a potential implantation-stage embryo-derived factor like hCG, however, with no overall increase in VEGF secretion appears intriguing.

Interestingly, we observed nuclear localization of VEGF protein in a subset of endometrial cells in the present experimental model. The phenomenon of nuclear localization of VEGF has previously been observed in a variety of experimental situations by other investigators, however, its biological implication is primarily speculative in nature at present (45, 46).

Our observation that VEGF synthesis in endometrial cells increased at a critical

concentration of hCG is intriguing. It is now well known that stage-age synchronized viable embryos secrete higher hCG compared with desynchronized and 'compromised' embryos (47). It is then possible that mid-luteal phase endometrial cells possess a critical threshold for hCG responsive elements that gives a default discrimination signal to the tissue about the state and stage of embryo growth. However, further studies are needed to explore this concept and to delineate relative tasks of mid-luteal phase endometrial epithelial and stromal cells in this process.

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