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IMMUNOHISTOLOGICAL LOCALISATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN HUMAN ENDOMETRIUM

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Abstract : Several polypeptide growth factors regulate epithelial and stromal development in endometrium under the influence of estrogen and progesterone, and thereby regulate growth and differentiation of endometrium during menstrual cycle. However, little is known about the angiogenic growth factors that may affect endometrial vasculature throughout each menstrual cycle. Vascular endothelial growth factor (VEGF) is suggestively an important angiogenic growth factor in the female reproductive tract. The aim of the present study was to immunolocalize and assess semi-quantitatively VEGF immunostaining in cells of proliferative phase (n=3), secretory phase (n=6) and hyperplastic (n=6) human endometrial samples. VEGF concentrations were significantly higher in glandular ($P<0.001$) and stromal ($P<0.01$) compartments of proliferative stage endometrium compared with those in secretory stage and hyperplastic endometrial samples, with no difference in the scores for glandular and stromal compartments between secretory stage and hyperplastic endometrial samples. Generally, glandular expression of VEGF was higher as compared to stromal compartment. Thus, it appears that endometrial VEGF expression and concentration are enhanced by estrogen, and may be correlated with neovascularization and increased vascular permeability during late proliferative period. Additionally, there was no enhancement in VEGF expression in hyperplastic glands, suggesting that regulation of glandular growth and that of angiogenesis in human endometrium operate through different mechanisms.

Key words : angiogenesis endometrium hyperplastic proliferative secretory VEGF

INTRODUCTION

Human endometrium undergoes cyclic changes during menstrual cycle. Ovarian steroid hormones play a critical role in the physiological processes of endometrial growth and differentiation as well as vascular remodelling (1). There is evidence to suggest that several cytokines under the regulation of steroid hormones play an

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integral role in the interaction between cells in endometrium (2). Cytokines have also been shown to be specifically involved in pathophysiology in various tissue systems (3). Vascular endothelial growth factor (VEGF) is one such cytokine. This dimeric growth factor is a specific mitogen for endothelial cells and thereby it influences angiogenesis (4). VEGF has been implicated in angiogenesis during tissue repair and in the metastasis of tumour cells (4). VEGF is secreted by endometrial cells and resident macrophages in primate endometrium and has been found to be elevated in conditions like endometriosis and endometrial carcinoma (5). In the present study, our aim was to immunolocalize and assess semi-quantitatively VEGF in cells of proliferative phase, secretory phase and hyperplastic human endometrial samples.

METHODS

Subjects

Endometrial tissue samples were obtained from 15 patients undergoing total hysterectomy for myoma uteri and some for conditions confined to the cervix alone.Their ages varied from 33 to 50 years. In each case a detailed menstrual history was available and only patients with no previous history of intrauterine device insertion were chosen for this study. Table I provides the details of the experimental samples used in the present study.

Processing of tissue samples

Hysterectomy specimens were obtained immediately after their removal in the operating room and were opened along the lateral border in order to obtain undistorted endometrial surfaces.

Tissue sections were taken longitudinally from endometrial mucosa. Tissue samples were then processed for chemical fixation in phosphate buffered neutral formaldehyde followed by paraffin embedding as described elsewhere (6). Paraffin embedded tissue samples were used for histology and immunohistochemistry (IHC).

Histology

Prior to IHC, tissue sections were stained in hematoxylin-eosin following routine procedure. The stained sections were examined in a Leica DMRD light microscope and were classified as normal or hyperplastic endometria. Histologic staging of normal endometria was performed according to the standard criteria of Noyes et al. (7). Hyperplastic endometria were classified based on architectural alterations of endometrial glands ranging from minimal to complex; glandular lining epithelia were devoid of cytological atypia. Occasionally, glands exhibited architectural alterations with complex intra- and extra-luminal epithelial buddings.

Immunohistochemistry

The procedural details of tissue processing and immunohistochemistry have been described earlier (6, 8). Briefly, tissue samples were fixed in phosphate-buffered neutral formaldehyde (4%) and embedded in paraffin wax. Tissue sections (5 µm) were deparaffinized and hydrated through graded ethanol to phosphate-buffered saline (PBS).

Endogenous peroxidase activity was then quenched with 0.3% hydrogen peroxide in methanol. Sections were incubated overnight at 4°C with the primary antibody. Primary antibody for VEGF was raised in goats and was obtained from R&D Systems (Minneapolis, MN, USA). Sensitivity (1:50, v/v) for VEGF immunostaining was precalibrated by diluting the stock (1 mg/ml) and performing 3–5 points titration and based on the information provided by the manufacturer. Final visualization was achieved using the ABC kit (Vector Laboratories, Burlinghame, CA, USA) and freshly made diaminobenzidine hydrochloride (Sigma Chemical Co., USA) with hydrogen peroxide as described previously (6, 8). Specificity of antibody liganding and visualization was assessed by omitting primary antibody, replacing primary antibody with unrelated immunoglobulins from same species, omitting secondary antibody and replacing labelled secondary antibody with unrelated immunoglobulins from same species and other species. The reagents were purchased from Vector Laboratories. All immunostaining procedures were performed in a single run. Duplicate sections were lightly counterstained with haematoxylin to facilitate the identification of cellular elements. The immunohistochemically stained sections were analysed microscopically to estimate morphometrically the areas of immunoprecipitation in glandular and stromal compartments in functionalis zones using a Leica microscope and a precalibrated computer assisted video image analysis system (QWIN-Quantimet 500C+, Leica, Cambridge, U.K.). The details of histometric measurements are given elsewhere (9). Briefly, glandular and stromal compartments at 25x were detected using an interactive planimeter analyzer only in cases where discernibility of these structures was distinct and immunopositive areas were measured in a particular compartment (segment) by detecting positive profiles in digitized images based on an optimized grey level threshold after shading correction and pixel calibration against standard provided by the manufacturer.

Statistical analysis

Statistical analysis of quantitative measurements was performed using the Kruskal-Wallis test followed by multiple comparision test (10). The probability level of $P = 0.05$ was taken as the limit of significance. The data are shown as means ±SEM.

RESULTS

Table I shows the histological staging of each endometrial sample. In three cases (cases #24, #26, and #33), late proliferative stage specific characteristics were observed. In six cases (cases #3, #7, #8, #13, #15, and #20), mid- to late secretory characteristcs were observed. Hyperplastic endometrial samples (cases #2, #4, #5, #18, #31, and #32) were also collected during mid- to late luteal period. In two cases, early adenomatous changes were observed, while in three cases adenomatous glands with hyperplastic epithelium outpouching and bridging were observed, and in one case mixed and diffuse hyperplasia was seen. Table II shows the morphometric analysis of immunopositive VEGF in glandular and stromal compartments of proliferative stage, secretory stage and hyperplastic human endometrium. As shown in Table II, VEGF

TABLE I : Details of patients and stages of endometrial samples.

Case number	*Age (yr)*	*Cycle day*	*Endometrial staging*
2	49	23	Hyperplastic
3	35	25	Secretory
4	42	15	Hyperplastic
5	50	24	Hyperplastic
7	44	18	Secretory
8	50	20	Secretory
13	38	22	Secretory
15	38	27	Secretory
18	44	20	Hyperplastic
20	33	24	Secretory
24	40	15	Proliferative
26	50	16	Proliferative
31	40	22	Hyperplastic
32	37	20	Hyperplastic
33	41	14	Proliferative

TABLE II : Morphometric analysis of immunopositive VEGF in human endometrium.

Group (Endometrial stage) (n)	*Area of immunopositive precipitation (per cent) (Mean ± SEM)*		*Statistical significance**
	Gland	*Stroma*	
Proliferative (3)	86.7±5.4	17.4±3.4	P < 0.001
Secretory (6)	19.3±2.7	3.3±0.7	P < 0.01
Hyperplastic (6)	21.2±2.8	3.4±0.7	P < 0.01
Statistical significance**	0.001	0.01	

*Gland vs. stroma. **Proliferative vs. secretory or hyperplastic.

concentrations were significantly higher in glandular (P<0.001) and stromal (P<0.01) compartments of proliferative stage endometrium compared with those in secretory stage and hyperplastic endometrial samples. However, there was no difference in the scores of VEGF in glandular and stromal compartments between secretory stage and hyperplastic endometial samples. In all groups, the glandular expression of VEGF was higher as compared to stromal compartment (Table II).

DISCUSSION

Ovarian steroids regulate growth and differentiation of the endometrium during menstrual cycle. It is now well substantiated that autocrine and paracrine functions of several polypeptide growth factors mediate the effects of estrogen and progesterone, especially in promoting epithelial and stromal development in endometrium. However, little is known about the angiogenic growth factors that may affect endometrial vasculature throughout each menstrual cycle. Fibroblast growth factors (acidic and basic) stimulate vascular endothelial cell growth and are present in human endometrium (11, 12). Still, they are unlikely to be principal regulators of cyclic changes in endometrial vasculature, because their expression does not change throughout the menstrual cycle, and actually increases in atrophic menopausal endometrium (13, 14). On the other hand, it has been suggested that vascular endothelial growth factor (VEGF) may be an important angiogenic growth factor in the female reproductive tract (15). VEGF is a heparin-binding dimeric glycoprotein with growth-promoting activity specifically for endothelial cells; it also increases vascular permeability. Five molecular species of VEGF have hitherto been identified (16).

The present study documents that levels of VEGF undergo dramatic changes during the menstrual cycle in the human, very high in late proliferative to early secretory

phases, while it was low during mid to late secretory period. However, endometrial production of VEGF has no clear-cut association with hyperplastic changes in the glandular compartment. Shweiki et al. (17) first described cyclic variation of VEGF mRNA in rodent endometrium. Based on several studies, it has been suggested that endometrial VEGF expression and concentration are enhanced by estrogen (18, 19). It is thus possible that neovascularization which is initiated in proliferative endometrium under estrogen dominance (20) is mediated by VEGF. Additionally, VEGF may also result in increased vascular permeability during proliferative period (7, 20). Whether different splice species of VEGF are differentially involved in different vascular responses in endometrium in a stage-specific manner during the menstrual cycle cannot be commented from the present study, because the antibody used in the present study detects all species of VEGF. Further mRNA studies are required to examine this possibility. It is interesting to note that consensus sequences for estrogen and progesterone response element are not found in the 3.4 kb 5'-promotor region of the human VEGF gene, however, several half-palindromes are present (21, 22). However, it is possible that estrogen may mediate an action on endometrial VEGF expression in a paracrine manner by stimulating other growth factors and cytokines (16). Studies using techniques of cell biology and molecular biology can be employed to decipher the mechanism of regulation of VEGF expression and production by ovarian hormones.

Although Charnock-Jones et al. (23) observed that VEGF mRNAs were mainly expressed by stromal cells during proliferative phase, and by glandular cells during secretory period in human endometrial samples, in the present study endometrial glands were found to discretely express VEGF in both proliferative and secretory phases of cycle, and its level was higher in glands compared with stromal compartment. Our observation corroborates well with the reports made by others for monkey and human endometrium (18, 19). Interestingly, there was no enhancement in VEGF expression in hyperplastic glands, suggesting that regulation of glandular growth and that of vascular growth and permeability in human endometrium operate through different mechanisms.

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