

Original Article

Increased expression of nestin and VEGF in endometrial polyps: an immunohistochemical study

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Abstract: Background: Pathogenesis of endometrial polyps have not been fully understood however the increased proliferation of blood vessels and fibrosis may play an important role in the pathogenesis of endometrial polyps. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and vascular function. Nestin is another molecule which has been reported to be associated with the process of angiogenesis. The aim of this study was to evaluate the expression of Nestin protein and VEGF in pathogenesis of endometrial polyps. Methods: A total of 20 women who had hysteroscopic and histologic confirmation of benign endometrial polyps and their normal endometrial tissue were recruited into the study. Immunohistochemical analysis for VEGF, Nestin and CD34 were performed on formalin fixed, paraffin-embedded tissue using the streptavidin-biotin-peroxidase technique. Angiogenesis was assessed by immunohistochemistry using monoclonal antibody against CD34. Results: Immunostaining of VEGF in glandular epithelial cells and stromal fibroblasts, and immunostaining of Nestin in newly formed vessels were determined. Immunopositivity staining of VEGF in endometrial polyps were significantly stronger than normal endometrium ($P<0.05$). Nestin expression was observed in vessels which were smaller than that of CD34-positive preexisting large blood vessels. Endometrial polyps demonstrated significantly greater expression of Nestin in proliferating endothelial cells, compared with control endometrium ($P<0.05$). Conclusion: This study suggests that the increased expression of VEGF and Nestin protein which are may have a role in the pathogenesis of endometrial polyps.

Keywords: Endometrial polyps, pathogenesis, angiogenesis, VEGF, nestin

Introduction

Endometrial polyps are pedunculated or sessile lesions which are composed of functional or basal endometrium or a combination of the two which includes variable amounts of glands, stroma and blood vessels. The prevalence of endometrial polyps is 2 to 23% among women investigated abnormal uterine bleeding. Although malignant changes are uncommon, it has been reported that polyps occurring in postmenopausal patients have a higher risk of associated endometrial neoplasia (5% of cases) [1].

Pathologic process leading to polyp formation is under special interest, the exact mechanism is still not fully understood however recent evidence shows that angiogenesis appears to play a crucial role in the pathogenesis of endometrial polyps [2, 3]. The fact that the increased

proliferation of blood vessels is a component of endometrial polyps highlights the importance of angiogenesis in pathogenesis of this disease. Vascular endothelial growth factor (VEGF) is one of the most important drivers of vascular formation as it is required to initiate the formation of new blood vessels by angiogenic sprouting or vasculogenesis [4]. The clinical importance of VEGF has been reported in both physiological and pathological angiogenesis of human endometrium [5-7].

Nestin is a class VI intermediate filament protein which is produced by stem/progenitor cells in the mammalian central nervous system (CNS) during development and is replaced by vimentin and glial acidic fibrillary protein (GFAP) during neurocytogenesis [8]. In adult nonneoplastic brain tissue nestin is rarely detected, although it is sometimes found in endothelial cells [9]. Nestin has recently received attention

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as a marker for proliferative endothelial cells in tissues undergoing neovascularization. Several studies proposed nestin as a reliable marker for detecting endothelial cells of newly formed microvessels [10-12].

In the light of these knowledges, we aimed to evaluate the expression of angiogenic markers, VEGF and Nestin in endometrial polyps.

Material and methods

Sample collection

Patients who had been performed hysteroscopic polyp excision and histologic confirmation of benign endometrial polyps were recruited into the study. Archived paraffin-embedded endometrial polyps samples were obtained from the Department of Pathology of Baskent University Hospital. Study group included premenopausal ($n = 20$) endometrial polyps and their control endometrial tissue ($n = 20$). We obtained the endometrial polyp and control endometrial tissue from the same subject. The age range of patients was 39-48 with a mean age of 40 years. All patients had been in premenopausal period and had not received exogenous hormonal therapy for at least 2 months before the procedure. Samples with evidence of endometritis, endometrial hyperplasia, or other pathologies such as myoma uteri or anomalies of uterine septum were excluded from the study. The same pathologist examined all of the endometrial samples.

Tissue microarray

Nestin, VEGF and CD34 expressions were evaluated in paraffin blocks which were constructed using tissue microarrays technique. Tissue microarray is a method of harvesting small-core biopsies from a range of standard histological sections and placing them in an array on a recipient paraffin block. With this technique, large numbers of small punched-out tissue cores from different cases can be analyzed in a single slide and immunohistochemical staining experiment. In this study, formaline-fixed, paraffin-embedded tissue blocks were retrieved from the archives of the Baskent University, the department of Pathology, and areas of containing both endometrial glands and vessel walls were identified on corresponding hematoxylin-eosine stained slides. The tissue blocks

were cored and transferred to a "recipient" block. Two cores were taken for each case and each core was about 0.6 mm diameter. After construction, 3 μ m thickness sections were obtained from "recipient" new paraffin blocks and affixed to the poly-L-lysine covered glass slides.

Immunohistochemistry

Sections were incubated at 56°C for 24 hours and were deparaffinized in xylene and in graded ethanol and placed in 0.5% hydrogen peroxide in methanol for 5 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by incubation in 0.01 M citrate buffer (pH 6.0) for 15 minutes in a microwave oven. Slides were rinsed 3 times in de-ionized distilled water and endogenous peroxidase activity was blocked during 30 minutes of incubation with 0.3% hydrogen peroxide and slides were washed again in PBS for 2-3 minutes. The sections were exposed to the primary antibody for 60 minutes at room temperature (Nestin antibody, 1:100 dilution, Santa Cruz; VEGF antibody, 1:100 dilution, Bio SB; CD34 antibody, 1:100 dilution, BioGenex). After incubation with primary antibody, slides were washed with PBS for 5 minutes. Biotinylated Goat Anti-Polyvalent (Lab Vision Corp., Fremont, CA) was applied and again washed in PBS. Then streptavidin peroxidase (Lab Vision Corp.) was applied and slides were incubated for 15 minutes at room temperature. The immunoreaction was visualized with DAB (Thermoscientific Dako, Glostrup, Denmark) as a chromogen. The slides were counterstained with Mayer's hematoxylin solution and mounted. All stages were carried out at room temperature to avoid drying.

Results

Assessment of VEGF expression

The staining of VEGF was detected in cytoplasm of glandular epithelium and stromal cells of endometrium. The glandular cell and stromal cell were scored separately for each core. VEGF score was calculated by multiplying the percentage of positively stained cells by their staining intensity to generate a final immunohistochemical score. This method has been validated in previous study [13]. The intensity of staining was graded as (0: negative, 1: weak

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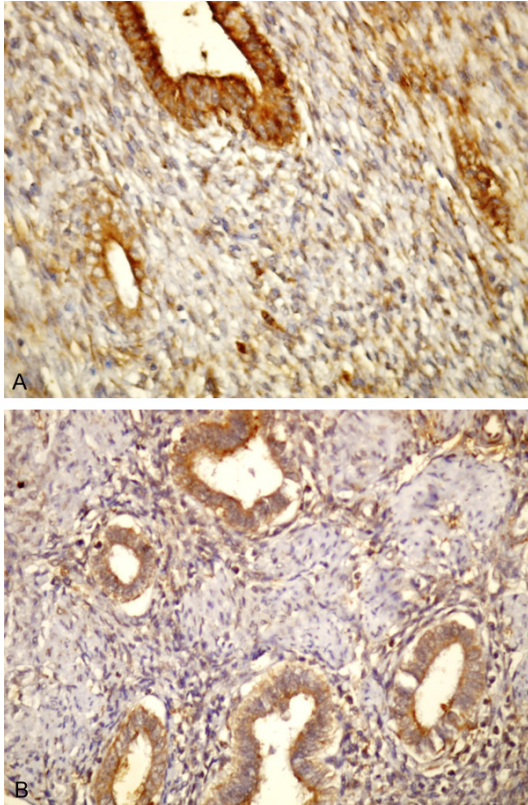


Figure 1. Immunohistochemistry of VEGF in endometrial polyp and normal endometrium. (A) shows strong glandular and stromal VEGF expression in the endometrial polyps and (B) shows weak VEGF expression in normal endometrium. Original magnification: (A and B), x400.

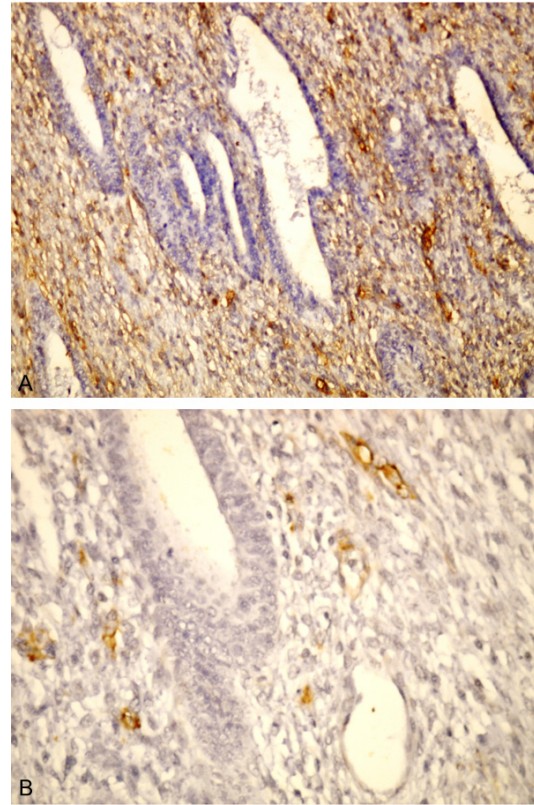


Figure 2. Immunohistochemistry of CD34 and nestin in endometrial polyps. CD34 and nestin immunoreactivities were detected in vascular endothelial cells. CD34 was detected in endothelial cells of large blood vessels, but not nestin (A and B, respectively). Original magnification: (A and B), x400.

staining, 2: moderate staining, and 3: strong staining) and the percentage of cells staining was graded as (0: negative, 1: positive staining in 25% of the cytoplasm of glandular epithelium or stromal cells, 2: positive staining in 26-50% of the cytoplasm of glandular epithelium or stromal cells, or 3: positive staining in 50% of the cytoplasm of glandular epithelium or stromal cells). The final scores between 0 and 2 were regarded as grade 1, scores of 3 and 4 as grade 2, and scores of 5 and 6 as grade 3. VEGF score was used in comparison of groups.

Assessment of vascularity and nestin expression

Nestin was densely positive at the endothelium of microcapillary vessels which we thought them newly formed vessels. In order to determine newly formed vessels, we performed CD34 immunostaining. CD34 expression has

been used as a marker which helps to compare the endothelial cells of newly formed blood vessels and preexisting one. Cytoplasmic staining was defined positive for CD34 immunostaining. Proliferating endothelial cells in areas of high vascularisation was determined using a $\times 40$ objective. The region of high vascular density within the polyps (vascular hot spot) was found according to previous report [14]. Nestin expression was evaluated semi-quantitatively using the following scale: -, negative; +, weak and moderate; ++, strong. To generate categorical variables for survival analysis, staining intensity was classified into 2 groups: "high" and "low".

Statistical analysis

A commercially available statistical package (SPSS15.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Chi-square test analysis of variance were applied, as appropri-

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ate, to compare continuous variables. Spearman rank of correlation was used to correlate VEGF, CD 34, and Nestin expressions. A value of P , 0.05 was considered statistically significant.

Results

VEGF staining, were significantly stronger in the endometrial polyps than in the control group which was the proliferative phase of those their normal endometrial tissue with statistical significance ($P < 0.05$). Endometrial polyps demonstrated predominately strong positive expression (3+) in the glandular epithelium and stroma for VEGF. However the expression of VEGF in the glandular epithelium of control endometrial tissue which is located surrounding endometrial polyps was mainly 1+ or negative, with stromal expression demonstrating a similar pattern. **Figure 1** shows immunohistochemical detection of VEGF in both endometrial polyp and normal endometrium with glandular and stromal staining.

Nestin expression was detected high intensity in proliferating endothelial cells of endometrial polyps. There were no staining of Nestin in the glandular epithelium or stroma. We found a significantly higher expression of nestin in endometrial polyps compare to normal endometrial tissue ($P > 0.05$) **Figure 2** shows the immunohistochemical staining of CD34 and Nestin in endometrial polyps (**Figure 2A, 2B**).

Discussion

The exact pathogenesis of the endometrial polyp formation is still under debate, several molecular mechanisms have been proposed the development of endometrial polyps. Endometrium is a dynamic tissue that undergoes regular cyclic proliferation and differentiation. Angiogenesis is associated with the physiological changes of endometrium [5]. It is mediated by the VEGF and its specific receptors. Previous studies have shown that VEGF have important role in several physiological functions of human endometrium, such as ovulation, periodical changes of the endometrium, embryo implantation and development [15]. Increased VEGF levels has also been reported in some pathological diseases of endometrium such as leiomyoma, endometriosis, endometrial cancer and endometrial polyps [16, 17]. In the present study we found that VEGF expression was high-

er in endometrial polyps compare to surrounding normal endometrium. Similar to us et al showed that VEGF immunostaining of glandular cells and stromal cells in the polyp was significant higher than the adjacent endometrium [11]. Hormonally dependent fluctuations in VEGF expression occur during the menstrual cycle. Knowledge on the variation of the VEGF expression in human endometrium during menstrual cycle is still limited and the results are controversial. Some studies revealed that VEGF expression in the glandular cells increases 3-5-fold from the early proliferative phase to late secretory phase [7, 18]. On the other hand, some studies reported that expression of VEGF significantly increased during the postmenstrual regeneration of the endometrium [19]. In this study, we recruited only patients with endometrial polyps who were premenopausal and in proliferative phases of their cycle and we obtained the endometrial polyp and control endometrial tissue from the same subject. We thought that our approach eliminates errors due to obtaining samples from the different stages of the menstrual cycle. In the current study, besides VEGF we used nestin antibody to determine angiogenesis in endometrial polyp. Nestin is an intermediate filament protein that is expressed in a variety of embryonic and fetal tissues. Recent studies showed that the expression of Nestin protein was found not only in immature neuroepithelial and glioma cells, but also in endothelial cells during active proliferation [10-12]. In the present study, we showed that Nestin expression was consistently present in all of endometrial polyps and it was interesting to note that the intensity of nestin expression was stronger particularly in the small capillaries than in the normal vessels, supporting the claim that nestin is a good marker for the newly developing endothelial cells. Indeed, nestin has been reported to be extensively expressed in developing blood vessels in a various of tumor tissues, before [20].

Authors thought that increased Nestin expression in a tumor might be a valuable predictor for tumor invasion and recurrence. To the best of our knowledge, our report is the first to deal with nestin in the evaluation of endometrial polyp angiogenesis.

On the basis of our findings, we thought that increased Nestin and VEGF expression in endometrial polyps might correlated endometrial polyps neogenesis through the proliferating the

vessel formation. The stimulus for angiogenesis in endometrial polyp formation is unknown, but authors have showed enzymes and cytokines such as cyclooxygenase (COX), matrix metalloproteinases (MMP), and interferon-gamma (IFN- γ) which may play role in cell proliferation and angiogenesis of endometrial polyp formation [21-23]. The fact that estrogen and progesterone act as modulators of endometrial proliferation and differentiation through their receptors, some investigators suggested that endometrial polyp is formed as a consequence of abnormal expression of estrogen and progesterone receptors [24, 25]. In addition, the expression of VEGF in the human endometrium is reportedly regulated by ovarian steroid hormones, especially estrogen level [26-28].

In the present study we did not investigate the ER, PR receptor status but from the points of literature increased VEGF might be under either oestrogen or progesterone receptor control. There is a need for further research to assess the correlation with VEGF and Nestin and hormone receptor expression in polyp development and growth. Another limitation of this study is the small number of included patients although many previous studies on endometrial polyps has been consisted of rather a few number of subjects, similar to us. Peng et al defined the differences among the various published reports on endometrial polyps [29].

In conclusion, the present study demonstrated higher expressions of VEGF and Nestin in endometrial polyp compare to control tissue. We thought that these results suggest angiogenic factors seem to be involved in the pathogenesis of endometrial polyps. To the best of our knowledge, this is also the first report concerning the Nestin as an new angiogenetic marker play role in endometrial polyp formation.

Disclosure of conflict of interest

None.

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