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Expression of vascular endothelial growth factor receptor-3 in the endometrium in menorrhagia

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Abstract. Angiogenesis is essential for endometrial growth and repair, and disruption of this process may lead to common gynecological disorders, including menorrhagia and endometriosis. We have recently shown that expression of vascular endothelial growth factor (VEGF)-A and its two main receptors, VEGFR-1 and -2, is increased in idiopathic menorrhagia (IM). The aim of this study was to determine the expression of VEGFR-3 in normal and IM endometrium. Endometrial biopsies from 24 patients with IM and 18 healthy and fertile women were used for immunohistochemistry assessments and image analyses of VEGFR-3 and CD34-stained endothelial structures. We found that weak to moderate expression of VEGFR-3 was present in stroma and glands throughout the menstrual cycle without differences between patients and controls. Capillaries expressed VEGFR-3 markedly, whereas arterioles and venules stained moderately to markedly. However, we observed that vascular expression of VEGFR-3 in capillaries was 1.6-fold higher in the IM group than in controls, when assessed as the number of stained capillaries per mm^2. There was also a 2.0-fold higher number of arterioles, which were VEGFR-3 positive in the IM group. There was no difference with regard to the menstrual cycle between patients and controls. Thus, human endometrium expresses VEGFR-3, and expression of this receptor is increased in idiopathic menorrhagia. These results indicate that VEGFR-3 may play a role in the abnormal endometrial angiogenesis of IM.

Introduction

Excessive menstrual bleeding, menorrhagia, is a serious healthcare problem accounting for >20% of gynecological outpatient visits (1). Although commonly associated with fibroids and carcinoma, ~50% of menorrhagia patients manifest no evidence of uterine pathology, i.e. idiopathic menorrhagia (IM). Several studies now suggest that the dysregulation of angiogenesis may be a feature of IM (2).

Endothelial cell proliferation, vascular morphogenesis and the maintenance of the integrity of blood vessels are controlled in part by the interaction of specific growth factors. Vascular endothelial growth factor (VEGF) is a key regulator of endothelial cell functions. It stimulates endothelial cell migration and promotes survival of newly formed vessels but increases vascular permeability (3). The VEGF family also comprises placenta growth factor, VEGF-B, VEGF-C, VEGF-D and VEGF-E, the effects of which are mediated by three cell-receptor tyrosine kinases: VEGFR-1, VEGFR-2 and VEGFR-3.

Vascular endothelial growth factor receptor-3 (VEGFR-3, Flt-4), a receptor for vascular endothelial growth factors (VEGFs) C and D, is specifically expressed on lymphatic endothelium and suggested to play a role in the maintenance of lymphatic endothelium and/or in lymphangiogenesis. In addition to being a ligand for VEGFR-3, VEGF-C has a high affinity for VEGFR-2, a receptor for VEGF-A. Like VEGF-A, VEGF-C is also able to stimulate the migration and the proliferation of endothelial cells of blood vessel origin *in vitro* and promotes blood vessel angiogenesis and increases vascular permeability in the adult *in vivo* (4).

In a previous study, we showed that IM is associated with a high expression of VEGF-A and VEGFR-1 and -2 in the human endometrium (5), which pointed to the possible existence of a discrete signaling chain of molecules, involved in the pathogenesis of IM.

The aim of this study was to investigate the expression of VEGFR-3 in the normal and IM endometrium in order to further clarify a possible significance of this receptor in the regulation of angiogenesis in the menorrhagic endometrium.

Materials and methods

Endometrial biopsies were obtained from 24 normal ovulating women (mean age 42 years) with histories of menorrhagia and 18 normal healthy ovulating age-matched

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women (mean age 41 years). Biopsies were obtained using a thin plastic catheter for endometrium samples (Pipelle Laboratoire C.C.D., Paris, France). All women were non-smokers and had not used drugs, hormonal or intrauterine contraception for at least three months prior to the biopsy sampling. Preoperative blood samples showed normal values for platelets, activated prothrombin thromboplastin time, INR, bleeding time and von Willebrand factor. In women with menorrhagia the uterine cavity was evaluated by hysteroscopy. These women had no endometrial pathology discernible by this procedure or routine histological examination. The stage of menstrual cycle was based on the date of last menstruation, analyses of oestradiol and progesterone and histological pattern of the biopsies. Eleven women with menorrhagia and 10 women from the control group were in the proliferative phase and 13 women with menorrhagia and eight from the control group were in the secretory phase.

The study was approved by the Ethics Committee of the Karolinska Hospital. All women gave their informed consent to the study.

Immunohistochemistry. Biopsies were processed as described previously (5). Primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used. The specificity of the used antibody was previously demonstrated by various blocking experiments (6). Moreover, the negative control was run without the primary antibody as well as without the secondary antibody. These procedures resulted in negative staining. Additionally, the primary antibody was replaced with a normal IgG antibody from the same species as the primary antibody. These procedures also resulted in a negative staining.

Adjacent sections were used in order to obtain information as to the co-localisation of vessels identified with the endothelial marker CD34 and those stained by the antibody to the VEGFR-3. Negative controls for CD34 were run without the primary antibody.

We used mouse anti-VEGFR-3 (Flt-4; #321, 4 μg/ml). Mouse anti-CD34 was purchased from Serotec Ltd. (Kidlington, UK). A biotinylated secondary antibody was applied for 20 min. For VEGFR-3 a rabbit anti-mouse antibody and for CD34 a mouse link #HK335-5M (BioGenex) were used. Thereafter, the avidin-biotin complex was added. After rinsing in PBS and developing in diaminobenzidine (DAB), the slides were counterstained with Harris' haematoxylin. Sections from a biopsy of endometrial carcinoma were used as a positive control since it comprised numerous blood vessels, including arterioles, capillaries and venules.

After coding of the slides, they were examined by three independent observers. Each observer examined the slides at least at two different occasions. The staining was graded as follows: 0 = no detectable staining, + = weak staining pattern, ++ = moderate staining pattern, and +++ = marked (strong) staining pattern. Differences in opinion between the observers were resolved at a discussion microscope.

Vessels were classified as follows: arterioles, vessels with a clearly visible cuff of 1-2 layers of smooth muscle cells in the tunica media; capillaries, thin-walled vessels consisting of a single layer of endothelial cells without any smooth muscle cells; and venules, vessels larger than the capillaries lined with endothelial cells and occasional pericytes or smooth muscle cells.

Microvascular density (MVD) and computer-assisted stereological analysis of immunoassayed blood vessels. MVD from five high-power randomly chosen microscopic fields (HPF) was assessed as described previously (5). Thereafter, two-dimensional quantitation of the number of vessels within a defined area that expressed the VEGFR-3 was performed with the unbiased counting frame proposed by Gundersen (8).

In the image analysis program five images were taken in a uniform random manner, always starting at the left upper side of the specimen. In each image, the numbers of stained arterioles, venules and capillaries were counted. The numbers of positively stained vessels are given per mm^2.

Images were obtained in a light microscope (Axioplan 2, Zeiss, Jena, Germany) and were captured with a 3-chip CCD RGB color camera (Sony DXC-9100P, Sony Corp., Japan) and downloaded into the computer (SUN SparcStation 20, Sun Microsystems Computer Corp., CA), equipped with a frame-grabber (Snapper-8, Active Imaging Ltd. Berkshire, UK). The images were then saved in 8-bit RGB-mode under the control of MicroGOP 2000s image analysis software (Context Vision, Linköping, Sweden).

Statistical analysis. The angiogenesis data did not consistently fulfill the assumptions necessary for using the analysis of variance and t-test; therefore, the non-parametric Kruskal-Wallis and Mann-Whitney tests were used. For the statistical correlations the Spearman test was used. Statistical analyses were performed with the Statistica® software package. $P<0.05$ was considered significant. Data are given as median values and the 95% confidence interval.

Results

Staining of VEGFR-3 in glands and stroma (Fig. 1; Table I). Immunoreactive VEGFR-3 was present in stroma and in the glandular epithelium throughout the menstrual cycle. VEGFR-3 expression was detected in glands, both in the apical and basal regions. The mean intensity was classified as moderate. The majority of staining in the stroma was localized in the blood vessels. In the avascular stroma a weak to moderate staining for VEGFR-3 was noted. There was neither a significant difference with regard to the menstrual phases, nor between patients and controls.

Staining of VEGFR-3 in the endometrial blood vessels (Figs. 1 and 2; Table II). VEGFR-3 showed marked endothelial expression in capillaries, arterioles and venules in both groups of subjects in proliferative as well as secretory phases, with no marked staining intensity differences between patients and controls.

When the number of stained vessels was compared, we observed a 1.6-fold higher number of capillaries, which were VEGFR-3 positive in the menorrhagia group than in controls. This difference was significant ($P=0.003$). There was also a significant difference between patients and controls that related to the menstrual phase. Thus, in the proliferative phase the difference was $P=0.02$ and in the secretory phase $P=0.03$ (Fig. 2).

xpression of VEGFR-3 in the endometrium.

	Staining intensity				
	Capillaries	Arterioles	Venules	Glands	Stroma
Patients					
Proliferative phase	+++	+++	++	++	+
Secretory phase	+++	++	++	++	+
Controls					
Proliferative phase	+++	+++	+++	+++	++
Secretory phase	+++	++	++	+++	+

The staining intensity was graded as: missing (0), weak (+), moderate (++) or marked (+++). Data are given as median values.

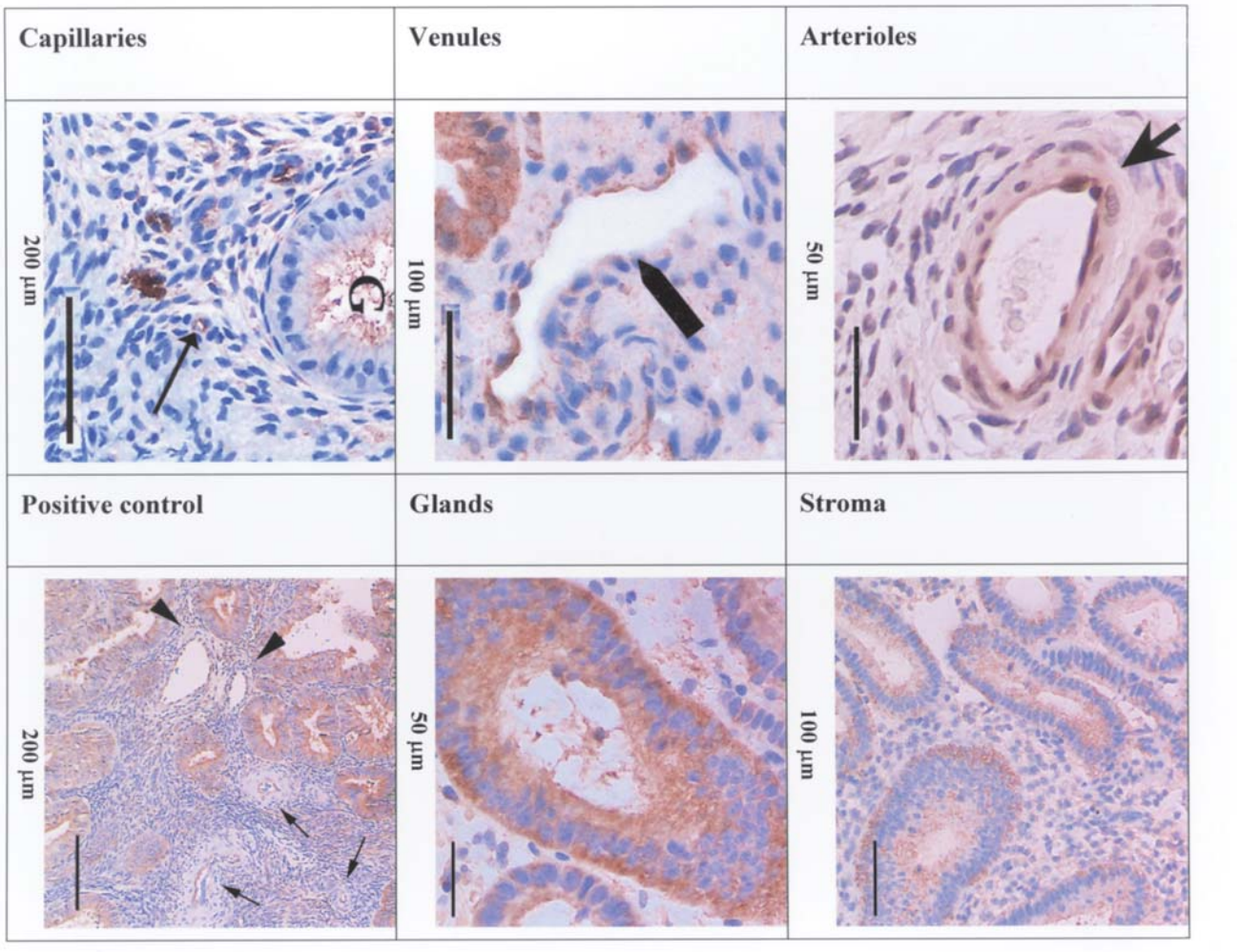

Figure 1. Micrographs of immunohistochemical staining of VEGFR-3 in menorrhagic endometrium. Arrows point to vessel structures. G, glands. Expression of VEGFR-3 in capillaries was 1.6-fold higher in the menorrhagia group. There was a 2.0-fold higher number of arterioles, which were VEGFR-3 positive in the menorrhagia group.

We observed that menorrhagia patients also showed significantly higher numbers of VEGFR-3-positive arterioles in the secretory phase than controls (P=0.03). We found no differences with regard to venules, mainly because very few were found.

Discussions

In the present study we focused on the expression pattern of VEGFR-3, the third and recently cloned member of the VEGFR family in human endometrium. We confirmed our

Table II. Expression of VEGFR-3 in the endometrial blood vessels.

	Arterioles No. of stained vessels/mm^2	Venules No. of stained vessels/mm^2
Controls	3 (2-7)	2 (1-3)
Proliferative phase	4 (2-10)	2 (0-4)
Secretory phase	2 (0-6)	1 (0-3)
Patients	7 (6-10)	2 (1-3)
Proliferative phase	8 (5-13)	2 (1-5)
Secretory phase	7 (4-10)	2 (0-2)

Data are given as median values and the 95% confidence interval for stained vessels per mm^2.

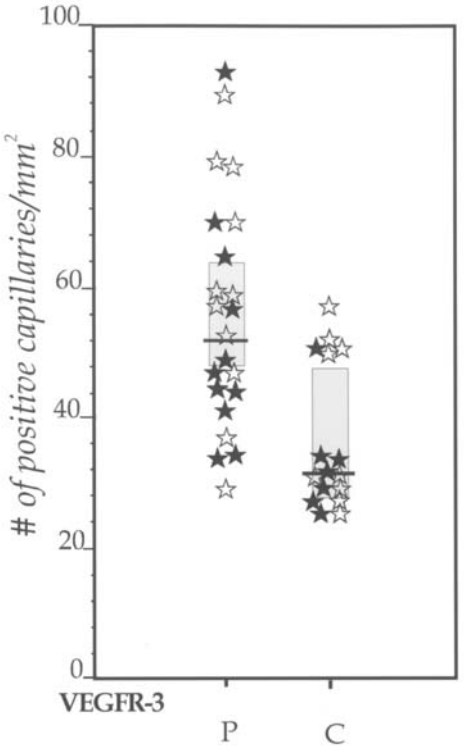

Figure 2. The expression of VEGFR-3 in capillaries in menorrhagia patients and healthy controls in endometrial sections. By means of the computerized software program we observed that vascular expression of VEGFR-3 in capillaries was 1.6-fold higher in the menorrhagia group, when assessed as the number of stained capillaries per mm^2 (p=0.001). Data are given as median values and the 95% confidence interval (CI) for stained vessels, as described in the Materials and methods section. The bars denote the median values. Shaded areas represent the 95% CI. Patients and controls in the secretory phase are depicted with filled symbols and in the proliferative phase with open symbols.

previous findings of VEGFR-3 expression in normal endometrium (7) and also, for the first time, showed expression of VEGFR-3 in menorrhagia.

We found that VEGFR-3 was expressed in the endometrial blood vessels. Moreover, the number of capillaries stained for VEGFR-3 was significantly higher in the menorrhagia group compared with controls. This finding suggests that VEGFR-3 is more actively expressed in capillaries in menorrhagic endometrium.

Partanen *et al* reported that VEGF-3 was expressed in many fenestrated endothelia (9). In our previous study, we showed that endometrial blood vessels were fenestrated and these fenestrations were more pronounced in women with idiopathic menorrhagia (Fertil Steril, in press). Our finding that numbers of capillaries stained for VEGFR-3 were significantly higher in the menorrhagia group suggests that VEGFR-3 plays a role in the transport functions of the discontinuous and more permeable endothelia. VEGF-C signaling via VEGFR-3 may be recruited by VEGF-A to act together with VEGF-C in one or more steps of the angiogenic cascade (10). This mechanism may be operative in endometrium in menorrhagia as we also observed increased levels of VEGF-A in these patients (7).

In this study, VEGFR-3 was detected in capillaries, venules and arterioles. There are a few studies concerning VEGFR-3 expression in the endometrium (11,12). Li *et al* demonstrated that expression of VEGFR-3 in the endometrium was observed in decidual NK cells (11). The difference between our study and those mentioned above is that we focused particularly on the specific distribution of VEGF receptors in not only capillaries, but also in venules and arterioles. It is conceivable that the stroma staining we observed partly reflects NK cells.

Expression of VEGFR-3 seems to be upregulated in pathological conditions, characterized by neovascularisation (13). Nilsson *et al* showed that hypoxic conditions potently stimulated the formation of an extensive vascular network during embryonal stem cell differentiation (14). Moreover, these morphological changes were correlated with activation of the VEGFR-3 gene. However, hypoxia in endometrium is less characterized. One hypothesis may be that hypoxia in endometrium in menorrhagia results in the upregulation of VEGFR-3.

In conclusion, we showed that VEGFR-3 was upregulated in menorrhagic endometrium. This finding may provide important insights into mechanisms underlining idiopathic menorrhagia.

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