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 mm². 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. 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 mm². 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. 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.

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. 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.

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, 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...
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 (Kidlington, UK). A biotinylated secondary antibody was applied for 20 min. For VEGFR-3 a rabbit anti-mouse antibody was used. The specificity of the used antibody was 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.

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, the proliferative phase the difference was P=0.02 and in the secretory phase P=0.03 (Fig. 2).
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
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 VEGF-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 VEGF-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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References