Introduction

Endometriosis is one of the most common gynecological diseases in women of reproductive age. The most common symptoms of endometriosis are pelvic pain and infertility, adversely affecting the quality of life. Its development is known to be highly dependent on angiogenesis. Angiogenesis, the formation of new blood vessels from pre-existing vessels, is key for both normal development and homeostasis as well as in certain pathological conditions. To maintain the endometrial tissues, angiogenesis that provides the substantial supply of oxygen and nutrition is critical. There are numerous endogenous regulators of angiogenesis. Among them, vascular endothelial growth factors (VEGFs) and their receptors are prime regulators of both physiological and pathological angiogenesis. The different VEGFs have overlapping but specific roles in controlling the growth of new blood vessels. The VEGF receptors transduce signals mediating endothelial cell proliferation, migration, organization into functional vessels, and remodeling of the vessel network.

We have previously reported that prostaglandin E2 (PGE2) biosynthesis was relevant to both the growth of endometrial tissue and angiogenesis in a mouse transplantation model. It was reported that receptor signaling of PGs, such as PGE2 enhanced angiogenesis in chronic inflammation and tumor microenvironment. In these pathological conditions, PG receptor signaling that elevates cAMP (cyclic adenosine monophosphate)
levels is relevant to the enhancement of angiogenesis. These results suggest that the angiogenic responses in the mouse transplantation model are highly regulated by endogenous VEGF.

In the present study, we tested the time course of the growth of endometrial tissues and the development of angiogenesis in the implanted mouse endometrial tissues, and evaluated the potential therapeutic significance of VEGF blockade. We previously reported that the signaling of a VEGF receptor, VEGF type 1 receptor (VEGFR 1) enhanced angiogenesis in a hind-limb ischemia model. We further tested whether or not VEGFR 1 signaling has a significant contribution to the growth of endometrial tissues and the development of angiogenesis using VEGFR 1 tyrosine kinase knockout mice (TK-/-). The results suggest that VEGF becomes a promising therapeutic target for the treatment of endometriosis.

Materials and Methods

Animals

Eight-week-old female C57BL/6 wild-type mice (WT) were purchased from CLEA Japan (Tokyo) and used as controls for VEGFR 1 tyrosin kinase knockout mice (TK-/-). We used the 8-week-old female TK-/- mice we developed previously. The knockout mice were backcrossed to the C57BL/6 background for more than 10 generations. All mice were housed in a limited access animal facility with temperature maintained at 25 ± 1℃ and relative humidity at 60% ± 5%. A 14-hour light/10-hour dark (6 AM to 8 PM) cycle was established by using artificial lighting. The study protocol was approved by the Animal Care and Use Committee at Kitasato University School of Medicine (3383, 2015-022).

Endometrial transplantation model

This protocol of endometrial transplantation was performed as previously described. Mice were bilaterally ovariectomized through paravertebral incisions. Postovariectomy, 100 mg/kg estradiol dipropionate in sesame oil (Aska, Tokyo) was injected subcutaneously into each mouse every week. Seven days after ovariectomy, the endometrial tissues were transplanted to the peritoneal cavity of the recipient mice, as described previously. Briefly, the uterine horns were removed and four round endometrial fragments (3-mm diameter) were trimmed off. The endometrial tissues were implanted into the peritoneal wall because the endometrial gland and peritoneum are in contact with it. WT or TK-/- endometrial fragments were implanted ectopically under the peritoneum of either WT or TK-/- mice. The implantation day was defined as day 0, and the mice were euthanized under anesthesia on days 7, 14, 21, or 28 after the implantation. The endometrial implants were removed to assess size, histological and immunohistochemical analyses, and analysis of gene expression by real-time reverse transcription polymerase chain reaction (RT-PCR).

Administrations of an anti-mouse VEGF neutralizing antibody

Mice were given an anti-mouse VEGF antibody (AF-493-NA; R&D Systems, Minneapolis, MN) by i.p. intra peritoneal injection, at a dose of 10 μg/mouse, every day for 2 weeks. Control mice were injected with control IgG normal goat IgG control (AB-108-C; R&D Systems) using an identical dosing regimen.

Histology

After removing the endometriotic lesions, they were immediately fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 hours and embedded in paraffin. Sections of 3-μm thickness were cut using a sliding microtome, deparaffinized in xylene. After dewaxing, endogenous peroxidases were quenched by incubation in 3% H2O2 buffer. Antigen was activated by microwave oven with 0.01 M sodium citrate buffer (pH 6.0). The sections were incubated overnight at 4℃ with the following primary antibodies: rabbit anti-CD31 polyclonal antibody (Ab28364; Abcam, Cambridge, MA, USA) at 1:800 dilution. After washing off the primary antibodies in phosphate buffer solution (PBS), they were immunohistochemically stained with Histofine Simple Stain MAX PO (Nichirei Bioscience, Tokyo). The sections were washed again, washing the off primary antibodies, stained with DAB (dimethylaminoazobenzene), for approximately 2 minutes, and then counterstained with Mayer's hematoxylin. As a negative control, sections were incubated identically without primary antibodies.

Immunofluorescence

Endometriotic lesions were immediately fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4℃ for 24 hours. Samples were placed in 7.5% sucrose/0.1 M phosphate buffer (pH 7.2) for 4 hours at 4℃ twice, 15% sucrose/0.1 M phosphate buffer (pH 7.2) overnight at 4℃, 4:1 solution of 15% sucrose/0.1 M phosphate buffer (pH 7.2) and optimal cutting temperature (OCT) compound for 4 hours at 4℃, and 1:1 solution of 15% sucrose/0.1 M phosphate buffer (pH 7.2) and OCT compound for 4 hours at 4℃. The samples were
embedded in OCT compound and frozen at -80°C. Then 8-μm sections were cut using a cryostat, the OCT compound was washed off in PBS, and then the sections were incubated in 1% bovine serum albumin (BSA)/PBS at room temperature for 1 hour overnight at 4°C to block non-specific binding, followed by incubation with the following primary antibodies: rabbit anti-VEGFR 1 polyclonal antibody (ab2350; Abcam, Cambridge, MA, USA) at 1:200 dilution, rat anti-CD31 monoclonal antibody (BD550274; BD Biosciences, Franklin Lakes, NJ, USA) at 1:200 dilution, rat anti-CD11b monoclonal antibody (BD550282; BD Biosciences) at 1:200 dilution, and goat anti-S100A4 polyclonal antibody (TA318024; OriGene Technologies, Rockville, MD, USA) at 1:200 dilution. After washing off the primary antibodies in PBS, the sections were incubated with the following secondary antibodies for 1 hour at room temperature: Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated donkey anti-rat IgG, and/or Alexa Fluor 594-conjugated donkey anti-goat IgG at 1:200 dilution. As a negative control, some sections were incubated in 1% BSA-PBS without any primary antibody. Images were observed and captured at ×400 magnification under a fluorescence microscope (Biozero BZ-9000; Keyence, Osaka).20,21

Determination of vessel density
Microvessel density (MVD) in the areas of most intense neovascularization (hot spots) in the endometrial implants was used as a measure of angiogenesis following methods previously described for tumor-associated angiogenesis.22,23 Briefly, blood vessels in the ectopic endometrium were stained with anti-CD31 antibody, and the areas of highest neovascularization were identified by scanning the endometrial tissues at low power (40 and 100 magnification). The individual microvessels within the area of maximal neovascularization were counted in one ×400 field. CD31 immunoreactive endothelial cells were clearly differentiated from the adjacent microvessels, stromal cells, and other connective tissue elements. MVD was expressed as the number of vessels/150 μm², and was calculated in the mean of microvessel counts at three different fields per section from each ectopic fragment.

Quantitative real-time RT-PCR analysis
Total RNA from endometriotic tissues was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions. RT-PCR and real-time RT-PCR to measure CD31 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were performed as previously described.24 The primer sequences used were as follows: CD31, 5’-CAGAGCCAGCAGTATGAGGAC-3’ (forward) and 5’-GCAACTATTAAGGTGCCGATG-3’ (reverse); GAPDH, 5’-ACATCAAGAAGGTGGTGATTGAAAGC-3’ (forward) and 5’-AAGGTGGAAGAGTGGGAGTATG-3’ (reverse).

Data analyses
Statistical analysis data were expressed as mean ± standard error of the mean (SEM). All statistical analysis was performed using JMP 10 (SAS Institute, Cary, NC, USA). Comparisons between the two groups were performed using Student’s t-test. Comparisons among multiple groups were performed using the Tukey-Kramer test. All comparisons were tested for equal variances. In case of unequal variances, comparisons were performed using Welch’s test or the Steel-Dwass test. A P value of <0.05 was considered statistically significant.

Results
Time course of growth of endometrial implants and angiogenesis
When WT endometrial fragments were implanted into WT mice receiving estrogen stimulation, the implanted endometrial tissues grew gradually showing a peak level on day 14, and the sizes of the implants decreased thereafter (Figure 1A, B). Judging from the reddish appearance of the implanted endometrial tissues on day 14, the tissues were well vascularized (Figure 1A). In fact, when we stained the endometrial tissues with CD31 antibody, neovasculized blood vessels were visualized throughout the experimental periods (Figure 2A). CD31-positive structures were reduced on day 21 and on day 28 (Figure 2A). When microvessel density was determined, the microvessel density in the implanted tissues was gradually increased by day 14 (Figure 2A, B). The increase on day 14 was significant, compared with microvessel density on day 0 (Figure 2B). Thereafter, the microvessel density in the implanted tissues was decreased on day 21 and on day 28 (Figure 2B).

VEGF neutralizing antibody effects on growth of endometrial implants and angiogenesis
To evaluate the role of VEGF in the growth of endometrial implants, we treated WT mice with VEGF neutralizing antibodies following the schedule described in the methods (Figure 3). When we evaluated the peak levels on day 14, VEGF antibody treatment significantly reduced the size of the implants (Figure 3A).
Figure 1. Time course of growth of endometrial implants

A. Typical appearance of endometriotic implants in WT → WT combination on days 7, 14, 21, and 28. Scale bars: 1 mm. B. Temporal changes in the size of endometrial implants in WT → WT combination. Data are expressed as the mean ± SEM from 6 to 22 mice. (Steel-Dwass test: **P < 0.01)

Figure 2. Time course of development of angiogenesis in endometrial implants

A. Typical CD31 immunostaining results from WT → WT combination on days 7, 14, 21, and 28. CD31 positive endothelial cells (arrows). Scale bars: 25 μm. B. Temporal changes in microvessel densities in endometrial implants from WT → WT combination. Data are expressed as the mean ± SEM from 6 to 22 mice. (Tukey-Kramer test, *P < 0.05)
microvessel density evaluated with CD31 immunostaining was also suppressed with a VEGF antibody on day 14 (Figure 3B). CD31 mRNA levels in the implanted WT endometrial tissues were significantly reduced with a VEGF antibody on day 14 (Figure 3C).

**VEGFR 1 signaling roles in growth of endometrial implants**

To separately evaluate the roles of VEGFR 1 in the implants and the hosts in growth of endometrial implants, we implanted WT endometrial tissues and TK endometrial tissues into the peritoneal cavities in either WT or TK-/- mice (Figure 4). When we implanted TK endometrial fragments into WT mice (TK-/- → WT implantation), the growth of the implants was not suppressed on day 14 after implantations in comparison to WT → WT implantation (Figure 4A, B). By contrast, the growth of implants in WT → TK-/- combination significantly suppressed in comparison to WT → WT implantation on day 14 (Figure 4A, B). The same degree of suppression was seen following the implantation of TK-/- → TK-/- (Figure 4A, B). These results suggested that the growth of endometrial fragments under estrogen stimulation was promoted by the cells expressing VEGFR 1 derived from the hosts.

**Roles of VEGFR 1 signaling in the enhancement of angiogenesis in endometrial implants**

To evaluate the involvement of VEGFR 1 signaling in the enhancement of angiogenesis in endometrial implants, we stained the immunoreactive CD31 in the implantation model using TK mice and their WT counterparts (Figure 5). When we implanted TK endometrial fragments into WT mice (TK-/- → WT implantation), the angiogenic responses in the implants was not suppressed on day 14 after implantations in comparison to WT → WT implantation (Figure 5A, B); however, the angiogenesis in the implants in WT → TK-/- combination significantly suppressed in comparison to WT → WT implantation (Figure 5A, B). The same magnitude of suppression was observed in the cases of implantation of TK-/- → TK-/- (Figure 5A, B). These results suggested that host-derived VEGFR 1 expressing cells facilitated proangiogenic responses in the endometrial fragments under estrogen stimulation.

**Figure 3.** Effect of a VEGF neutralizing antibody on growth of endometrial implants and angiogenesis

A. Size of endometriotic lesions in VEGF neutralizing antibody-treated mice (VEGFAb) and Control IgG-treated mice (Control) on day 14. B. Microvessel densities in implants on day 14. C. CD31 mRNA expressions in the implants on day 14. Data are expressed as the mean ± SEM from 3 to 4 mice. (Student's t-test, *P < 0.05, **P < 0.01)
Figure 4. Lack of VEGFR 1 signaling in the hosts restricts growth of endometrial implants

To evaluate separately the roles of VEGFR 1 in the implants and the host in growth of endometrial implants, we implanted WT endometrial tissues and VEGFR 1 TK-/- (TK-/-) endometrial tissues into the peritoneal cavities either in WT or in TK-/- mice. A. Typical appearance of endometrial implants in combinations of WT and TK-/- on day 14. Scale bars: 1 mm. B. Size of endometrial implants in combinations of WT and TK-/- on day 14. The mean endometrial lesion size in WT → WT mice on day 0 (dotted line). Data are expressed as the mean ± SEM from 9 to 10 mice. (Tukey-Kramer test, *P < 0.05, ***P < 0.001)

Figure 5. Lack of VEGFR 1 signaling in the hosts restricts development of angiogenesis in endometrial implants

To evaluate separately the roles of VEGFR 1 in the implants and the host in development of angiogenesis in endometrial implants, we implanted WT endometrial tissues and VEGFR 1 TK-/- (TK-/-) endometrial tissues into the peritoneal cavities either in WT or in TK-/- mice. A. Typical staining of CD31 in the endometrial implants in combinations of WT and TK-/- on day 14. Scale bars: 25 μm. CD31 positive endothelial cells (arrows). B. Microvessel density in endometrial implants in combinations of WT and TK-/- on day 14. The mean microvessel density in endometrial fragments isolated from Wild mice (dashed line). Data are expressed as the mean ± SEM from 8 to 10 mice. (Tukey-Kramer test, ** P < 0.001)
Expressions of VEGFR 1 in macrophages and fibroblasts in endometrial implants

We further identified the cell types expressing VEGFR 1 in the endometrial tissues on day 14 from WT → WT implantation experiments (Figure 6). A large population of the cells in the endometrial implants was positive to VEGFR 1 (Figure 6Ai, Bi, Ci). CD11b-positive cells were accumulated into the implants (Figure 6Aii), and most of these cells appeared to be VEGFR 1-positive judging from a merged image (Figure 6Aiii). The same was true in s100A4 staining (Figure 6Bii, Biii). By contrast, when we stained the endometrial tissues with an antibody against an endothelial marker, CD31 (Figure 6Cii, Ciii), about a half population of CD31-positive endothelial cells exhibited VEGFR 1-positive (Figure 6Ciii). These suggested that VEGFR 1-positive macrophages and/or fibroblasts enhanced the growth of endometrial tissues and angiogenesis in the implants.

Discussion

Peritoneal endometriosis is a significant debilitating gynecological problem of widespread prevalence. It is widely accepted that the pathogenesis of peritoneal endometriosis involves the implantation of exfoliated endometrium. Essential for its survival is the generation and maintenance of an extensive blood supply both within and surrounding the ectopic tissue. The vascular VEGF family of angiogenic molecules is believed as a major factor that control pathogenesis of endometriosis. VEGF was identified as a heparin-binding polypeptide mitogen with a target cell specificity restricted to vascular endothelial cells.25 Compared with other growth factors that induce angiogenesis, VEGF was solely acting on the vascular endothelial cells. In the present study, we clarified that VEGF enhanced the growth of endometrial tissues and the angiogenesis, judging from the results from the VEGF neutralizing antibody (Figure 3), and that one of the VEGF receptor subtypes, VEGFR 1 facilitated the growth of endometrial tissues and the angiogenesis in the implants in mouse endometriosis model (Figures 4, 5). Precise implantation experiments revealed that VEGFR 1 signaling in the host or host-derived cells or tissues in the implants were relevant to the growth of endometrial tissues and angiogenesis in the endometrial tissues. To our knowledge, this is the

Figure 6. Expressions of VEGFR 1 in macrophages and fibroblasts in endometrial implants

Endometrial tissues in WT → WT implantation experiments were stained with a VEGFR 1 antibody (Ai, Bi, Ci) and with antibodies against CD11b (Aii), S100A4 (Bii), or CD31 (Cii). In merged images, VEGFR 1+ cells with CD11b+(A), S100A4+(B), or CD31+(C) (arrows); VEGFR 1-cells with CD11b+(A), S100A4+(B), or CD31+(C) (arrowheads). Scale bars: 50 μm.
first report that described the importance of the host VEGFR 1 signaling in endometriosis.

We had previously tested that a VEGF type 2 receptor tyrosine kinase inhibitor, ZD6474 [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine], suppressed at least in part the growth of endometrial tissues and angiogenesis.8 We recently reported that angiogenic responses in hindlimb ischemia were enhanced by VEGFR 1 signaling but not by VEGFR 2 signaling, 15 suggesting that responsible receptors to ischemia and endometriosis were different. Since, this compound has some additional activity versus other tyrosine kinases, other receptor signaling may facilitate the growth of endometrial tissues and angiogenesis in the present model.

In the present experiments, VEGFR 1 expression was detected in macrophages and fibroblasts (Figure 6). Judging from the reduced growth of endometrial tissues and angiogenesis in the implants in which VEGFR 1 expressions in the host tissues are null, it is highly reasonable that VEGFR 1-expressing macrophages and fibroblasts were recruited from the host tissues. It was frequently reported that macrophages are relevant to the enhancement of angiogenesis in pathological conditions.26-28 We had reported previously that fibroblasts recruited from bone marrow accumulated in the stromal tissues up-regulated tumor-associated angiogenesis and tumor growth.12 Bone marrow transplantation will provide the further evidence that these cells are derived from bone marrow. In our preliminary experiments using GFP transgenic mice as a host, a large number of host-derived cells are detected in the endometrial implants, although the movement of endometrial tissue cells to the host peritoneal tissues is quite infrequent.

VEGFR 1 expressed on the vasculature may also participate in angiogenic responses and implant growth, although some of the vascular endothelial cells lack VEGFR 1 (Figure 6). It was known that VEGFR 1 signaling modulate VEGFR 2 signaling to enhance the angiogenesis.29 Synergistic effect of VEGFR 1 and VEGFR 2 may be present in this model, since blockade of VEGFR 1 was equal to that of VEGFR 2. Further, it is possible that VEGFR 1-expressing macrophages and fibroblasts migrated into the implants and secreted other proangiogenic factors than VEGF to enhance sprouting from host peritoneal tissues to the implants. Another possibility of vascular sprouting from the preexisting vascular beds in the implants themselves cannot be ruled out in the present experiments.

In conclusion, VEGF antibody treatment significantly reduced the size of the implants and angiogenic responses in the endometrial tissues. The cross transplantation experiments using TK-/- mice and WT mice revealed that VEGFR 1 signaling in the host or host-derived cells or tissues in the implants were relevant to the growth of endometrial tissues and angiogenesis. VEGFR 1 immunostaining suggested that VEGFR 1-positive macrophages and/or fibroblasts were accumulated in the endometrial implants, and possibly enhanced the growth of endometrial tissues and angiogenesis. These results indicate that VEGFR 1 blockade, using antibodies and small molecule kinase inhibitors will be promising for the treatment of endometriosis.

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References


