Original Article
Vascular endothelial growth factor receptor-2 inhibitor cediranib causes regression of endometriotic lesions in a rat model

Fang Liu1,2,3, Li Wang1,2,3, Xian-Xia Zhang1,2,3, Shu-Yun Min1,2,3, Yi-Xuan Liu1,2,3, Zhi Zuo1,2,3, Zhi-Xing Jin1,2,3, Zhi-Ling Zhu1,2,3

1Obstetrics and Gynecology Hospital, Fudan University, 419 Fang-Xie Road, Shanghai 200011, PR China; 2Department of Obstetrics and Gynecology of Shanghai Medical School, Fudan University, 138 Yi-Xueyuan Road, Shanghai 200032, PR China; 3Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, 413 Zhao-Zhou Road, Shanghai 200011, PR China

Received November 21, 2014; Accepted January 28, 2015; Epub February 1, 2015; Published February 15, 2015

Abstract: Vascular endothelial growth factor (VEGF) receptor-2 plays an essential role in angiogenesis, and it also expressed in the glandular epithelium and stromal cells of ectopic endometrium. Cediranib is a protein tyrosine kinase inhibitor that potently inhibits VEGF receptor-2, but there is no study about its effects on the endometriosis. We induced endometriosis on both sides of the abdominal wall in 20 female Sprague-Dawley rats and randomly divided them into 2 groups. They were administered: cediranib 4 mg/kg/day (group 1), equal saline (group 2) for 12 days. Then, the lesion volumes were calculated, and Masson trichrome was used to detect fibrosis. Angiogenesis was evaluated by CD-31 immunohistochemistry and serum VEGF levels. Proliferation was indicated by proliferating cell nuclear antigen immunohistochemistry. Apoptosis was measured by a TUNEL assay and cleaved caspase-3 immunohistochemistry. In the treatment group, the lesion volumes were smaller (P < 0.05), and the degree of fibrosis was greater. The microvessel density was lower (P < 0.05) than control, however, serum VEGF was up-regulated by a negative feedback mechanism (P < 0.01). In addition, proliferation was significantly suppressed (P < 0.01), and apoptosis in the lesions was more obvious in the treatment group. These data indicated that cediranib can inhibit development of endometriotic lesions in rats.

Keywords: Angiogenesis, cediranib, endometriosis, vascular endothelial growth factor receptor-2

Introduction
Endometriosis is defined as the appearance of endometrial glands and stroma outside the uterine cavity and is characterized by the presence of chronic pelvic pain and infertility [1-3]. About 6%-10% reproductive women suffer from this disease [4]. Hypotheses about its pathogenesis include retrograde menstruation, differentiation of epithelium, immune dysfunction, genetic predisposition to the condition, and environmental causes [5, 6]. However, these hypotheses can only partly explain the occurrence of endometriosis; the cause of endometriosis in most patients still remains unknown. Furthermore, the side effects of traditional medicine and recurrence after operation result in additional treatment difficulties.

Recently, it has become widely accepted that angiogenesis is necessary for the growth and survival of endometriotic lesions [7]. During laparoscopy, numerous peritoneal blood vessels are observed around active endometriotic lesions. Furthermore, among women with endometriosis, peritoneal fluid and endometrial vascular endothelial growth factor (VEGF) concentrations are increased [8]. Angiogenesis has become a promising target candidate for future endometriosis therapy.

VEGF and its receptors are major regulators in the formation of blood vessels. VEGF receptor-2 (VEGFR-2; KDR in humans, Flk-1 in mice) plays an essential role in angiogenesis during adulthood and is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability [9]. Further study indicated this receptor was expressed not only in vascular endothelial cells but also in certain tumor cells, and its activation can regulate cell proliferation, apoptosis,
Cediranib causes regression of endometriotic lesions in a rat model

invasion, and migration [10]. Recently, it has been reported that VEGFR-2 may be expressed in vascular endothelial cells, endometrial glandular epithelium, and stroma cells [11, 12]. Research using animal models of endometriosis and endometriosis patients also indicated that VEGFR-2 is related to this disease [13-17].

Cediranib (AZD2171) is an orally bioavailable protein tyrosine kinase inhibitor for the treatment of cancer. It was found to potently inhibit VEGF-A-induced VEGFR-2 phosphorylation in human umbilical vein endothelial cells and some tumor cells [18, 19]. Based on these facts, we used cediranib to treat endometriosis in rats and aimed to evaluate the effects of VEGFR-2 inhibition on peritoneal endometriosis.

Materials and methods

Animals

Female Sprague-Dawley rats (6-8 weeks of age) were obtained from Slac Laboratory Animal Co. Ltd (Shanghai, China). Experimental ani-

Figure 1. Growth of endometrial implant into a cystic structure: transparent cyst appeared at the site of transplantation; regression in treatment group. A: Control; B: Cediranib.

Figure 2. Histological examination revealed the presence of endometrial tissue containing stroma and glandular epithelium; A: Control; B: Cediranib; HE × 20.
Cediranib causes regression of endometriotic lesions in a rat model

mals, which were housed in groups of 5 per wire cage, were kept under standard laboratory conditions (12 hours of light, 12 hours of dark; 20-25°C) for 2 weeks to acclimatize to laboratory conditions. Standard rat feed and water were provided ad libitum.

First surgery: inducing the animal models of endometriosis

Rats were anesthetized with intraperitoneal administration of 0.35 ml/100 mg 10% chloralhydrate (Sinopharm, China). Before surgery, the abdominal skin was shaved, and antisepsis was obtained with 75% ethanol. A 1 cm ventral incision was made, and then 1 cm of the right uterine horn was excised and placed in normal saline. Each uterine horn was cut along the longitudinal axis, and then divided in half. Two squares of 5 × 5 mm open uterus were prepared. Then, the 2 squares were sutured to the inner surface of the abdominal wall (1 on each side), with the endometrium facing the peritoneal cavity, using a 6-0 nylon suture. After closing the abdominal muscle and skin, 40,000 units/kg penicillin was injected into the muscle.

Treatment

Twenty-four days after the operation was performed, animals were randomly divided into 2

Figure 3. Masson trichrome stain revealed the degree of fibrosis is deeper in treatment group, which blue stain area is bigger. A: Control; B: Cediranib; × 100.

Figure 4. Sections were stained with PCNA for the detection of proliferating cells in the endometrial stroma (red arrow) and glands (yellow arrow), endothelial cells (black arrow). A: Control; B: Cediranib; × 200.
Cediranib causes regression of endometriotic lesions in a rat model

Different groups. Group 1 (10 rats) received 4 mg/kg cediranib (Selleck, USA) dissolved in DMSO and then diluted (1:200) in normal saline. Group 2 (10 rats) received the same amount of normal saline including an equal volume of DMSO. The treatment was given by intragastric administration once daily for 12 days. The dose was determined based on a previous study [20]. During the treatment, 2 rats in group 1 died from postoperative obstruction.

Second surgery: taking the implant samples

Rats were anesthetized with 10% chloralhydrate, and then laparotomy was performed. During the operation, the implant volumes were calculated by measuring the dimensions and using the ellipsoid volume formula ($\pi/6 \times$ length $\times$ width $\times$ height). The investigator who measured the dimensions was blinded with to the treated rats and treatment. There were 11 endometriotic cysts in group 1 and 12 in group 2. After measurement, the implants, left uterus, and both ovaries were excised, fixed in 4% paraformaldehyde (Sinopharm, China), and embedded in paraffin wax for histopathological examination. Blood was taken from the heart and centrifuged to get blood serum.

**Histology of ovary and uterus**

Tissues were fixed in 4% paraformaldehyde for a minimum of 12 hours and then embedded in paraffin and cut into 5-mm sections. The paraffin sections were stained with hematoxylin and eosin for microscopic observation.

**Masson trichrome**

Paraffin sections were deparaffinized in xylene and rehydrated through graded concentrations

---

**Figure 5.** TUNEL analysis of the lesions. A: Positive control; B: Control; C: Cediranib; 1: apoptosis bodies were stained with FITC-12-dUTP; 2: cell nucleus were stained with DAPI; 3: merge two pictures before; x 200.
of ethanol. Sections were then stained with Masson trichrome.

**Immunohistochemistry**

Sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0) and applying high microwave irradiation for 20 minutes. The slides were allowed to cool to room temperature, endogenous peroxidase activity was inhibited with 3% H$_2$O$_2$ for 10 minutes, and nonspecific binding was blocked with normal goat serum for 15 minutes at room temperature. The sections were further incubated with primary antibodies against CD31 (1:100, rabbit polyclonal; Wuhan Goodbio Technology CO., LTD, China), proliferating cell nuclear antigen (PCNA; 1:200, mouse monoclonal; Santa Cruz Biotechnology Inc., CA, USA), and cleaved caspase-3 (1:800, rabbit polyclonal; CST, USA) overnight at 4°C and then placed for 45 minutes at room temperature. Slides were then incubated with horseradish peroxidase-labeled secondary antibodies (1:200, anti-rabbit; KPL, USA; 1:100, anti-mouse and 1:200, anti-goat; Genentech, China) for 45 minutes at room temperature. Then, visualization of the antigens was achieved with diaminobenzidine. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted.

**TUNEL assay**

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Company, Germany). Sections were deparaffinized and rehydrated as previously described. After washing with phosphate-buffered saline 3 times, all sections were incubated for 8 minutes in freshly prepared 0.1% Triton X-100 permeabilization solution with 0.1% citrate buffer and then washed with phosphate-buffered saline. TUNEL TdT enzyme reaction mixture (50 µL) was added to each sample and incubated for 1 h in a humidid-
Cediranib causes regression of endometriotic lesions in a rat model

ELISA

Blood samples were separated from heart blood and then centrifuged at 3,000 rpm for 15 minutes to separate serum. Serum VEGF was measured by enzyme-linked immune sorbent assay (ELISA) using the Rat VEGF ELISA kit (R&D System, USA), according to the manufacturer’s instructions.

Statistical analysis

Immunohistochemical staining was scored by integrated optical density using Image Pro-plus 6.0. This analysis was performed in 5 visual fields from each section at x 200 magnification. Data were presented as the mean ± standard deviation. Differences were compared by independent sample t-test using SPSS software (version 17.0). A P value < 0.05 was considered statistically significant.

Results

Volumes of endometriotic cysts

During treatment, 2 rats in group 1 (received 4 mg/kg cediranib) died from postoperative obstruction. There were 11 endometriotic cysts in group 1 and 12 in group 2, because there were no cysts formation on one side or both sides in some rats. All other uterine implants appeared as transparent cystic areas (Figure 1). Histological examination revealed the presence of endometrial tissue containing stroma and glandular epithelium (Figure 2). The length, width, and height of cysts were measured using calipers and volumes were then calculated. Compared with the control group, lesions in the cediranib group were smaller, and this difference was significant (P < 0.05).

Degree of fibrosis in endometriotic lesions

The results of Masson trichrome revealed that lesions from both groups were stained by blue pigments, but the area stained by blue pigments was larger in the cediranib group (Figure 3).

Proliferation and apoptosis of endometriotic lesions

To evaluate cell proliferation in the endometriotic tissue, we stained for the proliferation marker PCNA. The stroma cells were predominantly PCNA-positive in both groups. Compared with the control group, the number of PCNA-positive cells in the treatment group was significantly reduced (Figure 4). In addition, the proliferation index in the treatment group was significantly lower than that in the control group.

Apoptosis in the endometriotic lesions was assessed by TUNEL assay and cleaved caspase 3. Sections were stained with CD31 for the detection of the micro-vascular endothelium. A: Control; B: Cediranib; × 200.
Cediranib causes regression of endometriotic lesions in a rat model

pase-3 immunohistochemistry. In the TUNEL assay, apoptotic bodies were obvious in the treatment group, and they distributed in the endometrial stroma (Figure 5). Meanwhile, we also observed cleaved caspase-3-positive cells in the treatment group (Figure 6).

Effects of anti-angiogenesis

We used CD31 immunohistochemistry to measure microvessel density. When comparing the density of CD31-positive microvessels between the 2 groups, microvessel density in the treatment group was significantly reduced (Figure 7). During the treatment, 2 rats in group 1 died from postoperative obstruction. There were no endometriosis cyst in 3 rats, and these rats were excluded. Then we got serum from 7 rats in group 1 and 8 rats in group 2. Serum VEGF was measured at the same time, and the treatment group had significantly increased levels of serum VEGF (Figure 8).

Discussion

VEGF, a major regulator of angiogenesis, binds and activates 3 receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3. In the embryo, VEGFR-1 plays a negative role in angiogenesis, most likely by trapping VEGF-A. VEGFR-2 is a direct signal transducer for pathological angiogenesis in cancer and diabetic retinopathy; thus, VEGFR-2 itself and its signaling pathway appear to be critical targets for the suppression of these diseases. VEGFR-3 plays an important role in lymphangiogenesis [9, 21]. Hence, we chose VEGFR-2 as a target in this study. It is generally accepted that the process of angiogenesis is essential for further implantation and development of endometriosis; therefore, inhibition of angiogenesis may offer a new opportunity for treatment. It has been reported that numerous compounds have anti-angiogenic effects on endometriotic lesions, such as growth factor inhibitors, endogenous angiogenesis inhibitors, fumagillin analogues, and statins [22].

Cediranib (AZD2171) is a protein tyrosine kinase inhibitor used in phase 2 study of many diseases, including ovarian, biliary tract, breast, prostate and cervical cancer. The common adverse events were fatigue, diarrhea and hypertension, but all of these side effects can
Cediranib causes regression of endometriotic lesions in a rat model

be manageable [20, 23]. To the best of our knowledge, this is the first study investigating the effect of cediranib on surgically induced endometriosis in rats.

In the present study, we demonstrated that treatment with cediranib significantly reduced lesion size, and development of endometriosis was severely decreased as a consequence. These results are in agreement with previous research which used similar drugs, such as sorafenib and sunitinib [24, 25]. Furthermore, we observed that cediranib treatment resulted in the reduction of microvessel density in experimental animals. This indicated that inhibition of angiogenesis may lead to regression of lesion size, which was previously proposed in many studies [26-28]. In our study, the VEGF level in serum increased significantly. This can be explained by a negative feedback mechanism. Cediranib inhibits the activation of VEGFR-2, and then more VEGF stimulation is needed to meet the requirement of vascular endothelial cells growth. However, the extent of receptor inhibition was greater than the extent of VEGF elevation. Unfortunately, we only measured serum VEGF levels and did not assess levels in peritoneal fluid and endometriotic lesions.

Endometriosis is characterized by fibrous tissue surrounding the endometrial glands and stroma [29]. Excess fibrosis may lead to scarring, adhesion, chronic pain, and altered tissue function [30]. Some scholars have suggested anti-fibrosis treatment as a new endometriosis therapy [31, 32]. In our study, there was greater extent of fibrosis after treatment, but we did not observe severe adhesion during the progress of the second operation. We considered this fibrosis resulted from hypoxia due to inhibition of angiogenesis. This was consistent with fibrosis occurring in ischemic kidney injury [33, 34].

Cediranib treatment has also been shown to have an effect on the proliferative activity of endometriotic lesions. We found significantly fewer PCNA-positive cells in the treatment group than in the control group. However, most PCNA-positive cells were stromal cells in both groups, and few glandular cells were stained. In the TUNEL assay, we found more apoptotic bodies in the treatment group than in the control group, and they were mainly distributed in endometriotic stromal cells. Furthermore, we detected cleaved caspase-3; activation of caspase-3 plays a critical role in the regulation of cell apoptosis. It is known that hypoxia can induce many types of cellular apoptosis [35, 36]. Cediranib inhibited microvessel formation, resulting in tissue hypoxia. At the same time, it has already demonstrated that in vascular endothelial cells and some tumor cells, activation of VEGFR-2 can activate Erk1/2 and p38 to regulate cell proliferation and migration [9, 10]. In our research, we found glandular cells and parts of stromal cells also expressed VEGFR-2, consistent with previous research [11, 12]. An in vitro study is needed to confirm the direct effects of this receptor on endometriotic cells.

In summary, we demonstrated that cediranib caused regression of endometriotic implants in rat models. This was associated with a decrease in microvessel density and proliferative activity and an increase in fibrosis and apoptosis. The mechanism of this drug was previously unclear. Further studies are needed to clarify the direct and indirect effects of this drug on endometriotic cells and angiogenesis. In addition, using rat model with auto-transplanted uterine tissue is the limitation of our research. There must be some difference between the rats and endometriosis women. Transplanting endometriosis lesion from patients to nude mice may be a better model to further study.

Acknowledgements

This study was supported by the Shanghai Committee of Science and Technology (Grant Number: 12401902200).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi-Ling Zhu, Obstetrics and Gynecology Hospital, Fudan University, 419 Fang-Xie Road, Shanghai 200011; Department of Obstetrics and Gynecology of Shanghai Medical School, Fudan University, 138 Yi-Xueyuan Road, Shanghai 200032; Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, 413 Zhao-Zhou Road, Shanghai 200011, PR China. E-mail: zhilingzhu88@163.com

References

[1] Rogers PA, D’Hooghe TM, Fazleabas A, Gargett CE, Giudice LC, Montgomery GW, Rombauts L,
Cediranib causes regression of endometriotic lesions in a rat model


Cediranib causes regression of endometriotic lesions in a rat model


