Effect of antiangiogenic treatment on peritoneal endometriosis-associated nerve fibers

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Objective: To investigate the effect of antiangiogenic treatment on experimental endometriotic lesion nerve fibers.
Design: Heterologous mouse model of endometriosis.
Setting: University Institute IVI, University Hospital La Fe.
Animal(s): Ovariectomized nude mice (n = 16) receiving human endometrial fragments from oocyte donors (n = 4).
Intervention(s): Endometrium fragments stuck in the peritoneum of 5-week-old female nude mice treated with vehicle (n = 8) and antiangiogenic agent cabergoline (n = 8; Cb_2, 0.05 mg/kg/day) for 14 days.
Main Outcome Measure(s): Immunofluorescence analysis of von-Willebrand factor (vWF) and vascular smooth muscle cells (αSMA) for evaluating the number of immature blood vessels (IBV) and microvascular density (MVD); immunochemical analysis of protein-gene product 9.5 (PGP 9.5) to assess nerve fibers density (NFD), and blue toluidine staining to confirm presence of mast cells and macrophages in endometriotic lesions.
Result(s): All the results were quantified by morphometric techniques. The IBV, NFD, and number of macrophages and mast cells were statistically significantly decreased in the Cb2-treated group when compared with controls.
Conclusion(s): Antiangiogenic treatment statistically significantly diminishes new blood vessel formation after macrophage, mast cell, and nerve fiber reduction, providing a rationale to test antiangiogenic agents as a novel therapeutic approach to severe pelvic pain associated with human peritoneal endometriosis. (Fertil Steril® 2012;98:1209–17. ©2012 by American Society for Reproductive Medicine.)
Key Words: Angiogenesis, dopamine agonists, endometriosis, nerve fibers, vascular endothelial growth factor

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Endometriosis, an estrogen-dependent inflammatory disease characterized by the presence of endometrial tissue outside the uterus, predominantly affects women of reproductive age. The real incidence of endometriosis is unknown, but it is estimated that this pathology is present in more than 15% of reproductive age women and in up to 50% of women with pelvic pain (1). Pain, in the form of dysmenorrhea, dyspareunia, or dyschezia, is the most common symptom in patients with endometriosis and may be due to nociceptive, inflammatory, or neuropathic mechanisms. Surgical resection of endometriotic lesions provides temporary relief in terms of reduced chronic pelvic pain and severe dysmenorrhea (2), but the recurrence rate at 2 years after surgery is as high as 75% of cases. Thus, medical treatment is employed at any stage of the disease in many patients worldwide.

The presence of nerve fibers in the peritoneal endometriotic lesions (3–8) and eutopic endometrium of women with endometriosis (9, 10) has received much attention recently. Nerve fibers in peritoneal lesions are located in or near endometriotic stromal cells and are colocalized with

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immature blood vessels (5, 6). These findings suggest that human peritoneal endometriotic lesions are innervated by nerve fibers that may interact with each other and with many active molecules released by endometriotic lesions to cause pain and local tenderness (11). Studies have shown that nerve fiber density (NFD) is significantly greater in the peritoneal lesions of patients with painful endometriosis than in patients with asymptomatic disease, which suggests a direct association between pain and NFD (3, 12).

Some current treatments for endometriosis, such as combinations of oral contraceptives and progestogens, significantly decrease NFD in the endometrium and myometrium in women with endometriosis (13, 14). Steroids act via a different route and have no effect on vascular endothelial growth factor (VEGF) expression, so they cannot be employed in all patients. Thus, the search for new routes in the medical treatment of endometriosis must continue.

Different proteins and growth factors have been described as regulators of nerve fibers growth. Vascular endothelial growth factor, produced by macrophages that are elevated in the peritoneal fluid and endometriotic lesions of women with endometriosis, has been reported to act as a neurotrophic factor, stimulating nerve-fiber growth (15). These findings suggest that VEGF may also play a role in increasing NFD in peritoneal endometriotic lesions (8). The role of VEGF in endometriosis-related angiogenesis is well established (16, 17). Expression of VEGF is increased in active red lesions (18, 19) and deep infiltrating endometriosis (20), which are characterized by an active angiogenic process and a rich vascular net formed by immature blood vessels.

In previous studies, we targeted VEGF in an experimental endometriosis model that examined the dopamine/dopamine receptor 2 (DRD2) pathway, whose activation is involved in the regulation of angiogenic events mediated by VEGF/VEGF receptor 2 (KDR) signaling (19, 21). By targeting the VEGF system, we were able to inhibit the angiogenic process in endometriotic lesions and reduce the number of immature blood vessels (21). This concept has also been successfully addressed in humans (22). Moreover, it is well-known that neovascularization plays an important role during neurogenesis (5). Based on this background, we hypothesized that the antiangiogenic properties that dopamine agonists exert on endometriotic lesions targeting VEGF could also diminish NFD through the inhibition of new blood vessel development in endometriotic lesions.

MATERIALS AND METHODS

This study was approved by our institutional review board, and informed consent was obtained from patients before the collection of biopsy samples. Similarly, all the procedures employing animals were performed according to European Directive 86/609/CEE and NIH Guidelines for the Care and Use of Laboratory Animals.

Heterologous Mice Model of Endometriosis

Sixteen 5-week-old ovariectomized female nude mice (Hsd: athymic Nude-nu; Harlan Ibérica S.L.) were used to develop the endometriosis model, as previously described elsewhere (19). Sixty-day-release sterile capsules containing 18 mg of 17β-estradiol (E_2) (Innovative Research of America) were placed subcutaneously in the neck of each animal. Four days later, fresh human endometrial biopsy samples (employing a Pipelle cannula) were obtained at ovum pickup from oocyte donors (n = 4, age range: 18 to 34 years) with normal menstrual cycles and no history of endometriosis. Informed consent was obtained before the human endometrial collection. Although names were kept confidential, patient age, cycle stage, and medication history were made available. The biopsy samples were placed in a prewarmed, sterile phosphate-buffered saline (PBS) solution at pH 7.4 and transported to the laboratory, where the specimens were cut into pieces of approximately 2 × 3 mm. A part of each biopsy sample was fixed in 4% buffered formaldehyde and embedded in paraffin for histologic confirmation of the proliferative phase by use of established criteria (23) and to analyze the presence or absence of nerve fibers by immunohistochemical analysis.

The remaining tissue was fixed in the peritoneum of each mouse (four human endometrial fragments per mouse) using an n-butyl-ester cyanoacrylate adhesive (3M Animal Care). Human endometrial samples from each individual donor were used in two animals of each experimental group, and four implants were introduced per animal. Three weeks after establishment of experimental lesions, the animals were divided into two experimental groups. Vehicle solution (1:6 alcohol in a sterile water mixture) was used to dilute Cb2 (Pfizer Laboratories), and this solution was administered daily and orally by gavage at doses of 0.00 mg/kg (control, n = 8) and 0.01 mg/kg (n = 8) for 14 days. Two weeks after Cb2 treatment, animals were killed, and the endometriotic implants were examined under a binocular microscope.

Histologic Evaluation

Lesions were fixed in 4% neutral buffered formalin overnight at 4°C before being routinely embedded in paraffin wax and cut into 4-μm serial sections. Four to five noncontiguous sections from each specimen were stained with hematoxylin and eosin (Sigma) and examined microscopically for the presence of the histologic hallmarks (glands and stroma) of endometriosis. Only active lesions, the ones that presented both glandular and stromal elements, were included in this study. Lesions that did not show both elements, presenting an atrophic epithelium surrounded by fibrotic tissue instead of stroma, were considered inactive lesions and discarded. A morphologic study was performed by blue toluidine staining to study the mast cell and macrophage localization in endometriotic lesions.

Immunofluorescence Staining and Laser Scanning Confocal Microscopy

A double-fluorescent was applied as previously described elsewhere (20). Human and murine endothelial cells were identified by use of a polyclonal rabbit anti-von Willebrand factor (vWF, Dako Corp.) conjugated with Zenon Alexa Fluor-647 (Molecular Probes), used according to the manufacturer's instructions. Rabbit anti-mouse immunoglobulins (Dako Corp.) were used as the negative control. Human