Growth-associated protein 43–positive sensory nerve fibers accompanied by immature vessels are located in or near peritoneal endometriotic lesions

Sylvia Mechsner, M.D.,[a] *Jessica Schwarz,*[a] *Johanna Thode,*[a] *Christoph Loddenkemper, M.D.,*[b] *David S. Salomon, Ph.D.,*[c] *and Andreas D. Ebert, M.D., Ph.D.*[a,d]

[a] Endometriosis Research Center Berlin, Department of Gynecology, and [b] Institute of Pathology, Consultation and Reference Center for Lymph Node, Pathology and Hematopathology, Charité, Campus Benjamin Franklin, Berlin, Germany; [c] Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and [d] Endometriosis Center Berlin Level III, Vivantes Humboldt-Klinikum, Berlin, Germany

Objective: To investigate the topographical relationship between nerve fibers and peritoneal endometriotic lesions and to determine the origin of endometriosis-associated nerve fibers.
Design: Retrospective nonrandomized study.
Setting: University hospital endometriosis research center.
Patient(s): Premenopausal women with histologically confirmed endometriosis were selected (n = 73). Peritoneal endometriotic lesions (n = 106) and unaffected peritoneal biopsies from patients without endometriosis (n = 9) were obtained.
Intervention(s): Immunohistochemistry was used to study the expression of neurofilament, substance P, smooth muscle actin, von Willebrand factor, growth-associated protein 43, nerve growth factor, and neutrophin-3 in peritoneal endometriotic lesion samples from women with symptomatic endometriosis and in peritoneal samples from women without endometriosis.
Result(s): Pain-conducting substance-P–positive nerve fibers were found to be directly colocalized with human peritoneal endometriotic lesions in 74.5% of all cases. The endometriosis-associated nerve fibers are accompanied by immature blood vessels within the stroma. Nerve growth factor and neutrophin-3 are expressed by endometriotic cells. Growth-associated protein 43, a marker of neural outgrowth and regeneration, is expressed in endometriosis-associated nerve fibers but not in existing peritoneal nerves.
Conclusion(s): The data provide the first evidence of direct contact between sensory nerve fibers and peritoneal endometriotic lesions. This implies that the fibers play an important role in the etiology of endometriosis-associated pelvic pain. Moreover, emerging evidence suggests that peritoneal endometriotic cells exhibit neurotrophic properties. (Fertil Steril® 2007;88:581–87. ©2007 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, sensory nerve fibers, pelvic pain, neurotrophism, vascularization, GAP-43, angiogenesis, neurogenesis

Endometriosis is a chronic, benign, estrogen-dependent gynecological disease that is associated with infertility and pelvic pain. It affects millions of reproductive-aged women worldwide (1). The severity of endometriosis-associated pelvic pain does not always correlate well with the disease stage or lesion site (2–7).

This lack of a correlation may be a result of variations in the metabolic activity of the endometriotic implants at different disease stages (2). In this context, pain-associated prostaglandins such as prostaglandins E_2 and $F_{2\alpha}$ are expressed by endometriotic implants and can activate peritoneal nociceptors (2, 8–10). In addition, several other pain-mediating substances such as histamine, kinins, and interleukins are synthesized by endometriotic implants and may thus be involved in pain mediation (11). However, no correlation has been found between the morphology of peritoneal endometriotic implants and the severity of endometriosis-associated pelvic pain (4, 6, 7, 12).

The pathophysiological basis for peritoneal endometriosis-associated pelvic pain is not known. Recently, three candidate genes possibly associated with deep infiltrating endometriosis-related pain were identified by DNA microarray analysis using laser capture microdissection (13). Up-regulation was

Received November 14, 2006; revised and accepted December 27, 2006.
Supported by the Stifterverband für die Deutsche Wissenschaft (A.D.E.), Essen, Germany.
Reprint requests: Andreas D. Ebert, M.D., Ph.D., Department of Obstetrics and Gynecology, Vivantes, Humboldt-Klinikum, Am Nordgraben 2, 13509 Berlin, Germany (FAX: 49-30-4194-1262; E-mail: andreas.ebert@vivantes.de).

demonstrated for the tyrosine kinase receptor B in epithelial cells and also for the serotonin transporter and the mu opioid receptor in stromal cells (13).

Previous studies comparing peritoneal samples from patients with and without endometriosis did not show differences in the mean nerve score (14). No direct contact was found between nerve fibers and endometriotic lesions, but the closest nerve fibers did tend to be nearer to the endometrial glands in women with pelvic pain than in those without it (11, 14). This led to the assumption that the presence of nerve fibers in the peritoneum is not related to endometriosis-associated symptoms (11, 14). Investigations in a rat model of surgically induced endometriosis recently revealed that ectopic endometrial growth induces its own autonomic and sensory innervation (15). In rodents, it recently has been shown elsewhere that ectopic implants develop a sensory and sympathetic nerve supply (16). However, the morphological relationship between peritoneal nerve fibers and endometriotic implants has not yet been clarified in human beings.

To systematically investigate the topographic relationship between peritoneal nerve fibers and peritoneal endometriotic lesions, an immunohistochemical analysis was performed to examine 106 peritoneal endometriotic lesions of 73 patients with symptomatic endometriosis and the peritoneum of 9 patients without endometriosis. Nerve fibers found to be in close contact with the endometriotic implants were identified as afferent fibers.

Additional analyses were performed to investigate the maturation status of the nerve fibers and the accompanying blood vessels. Finally, expression of nerve growth factor (NGF) and neurotrophin (NT-3) was evaluated in endometriotic stromal cells and epithelial cells. Our data provide evidence that endometriosis may be related to neurotrophic events.

MATERIALS AND METHODS

Tissue Samples

Biopsies were obtained from 73 patients who underwent diagnostic laparoscopy or laparotomy for symptomatic endometriosis (pelvic pain, dysmenorrhea, and dyspareunia). All women were premenopausal, with a mean age of 32 years (range, 19–50 y). Forty-seven women had regular menstrual cycles without hormone treatment for ≥ 2 months before surgery. Twenty-two were on hormones at the time of surgery (oral contraceptives, P-only pill, GnRH agonists). In four cases, no information was available on hormone treatment. The respective phases of the cycle were defined by correlating the date of the last menstrual period with histological findings from endometrial samples (microcurettage), applying an established histological dating method (17). The disease was staged during surgery according to the revised classification of the American Society of Reproductive Medicine (I = 30, II = 16, III = 14, and IV = 13) (18).

We studied 106 typical black endometriotic lesions containing glandular and stromal cells. The lesions were excised from peritoneum of the bladder (n = 38), the pouch of Douglas (n = 18), the ovarian fossa (n = 16), the lateral pelvic wall (n = 13), the uterosacral ligament (n = 11), the round ligament (n = 1), and the serosa of the uterus and adnexae (n = 9). All peritoneal biopsies had subperitoneal fat at the lower border.

In addition, control biopsies were taken from the peritoneum of the bladder (right side, close to the round ligament) or the pouch of Douglas in cases of laparoscopically assisted vaginal hysterectomy or unilateral adnexectomy from patients without endometriosis (n = 9). All patients gave consent for these biopsies, and the study was approved by the institutional review board.

Immunohistochemistry and Evaluation of Staining

All tissues that were excised were immediately fixed in 4% buffered formalin for 12 hours and then embedded in paraffin. Serial sections that were 1–2 μm thick were cut for hematoxylin-eosin staining and immunohistochemistry.

Neurofilament analysis (n = 106) After deparaffinization in xylene (twice for 5 min each at room temperature [RT]) and rehydration (10 min at RT in acetone and acetone–tris (hydroxymethyl aminomethane–buffered saline [TBS], 1:1, and TBS)), a heat-induced epitope retrieval (HIER) procedure was performed by heating the probes in citrate buffer (0.1 M citric acid and 0.1 M sodium citrate, pH 6.0, microwaved at 700 W for 17 min). After rinsing with TBS, the sections were blocked with 10% fetal calf serum for 30 minutes at RT. The sections were then incubated for 1 hour at RT with monoclonal mouse anti-neurofilament (anti-NF) antibody (clone 2F11, dilution 1:50; Dako, Hamburg, Germany). Rinsing with TBS was followed by application of the labeled streptavidin–biotin kit (Dako). Fuchsin substrate (Dako) was used to visualize the specific immunoreactive staining. Peripheral nerve tissue was taken as a positive control. Samples of normal peritoneum were serially sectioned and examined to exclude the presence of endometriosis.

Neurofilament–substance P double staining (n = 26) The HIER procedure was performed after deparaffinization in xylene and rehydration. After rinsing with TBS, the sections were blocked with 10% fetal calf serum for 30 minutes at RT and then incubated for 1 hour at RT with monoclonal mouse anti-NF. After rinsing with TBS, the Cy2-conjugated donkey anti-mouse antibody (dilution 1:100; Dianova, Hamburg, Germany) was applied for 45 minutes. After additional rinsing with TBS, rat anti–substance P (dilution 1:50; Santa Cruz Biotechnology Inc, Santa Cruz, CA) in background-reducing buffer (Dako) was applied for 1 hour at RT. The sections were then incubated for 1 hour at RT with rhodamine red–conjugated donkey anti-rat antibody (dilution 1:100; Dianova). Colon with myenteric plexus was used as a positive control.

Smooth muscle actin–vWF double staining (n = 40) The HIER procedure was performed after deparaffinization in xylene and rehydration. After rinsing with TBS, the sections were incubated with proteinase K (Dako) for 3 minutes