ORIGINAL ARTICLE: ENDOMETRIOSIS

Important role of collective cell migration and nerve fiber density in the development of deep nodular endometriosis

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Objective: To evaluate deep nodular endometriotic lesions induced in baboons over 12 months and analyze collective cell migration and nerve fiber density.
Design: Morphologic and immunohistochemical analysis of endometriotic lesions induced in baboons over the course of 1 year.
Setting: Academic research unit.
Animal(s): Three female baboons (*Papio anubis*).
Intervention(s): Recovery of induced deep nodular endometriotic nodules from baboons.
Main Outcome Measure(s): Evaluation of the morphology of glands by analysis of the center of lesions and the invasion front; immunohistochemical staining with Ki67, E-cadherin, and β-catenin for investigation of mitotic activity and cell-cell junctions, and with protein gene product 9.5 and nerve growth factor (NGF) for study of nerve fiber density (NFD).
Result(s): All (100%) of the lesions were invasive 1 year after induction, compared with 42.29% after 6 months. Glands from the invasion front showed significantly reduced thickness but significantly higher mitotic activity. E-Cadherin and β-catenin expression were similar between the center and front. NFD was significantly higher in lesions induced after 1 year than after 6 months, and NGF expression was significantly lower in 1-year lesions than in 6-month lesions.
Conclusion(s): Nodular endometriotic lesions induced in the baboon model were found to be significantly more invasive and innervated after 12 months than after 6 months. The invasive phenotype was highly expressed in glands at the invasion front, and our study suggests that nerve fibers play a role in the development of lesions as observed in women. (Fertil Steril® 2017;■:■–■. ©2017 by American Society for Reproductive Medicine.)
Key Words: Deep nodular endometriosis, baboon model, collective cell migration, nerve fibers, nerve fiber density, invasion

Endometriosis is one of the most frequently encountered benign gynecological diseases, known to occur in 7%–10% of women of reproductive age (1, 2). It is now well established that three different forms of endometriosis can occur in the pelvis: peritoneal endometriosis, ovarian endometriosis, and deep endometriotic nodules of the rectovaginal septum (3). Most deep lesions originate from the posterior part of the cervix and secondarily infiltrate the anterior wall of the rectum (4, 5). Anaf et al. were the first to pinpoint a close histologic relationship between nerves and endometriotic foci, and between nerves and the fibrotic component of nodules (6). The pathogenesis of these endometriotic nodules (whose histology reveals typical features of adenomyosis with smooth muscle hyperplasia and fibrosis) remains unclear and probably differs from the pathogenesis of peritoneal and ovarian endometriosis (3).

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R.O. and J.G.-S. should be considered similar in author order.
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Some authors have reported high rates of spontaneous and induced peritoneal endometriosis in baboons (7, 8), but we found only 4.8% and 20.7% of spontaneous and induced endometriosis, respectively, with the use of the same method, suggesting that baboons are able to cleanse and renew their peritoneum (9). Based on our previous observational studies of iatrogenic adenomyosis in humans (10, 11), we developed the first nonhuman animal model for nodular endometriosis (12). Although grafting endometrium or myometrium alone did not induce any endometriosis at all, when endometrium was associated with submyometrial layers, including the junctional zone (JZ), we observed a 100% rate of endometriosis induction, highlighting the importance of the JZ in the development of nodular endometriosis in this model. Nerve growth factor (NGF) expression was significantly higher in invasive lesions than in noninvasive lesions, but nerve fiber density (NFD) was similar in both groups (13). We also found altered morphology, increased mitotic activity, and fewer adhesion molecules in invasive glands present in induced nodular endometriosis, particularly along the invasion front, suggesting that collective cell migration is involved in the invasion process of deep endometriotic lesions induced in a baboon model (14).

The goal of the present study was to determine if nerves still grow in invasive and noninvasive lesions >6 months after grafting and to evaluate the role of the lesion environment in neuroregeneration. We explored the kinetic factors by analyzing collective cell migration and innervation in nodular endometriotic lesions induced after 12 months compared with 6 months (12, 13) and investigated morphology, mitotic activity, expression of adhesion molecules, NFD, and NGF. Because invasion was observed mainly in the bowel and cervix in our previous studies (12–14), we decided to focus on retroperitoneal pelvic areas close to the ureters.

MATERIALS AND METHODS

Extrapolating from previous studies (12), we estimated that we could obtain 15 induced nodular endometriosis specimens with the use of only three baboons by grafting fragments containing the JZ. This allowed us to reduce the number of animals, in line with the principles of ethical conduct in animal research, as well as reducing overall costs. Three female baboons (*Papio anubis*) were studied at the Institute of Primate Research (IPR), Nairobi, Kenya. Approval was obtained from the Institutional Scientific Evaluation and Review Committee and the Animal Care and Use Committee of the IPR. All of the baboons tested negative for common pathogens (bacterial and viral infections as well as parasites) and were screened for tuberculosis, simian T-lymphotropic virus 1, and simian immunodeficiency virus. The animals were housed in single cages and fed commercial monkey pellets (Gold Star Products) and seasonal fruit and vegetables with free access to water. A comparison was made with lesions identified in previous studies (13, 14), where deep endometriotic nodules were induced in ten baboons and recovered 6 months later.

Induction of Deep Nodular Endometriosis

The baboons initially underwent median laparotomy. After complete pelvic and abdominal exploration, no spontaneous endometriosis was found in any of the three animals. Bilateral salpingo-oophorectomy was performed to avoid individual variations in hormone secretion, and hormone replacement therapy was initiated on the same day (E_2 valerate, 2 mg/d orally; Progynova; Bayer Schering Pharma Berlin). Hormone replacement therapy was administered daily with food under the supervision of a designated technician and controlled by a veterinarian. Uterine biopsies were obtained by means of a cold knife.

Biopsies of 1 cm^3 containing endometrium and myometrium were grafted beneath the peritoneum in five different sites in the pelvic area: upper and lower parts of both uterosacral ligaments close to the ureters ($n = 4$) and rectovaginal septum ($n = 1$). The grafting process involved opening the peritoneum with cold scissors, placing the biopsies in the relevant sites, and then closing the peritoneum by means of simple suture with the use of absorbable 3/0 Vicryl FS2 (Johnson and Johnson). The biopsy itself was not sutured in any case. When uterine biopsies were placed close to the ureters on both sides, it is important to note that ureterolysis was not performed, because we did not want to facilitate invasion of the ureters. After 1 year, laparotomy was performed to recover the lesions. All induced lesions were identified and photographed before complete excision, then measured, fixed in formalin, and embedded in paraffin. All sections were assessed by two investigators (O.D. and R.O.) blinded to lesion phenotypes. Fifty to 200 consecutive serial sections of each lesion were evaluated up to the invasion front. Histologic analysis was performed over the entire lesion and where glandular density was most concentrated. All sections were scanned with the use of the Leica SCN400 scanner (Leica Biosystems Wetzlar), and image acquisition was achieved with the use of the Tissue IA system (Leica Biosystems Dublin). Image J software was applied for morphologic analysis and immunohistochemical quantification, using the color deconvolution plugin to isolate the diaminobenzidine (DAB) channel.

Morphologic Analysis

The total size of each lesion was determined immediately after its extraction by measuring three dimensions in the corresponding *x*, *y*, and *z* axes. Hematoxylin-eosin staining was carried out for microscopic analysis of the lesions: surface area (mm^2), glandular density (glands/mm^2), and surrounding organ invasion. In line with our previous publications (12, 13), invasion was defined as the presence of glands and stroma inside surrounding organs, as detected with the use of serial sections (50–200 sections). In invasive lesions, a morphologic distinction was made between the center of the lesion (trailing edge) and the invasion front (leading edge). Well circumscribed lesions were classified as noninvasive lesions. Gland thickness was calculated by measuring the distance between the external (basal) and internal (apical) parts of the gland. To avoid artifacts due to sectioning, we excluded glands without a lumen and areas with multilayer cells.