Effect of oxygen tensions on the proliferation and angiogenesis of endometriosis heterograft in severe combined immunodeficiency mice

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Objective: To investigate the effects of oxygen on the proliferation and angiogenesis of endometriosis in vivo.
Design: Animal studies.
Setting: Animal research facility.
Animal(s): Thirty-six female severe combined immunodeficiency (SCID) mice, implanted with eutopic endometrium from seven endometriosis patients.
Intervention(s): Human eutopic endometrial tissues were randomized to normoxia, hyperoxia, or hypoxia pretreatment and were subcutaneously implanted into estrogen-treated ovariectomized SCID mice.
Main Outcome Measure(s): The growth and quality of the implants were measured, and the expression of proliferation- and angiogenesis-associated markers (i.e., Ki67, CD31, vascular endothelial growth factor, and hypoxia-inducible factor-1α) were assessed using immunohistochemistry and Western blot analyses.
Result(s): The growth curves of the implants were distinct with different oxygen pretreatments. The growth of the implants of the hypoxia group was significantly increased compared with the normoxia group, but the growth of the implants of the hyperoxia group was significantly decreased compared with the normoxia group. Microscopic examination indicated that lesions with hyperplastic cylindrical glandular epithelium were surrounded by the endometrial stroma in the hypoxia group, but the glandular epithelium was partially depauperate in the hyperoxia group. The expression of Ki67, CD31, vascular endothelial growth factor, and hypoxia-inducible factor-1α in the hypoxia-pretreated implants was significantly higher compared with the hyperoxia or normoxia groups.
Conclusion(s): Oxygen can alter the growth patterns of endometriosis implants in a SCID mouse model. Hypoxia pretreatment promoted the proliferation and angiogenesis of endometriosis, whereas hyperoxia pretreatment exhibited the opposite effect. (Fertil Steril® 2014;101: 568–76. ©2014 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, oxygen tension, SCID mouse, proliferation, angiogenesis

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Endometriosis is a common gynecologic disorder characterized by the growth of endometrial tissue outside the uterine cavity. Several studies have suggested that this disorder affects 6%–10% of all females and 35%–50% of females with pelvic pain and infertility (1). However, the precise mechanism underlying its etiology remains unknown, and supporting evidence is mostly indirect; it is believed that most endometriotic lesions originate from the shed endometrium, which enters the abdominal cavity via the fallopian tubes during menstruation. For example, endometriosis only occurs in primate species that menstruate, and angiogenesis plays an essential role in the growth and survival of endometriotic lesions implanted on the peritoneal surface after retrograde menstruation (2). In addition, most females demonstrate reflux menstruation into the peritoneal cavity, but endometriosis occurs in only 5%–10% of females with retrograde menstruation, and affected females

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may have other pathophysiologic dysfunctions that interfere with the lesions (3).

Recently, tumor microenvironment studies have suggested that hypoxia is a key to tumor angiogenesis, tumor cell motility, and metastasis and cancer stem cell selection (4). Similarly, endometriosis lesions have the capacity to adhere, attach, initiate angiogenesis, and implant similar to malignant neoplasms (5). Furthermore, human endometrial stromal cells have been cultured under hypoxic and normoxic conditions and treated with the hypoxia-mimicking agent cobalt chloride. Hypoxia simultaneously induced the expression of messenger RNA (mRNA) and the production of vascular endothelial growth factor (VEGF) in a time-dependent manner, and the highest expression of hypoxia-inducible factor (HIF)-1α protein was observed at 6 hours (6). Moreover, VEGF is one of the most important agents affecting angiogenesis and can induce endothelial proliferation and capillary loop formation, and consequently new blood vessel formation can increase capillary permeability (7). Furthermore, HIF-1 serves as a transcription factor involved in cellular adaptation to hypoxia, and HIF-1 becomes active in hypoxia and, subsequently, controls a large number of angiogenesis-involved genes (8).

Currently laparoscopic surgical removal of lesions is the mainstay of treatment for endometriosis, but the estimated recurrence rate remains at >50% at 5 years after laparoscopic surgery (9). However, carbon dioxide, the most common gas used for pneumoperitoneum, primarily induces local peritoneal environmental hypoxic alterations (10). Because hypoxia promotes the expression of VEGF and HIF-1α, we hypothesized that hypoxia might be a critical parameter for the angiogenesis of endometriosis and is related to the high recurrence rate after laparoscopic surgery.

To investigate the effect of different oxygen tensions on the in vivo growth and angiogenesis potential of human endometrial implants, we pretreated the eutopic endometrium from patients with endometriosis with brief periods of normoxia, hyperoxia, or hypoxia and subsequently implanted the grafts subcutaneously into severe combined immunodeficient (SCID) mice. The SCID mouse endometriosis model is an established in vivo model that enables the detailed analysis of specific histologic, functional, and biochemical properties of human endometriosis (11). Previously our research group demonstrated that secretory human eutopic endometrium developed a high survival rate of implantation with estrogen administration for 4 weeks in a SCID mouse model (12). CD31 is commonly used as an immunohistochemical marker for microvessel density (MVD), and proliferating cells can be identified by studying the nuclear or perinuclear expression of Ki67, a protein that is expressed during all phases of the cell cycle, except the G0 phase (13). For this reason, we focused on morphology and semiquantitatively analyzed the expression and distribution of VEGF, HIF-1α, CD31, and Ki67 as angiogenesis and proliferation correlation factors of endometrial implants.

MATERIALS AND METHODS

Animals

The guidelines for animal care and use were approved by the People's Liberation Army General Hospital Committee on Animal Research. Thirty-six mature female SCID mice, weighing 20–25 g, were purchased from the Academy of Military Medical Sciences. Animals were maintained in microisolator cages and housed in a separate barrier facility in a well-controlled pathogen-free environment, where the ambient temperature was monitored and the cycles of light and darkness were regulated. Mice were fed ad libitum with laboratory chow and water.

Experimental Subjects and Tissue Collection

This study was approved by the Institutional Ethical Review Committee, and informed consent was obtained from the patients. Secretory eutopic endometrial tissues (n = 7) were acquired at the time of surgery from women undergoing laparoscopic resection of endometriosis in our department. All patients (aged 32–48 years) were classified with stage IV endometriosis, according to the revised classification by the American Society for Reproductive Medicine. The patients demonstrated regular menstrual cycles and had not received any hormone therapy or other medications that might affect the results of this study within the last 3 months. Fresh endometrial biopsies were washed to remove any residual blood and mucus and collected in cold serum-free Roswell Park Memorial Institute (RPMI) 1640 medium. The tissue was sectioned into 1- to 2-mm^3 pieces and suspended in culture dish with Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum. At the same time, duplicates were fixed in formalin and processed to histologically confirm the early secretory phase.

Human Endometrial Fragment Exposure to Different Oxygen Tensions

Tissue exposure to different oxygen tensions was performed according to a previously reported method with some modifications (14, 15). The tissue fragments were randomly pretreated in a humidified atmosphere for normoxia (5% CO_2, 95% air), hyperoxia (95% O_2, 5% CO_2), and hypoxia (5% O_2, 95% CO_2) and were obtained in a tightly sealed modular chamber (Billup-Rothberg) at 37°C. Next the pretreated fragments were kept at 2.0 kPa for 2.5 h (the average time of the laparoscopic operation).

Implantation of Endometriosis Fragments in SCID Mice

All of the mice had been ovariectomized at least 2 weeks before tissue implantation. The mice were randomly implanted with one type of sample. Samples with a volume of 30 mm^3 were implanted into a single SC pocket created in the abdominal walls of the mouse along the ventral midline just below the umbilicus. All surgical procedures were performed under general anesthesia using pentobarbital (0.06 mg/g; Shanghai New Asia Pharmaceutical). Twenty-four hours after implantation, estradiol benzoate (0.02 mL, 1 mg/mL, Shanghai General Pharmaceutical) was injected IM every day to promote implant growth.