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Pregnancy affects morphology of induced endometriotic lesions in a mouse model through alteration of proliferation and angiogenesis

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ABSTRACT

Objective: Pregnancy is known to alleviate the symptoms of endometriosis and is also known to be a pro-angiogenic condition affecting blood and lymphatic vessels. However, angiogenesis actively participates in the development of endometriosis. The objective of our study was to study the impact of pregnancy on endometriotic tissue.

Study design We performed a cross-sectional, control versus treatment study in a mouse model of endometriosis. Thirty-one female C57Bl6 mice were mated and became pregnant and 31 females were not mated and served as control. Intraperitoneal endometriotic lesions were surgically induced in C57Bl6 mice which were subsequently mated or not (group P: pregnant, group NP: non-pregnant). P and NP mice were sacrificed on day E15.5 of the pregnancy of P mice and lesions were harvested. Lesions were weighed and analyzed by histology, immunohistology, flow cytometry and real-time quantitative RT-PCR (qRT-PCR).

Results: Pregnancy reduced lesion weight, decreased the proportion of cystic component (0.02 vs. 0.4; $p < 0.001$) and modified the architecture of peritoneal endometriotic lesions. Pregnancy also increased cell proliferation in both stromal and glandular tissue as shown by the increase in Ki 67-positive cells in the P group (glandular: 19 vs. 3.9%, $p < 0.001$; stromal: 8.7 vs. 3.3%, $p < 0.01$). Finally, pregnancy increased angiogenesis in endometriotic lesions as indicated by an increased microvessel density (CD-31 and LYVE-1 stainings: respectively 2.2 vs. 5.1%, $p < 0.01$ and 0.4 vs. 0.9%, $p < 0.001$), an increased number of LYVE1 positive cells evaluated by flow cytometry (18.9 vs. 4.6%, $p < 0.05$) and a rise in VEGF-A, -R2 and -R3 RNA expression shown by qRT-PCR ($p < 0.001$; $p < 0.01$; $p < 0.05$).

Conclusion: These challenging results provide insight in understanding the pathophysiology of endometriosis and evoke a correlation between lesion architecture and symptomatology.

Introduction

Endometriosis, defined by the presence of endometrium-like tissue outside the uterine cavity, affects about 10% of reproductive-aged women and up to 50% of infertile women [1]. Symptoms associated with endometriosis include dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility. Although the pathogenesis of the disease is not fully understood, it has been demonstrated that steroid hormones and angiogenesis play a crucial role in its development [2–5]. Indeed, endometriosis is a typical angiogenic disorder [6–8].

It was first suggested that pregnancy had a positive effect on endometriosis in 1959 [9,10]. This assumption was based on observations that endometriotic symptoms improved during pregnancy. Reduction in symptoms might be subsequent to anovulation and amenorrhea preventing bleeding of endometriotic tissue but also related to various metabolic, hormonal, immune and angiogenesis changes associated with pregnancy [11,12]. Moreover, experimental studies demonstrated that progestagens administration inhibited estrogen-induced development of endometriosis and this observation led to the use of combined hormonal therapies to relieve symptoms [9,13–15].

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Human placenta secretes a number of pro-angiogenic factors regulating placental vessel formation and maternal vascular adaptation to pregnancy [16,17]. Vascular changes induced by pregnancy are well illustrated by the high incidence of spider angiomas, palmar erythema and gingival hyperplasia which regress spontaneously after delivery [18]. Moreover, experimental studies in mice showed that lymphatic and blood angiogenesis increased during pregnancy in mice with melanoma [19] and that fetal progenitor cells play a role in facilitating the wound healing process [20].

Finally, the effect of pregnancy on endometriosis is poorly understood. On one hand the relief of symptoms during pregnancy suggests an inhibition of endometriotic lesions, and on the other hand, angiogenesis enhancement linked to pregnancy may contribute to endometriosis development. Therefore, the aims of our study were to evaluate the effect of pregnancy on endometriotic lesions (cell proliferation, lymphatic and blood angiogenesis) in a mouse model of surgically induced endometriosis.

Materials and methods

Animals

All experiments were conducted according to the European Communities Council Directive (2010/63/UE) for the care and use of animals for experimental procedures and complied with the regulations of the French Charles Darwin Ethics Committee in Animal Experiments registered at the "*Comité National de Réflexion Ethique sur l'Experimentation Animale*" (Ile-de-France, Paris 5). All efforts were made to minimize pain according to best standard procedures.

Five-week-old (recipients) and 9-week-old (donors) female C57Black/6 (C57BL/6) mice (Janvier®) were used for the study. They were housed five per cage in a temperature-controlled environment under a 12 h/12 h light–dark cycle and received standard pellet food and water ad libitum.

Induction of peritoneal endometriosis

Endometriosis-like lesions were induced using a surgical technique modified from Vernon and Wilson and Cummings and Metcalf [21,22]. For syngeneic transplantation, 9-week-old C57BL/6 donor female mice were anesthetized by isofluorane inhalation (Aerane, Baxter). Both uterine horns were removed and transferred to a Petri dish containing Dulbecco's modified Eagle's medium (10% fetal calf serum, 100-U/ml penicillin, and 0.1-mg/ml streptomycin (Gibco®, Life Technologies) at 37 °C. The uterine horns were then opened longitudinally with micro-scissors under a stereomicroscope, and 2-mm tissue samples were removed using a dermal biopsy punch (Kai Medical®). Two tissue samples were transplanted with a 7-0 polypropylene suture (Prolene; Ethicon Products®) to the right and left abdominal walls of 5-week-old recipient female C57BL/6 mice after a 10–15-mm midline incision initiated 1 cm above the urethral opening. In accordance with a recent publication on the mouse model, the perimetrium of the 2-mm tissue samples was sutured to the peritoneum [23]. Finally, the peritoneum was closed with a 7-0 polypropylene suture and the skin with 4-0 polypropylene suture.

Study design

The study population was composed of 62 female C57BL/6J mice with surgically induced endometriosis.

Ten days after surgery, half of the transplanted mice were mated with syngeneic C57BL/6 males overnight. This led to two groups: 31 recipient pregnant mice (P group) and 31 matched recipient non-pregnant control mice (NP group) grafted the same day. The mice in both the groups were sacrificed at day 15.5 of gestation (E 15.5).

Histology

Endometriotic lesions were excised, weighed and analyzed.

Formalin-fixed specimens of endometriotic lesions were embedded in paraffin. Sections of 4-μm thickness were cut and stained with hematoxylin and eosin according to standard procedures and observed under microscope (Nikon eclipse 90i, Japan).

The ratio of cystic lesion was calculated as being the cyst area/total area of the endometriotic lesion.

Immunostaining

Immunofluorescence staining was performed on frozen sections in order to assess angiogenesis and cell proliferation at tissue level. After permeabilization (Triton X-100), sections were blocked using 20% normal goat serum. For the immunofluorescent microscopic detection of microvessels in endometriotic lesions, sections were stained with a rabbit purified anti-mouse antibody against the endothelial cell marker CD31 (1:40; BD Pharmingen, USA). For the detection of lymphatic vessels in lesions, sections were stained with a rat anti-mouse antibody against the lymphatic cell marker lymphatic vessel endothelial hyaluronan receptor (Lyve-1) (1:200; Abcam, UK). To investigate the proliferation of epithelial and stomal cells, sections were stained with a polyclonal goat anti-rabbit antibody against Ki67 (1:200; Abcam, UK). The secondary antibodies were the goat anti-rabbit IgG labeled with Alexa 488 and the goat anti-rat IgG labeled with cyanine 3 (1:1000; Invitrogen). Nuclei were counterstained with 0.3 μg/ml 4′,6-diamidino-2-phenylindole (DAPI).

Quantification of angiogenesis

Frozen tissues were double stained for Lyve-1 and CD31. Sections were then examined with a fluorescent microscope (Leica®, Deerfield, IL) and images were captured using a digital camera (SPOT®; Media Cybernetics, Silver Spring, MD). Total area of each section stained with DAPI was carefully delineated and measured using Image J° software. Total area labeled with the specific vascular antibody was measured using the same software. The calculation of the labeled vascular area related to the total section area gave the related area occupied by vessels. This analysis was performed blinded to pregnancy status.

Proliferation analysis

Tissues were double stained with DAPI and Ki67 antibody. The fraction of Ki67-positive stromal and glandular cells (in % of all cells counted thanks to DAPI counterstaining) was assessed in each section.

Flow cytometry

Flow cytometry was used to confirm the presence of the angiogenesis at cellular level and to quantify it. Tissues were digested in collagenase I (Life Technologies, UK) for 1 h at 37 °C, passed through a 40 μm cell strainer (BD Pharmingen, USA) to obtain a single-cell suspension. Antibodies used were directed against the lymphatic cell marker LYVE1 APC (1:100; eBiosience)