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Ovarian-like differentiation in eutopic and ectopic endometrioses with aberrant FSH receptor, INSL3 and GATA4/6 expression

Baptiste Fouquet [a], Pietro Santulli [a,1], Jean-Christophe Noel [b], Micheline Misrahi [a,*]

[a] Faculté de Médecine Paris Sud, Univ Paris Sud, Université Paris Saclay, Hôpital Bicêtre, Le Kremlin Bicêtre, France
[b] Erasme University Hospital, Department of Pathology, Université Libre de Bruxelles, Belgium

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ABSTRACT

Endometriosis, the hormone-dependent extrauterine dissemination of endometrial tissue outside the uterus, affects 5–15% of women of reproductive age. Pathogenesis remains poorly understood as well as the estrogen production by endometriotic tissue yielding autocrine growth. Estrogens (E2) are normally produced by the ovaries. We investigated whether aberrant "ovarian-like" differentiation occurred in endometriosis.

69 women, with ($n = 38$) and without ($n = 31$) histologically proven endometriosis were recruited. Comparative RT-qPCR was performed on 20 genes in paired eutopic and ectopic lesions, together with immunohistochemistry. Functional studies were performed in primary cultures of epithelial endometriotic cells (EEC).

A broaden ovarian-like differentiation was found in half eutopic and all ectopic endometriosis with aberrant expression of transcripts and protein for the transcription factors GATA4 and GATA6 triggering ovarian differentiation, for the FSH receptor (FSHR) and the ovarian hormone INSL3. Like in ovaries the FSHR induced aromatase, the key enzyme in E2 production, and vascular factors in EEC. The LH receptor (LHR) was also aberrantly expressed in a subset of ectopic endometriosis (21%) and induced strongly androgen-synthesizing enzymes and INSL3 in EEC, as in ovaries, as well as endometriotic cell growth. The ERK pathway mediates signaling by both hormones. A positive feedback loop occurred through FSHR and LHR-dependent induction of GATA4/6 in EEC, as in ovaries, enhancing the production of the steroidogenic cascade.

This work highlights a novel pathophysiological mechanism with a broadly ovarian pattern of differentiation in half eutopic and all ectopic endometriosis. This study provides new tools that might improve the diagnosis of endometriosis in the future.

1. Introduction

Endometriosis, characterized by the extrauterine dissemination of endometrial tissue, is a major public health problem affecting 5–15% of women of reproductive age. It has an incidence of up to 50% in women seeking treatment for pain and infertility, places a large economic burden on countries throughout the world and is functionally debilitating for affected women. The pathogenesis of endometriosis is still poorly understood but it is known to be a hormone-dependent disease. The survival and proliferation of lesions is dependent on estrogens (E2) produced by endometriotic implants which acquire aberrant endocrine function. E2 induces autocrine and paracrine growth pathways. This chronic disease is often diagnosed late, about 7–10 years after the apparition of the first symptoms during surgery [1–4] when lesions have already disseminated in the pelvis. There is no early non-invasive marker predicting the development of the disease. Endometriosis is considered benign disease, but has features common with cancers, such as progressive estrogen-dependent proliferation, peritoneal and pelvic invasion, associated with chronic inflammation and angiogenesis [1,5]. The primary mechanism of action of hormone therapy for endometriosis is the inhibition of estrogen production [2,3,6].

The mechanisms underlying the abnormal production of estrogens by endometriotic tissue are poorly understood. Inflammation is thought to be responsible for overexpression of the Steroidogenic Factor-1 (SF1) transcription factor and in situ E2 production in endometriosis [1]. However, E2 is normally produced by the ovary. The FSH receptor (FSHR) and LH receptor (LHR) play a critical role in this process by inducing respectively aromatase, which converts androgens into

Abbreviations: CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; CYP17, Cytochrome P450 Family 17 Subfamily A Member 1; CYP19A1, Cytochrome P450 Family 19 Subfamily A Member 1; EEC, Epithelial Endometriotic Cells; EGVEGF, Endocrine Gland-derived vascular endothelial growth factor; GATA4/6, GATA binding protein 4/6; INSL3, Insulin Like 3; LHR, Luteinizing Hormone Receptor; FSHR, Follicle Stimulating Hormone Receptor; PTGER, Prostaglandin E Receptor; PTGS2, Prostaglandin-Endoperoxide Synthase 2; RT-qPCR, Reverse Transcription quantitative Polymerase Chain Reaction; SF1, Steroidogenic Factor-1; VEGF, Vascular Endothelial Growth Factor.

* Corresponding author.
E-mail address: micheline.misrahi@aphp.fr (M. Misrahi).
[1] Present address: Groupe Hospitalier Cochin-Saint Vincent de Paul, Service de Gynécologie Obstétrique, et Université Paris Descartes, Sorbonne Paris Cité, Inserm, Unité de recherche U1016, Institut Cochin, INSERM CNRS (UMR 8104), Paris, France.

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estrogens, and steroidogenic enzymes responsible for the synthesis of androgens, which serve as substrates for aromatase [7]. This raises questions about the role of the FSHR and LHR in controlling endometriotic steroidogenesis and whether more general aberrant "ovarian-like" differentiation occurs in endometriosis.

Angiogenesis of the endometriotic cells in implanted places appears to be essential for the survival and development of endometriotic cells, as already reported for tumor growth [2]. FSHR is also expressed in tumor blood vessels [8] and most metastases [9]. It is thought to stimulate angiogenesis, thereby favoring tumorigenesis.

We investigate a possible "ovarian-like" differentiation in endometriosis. We thus studied FSHR and LHR expression in paired eutopic endometrium and ectopic lesions of patients with endometriosis by RT-qPCR and immunohistochemistry using monoclonal antibodies. To understand the aberrant expression of receptors we also studied the expression of other markers of ovarian differentiation and transcription factors necessary for gonadotropin receptor onset in the ovary. We also perform functional studies in primary cultures of epithelial endometriotic cells (EEC).

2. Materials and methods

2.1. Patients and study design

All the patients studied in this work have been described in a previous study [10]. The cohort recruited in this study consisted of 69 women with ($n = 38$) and without ($n = 31$) macroscopic endometriotic lesions already described [10]. The patients and controls groups were homogenous for age, gestity, parity, and phase of the menstrual cycle with the same number of patients being in proliferative or secretory phases [10]. All samples were histologically characterized for patients and controls. Patients with endometriosis provided both eutopic and ectopic endometrium. The samples were selected from 38 patients afflicted with painful endometriosis and operated for complete surgical exeresis of all endometriotic lesions. Endometriosis was categorized according to a previously published surgical classification based on the location of the worst endometriotic lesion location: superficial (SUP), endometrioma (OMA) ($n = 19$), and deeply infiltrating endometriosis (DIE) ($n = 19$). Endometriosis was scored according to the revised American Fertility Society classification [10]. Control endometrial specimens were collected from 31 patients without any macroscopic endometriotic lesion as checked during a thorough surgical examination of the abdominopelvic cavity. Indications for surgery in controls were the following: tubal infertility, non endometriotic ovarian cysts, or uterine myoma. The local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale of Paris- Cochin) approved the study protocol. All patients gave an informed written consent.

2.2. Total RNA extraction, reverse transcription and RT-qPCR

We used 47 tissue samples (32 paired eutopic and ectopic endometrioses, and 15 controls) for RT-qPCR on 17 genes of interest and 3 control genes. The characteristics of these selected patients have been described [10]. After surgical resection, samples of disease-free control biopsy specimens and eutopic or ectopic endometrium biopsy specimens were immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and spectrophotometry. We generated cDNA by reverse transcribing 4 μg of total RNA with random primers and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. We treated the cDNA with RNase before RT-PCR. The primers used for RT-PCR analysis were selected with PRIMER3 software, on the basis of published sequences. The primers were produced by Eurogentec (Angers, France). Quantitative PCR was carried out on a 96-well Light Cycler 480 machine (Roche Diagnostics, Manheim, Germany), with a Light Cycler 480 SYBR Green I Master kit (Roche). Results were analyzed with Light Cycler software, by the three-fit point method. Relative abundances in endometriotic tissues were calculated by the $-2\Delta\Delta Ct$ method with respect to the mean for the three control genes (mean for 3 housekeeping genes SDHA, HPRT and HISTH1A genes) and normalization with the mean value for a pool of genes from control endometria. We also studied FSHR and LHR expression in 12 additional patients with endometriosis and six supplementary controls from the same cohort already described [10]. Sequences of primers are available on request.

2.3. Immunohistochemistry

Immunohistochemistry was performed on 10 matched ectopic endometriosis and 10 control disease-free endometria (for each 5 in proliferative phase and 5 in secretory phase). The patients belong to a cohort already described [10]. A previously described antigen retrieval method was used [11]. Briefly, the samples were embedded in paraffin and 4-μm sections were cut sequentially and mounted on SuperFrost treated slides (Menzel-Glasse, Braunschweig, Germany). The slides were allowed to dry overnight at 37 °C. The paraffin was then eliminated by immersion in xylene and the sections were rehydrated in a graded series of ethanol solutions. For epitope retrieval, the slides were immersed in citrate buffer pH 6.0, in a water bath at 95 °C to 99 °C for 60 min. The slides were then allowed to cool in the buffer for 20 min at room temperature. H_2O_2 (0.3%) was added to the slides, which were then incubated at room temperature for 30 min. They were then incubated with the primary antibodies for 2 h. The NovolLink max Polymer Detection System (Newcastle, UK) was used for the subsequent steps, according to the manufacturer's instructions. Diaminobenzidine-hydrogen peroxide or aminoethyl carbazole (Clinisciences,FR) were used for chromogenic development. The slides were lightly counterstained with hematoxylin, dehydrated, and covered with a coverslip. For control IgG, replacement of the specific primary antibody was performed on serial sections incubated with corresponding pre-immune immunoglobulins (mouse and rabbit IgG, Sigma Aldrich) at the same concentration used for the specific primary antibody.

We controlled also for nonspecific binding of the primary antibody preparation, by replacing the first layer of serial sections with non specific slides. Normal human ovarian granulosa cells and/or theca cells served as positive controls. The following antibodies were used: monoclonal antibody against LHR (clone 29, dilution: 1/200) [12], monoclonal antibody against FSHR (clone 323, dilution: 1/300) [12], and polyclonal antibodies against aromatase (H4, dilution: 1/50) (Acris Antibodies, San Diego, USA), CYP 17 *A*1 (M-80-SC/66850, dilution: 1/100) (Santa Cruz Biotechnology Inc., USA), CYP11A1 (13363-1-AP, dilution 1/100) (Proteintech, USA) , INSL3 (ab65981, dilution 1/100) (abcam, FRA), GATA6 (ab22600, dilution 1/100) (abcam, FRA), and GATA 4 (SC:25310, dilution: 1/50) (Santa Cruz Biotechnology Inc., USA).

2.4. Primary cultures

Primary cultures of epithelial endometriotic cells (EEC) from women with and without endometriosis were obtained from 12 consecutive patients, as previously described [10,13]. All cells were cultured and treated with hCG or rFSH as described [13–15]. Briefly, for each of these patients, a surgical biopsy was performed from DIE. Specimens were collected under sterile conditions and transferred, in DMEM (Dulbecco's modified Eagle medium, Gibco Invitrogen, Cergy Pontoise, France), to the laboratory, where they were prepared for cell extraction or in vivo experiments within 30 min. Biopsy specimens were rinsed, minced into small pieces, digested with dispase and collagenase (2 mg/ml, Gibco Invitrogen, Cergy Pontoise, France) for 1 h at 37 °C and separated by serial filtration. Red blood cells were removed by