

Detection of aromatase in human endometrial tissue cultured in three-dimensional fibrin matrix in vitro

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Abstract

Background: Endometriosis is the presence of endometrial glandular and stromal cells outside of the uterine cavity. Our previous study showed that in vitro culture of human endometrial tissue in a three-dimensional (3D) fibrin matrix could mimic the early stages of endometriosis with invasion, gland and stroma formation and sprouting of new vessels.

Objective: The objective of the present study was to evaluate the expression of aromatase in in vitro cultured endometrial fragments.

Materials and Methods: Endometrial samples obtained from the fundus of the uterine cavity of ten normal ovulating women on cycle days 19-24 that referred to Toronto Center for Advanced Reproductive Technology (T.C.A.R.T) for infertility treatment and cultured in vitro by a three-dimensional fibrin matrix culture system. Cultures were performed in 24-well culture plates.

Results: After 4 weeks, the tissues were fixed and blocked for immunostaining. They were stained by anti-aromatase antibodies. Although a fine anti-glycodelin positive glandular epithelium and anti-cyclooxygenase-2 (COX-2) positive new vessels were detected and reported in our previous study, we were unable to detect any aromatase activity in the cultured fragments.

Conclusion: No aromatase activity was detected in the 3D cultured endometrial tissue. Although it is reported that aromatase is expressed in endometrial disease, it is possibly not expressed in this phase in normal endometrium.

Key words: Endometriosis, In vitro culture, Three-dimensional culture, Aromatase.

Introduction

Endometriosis is the presence of endometrial glandular and stromal cells outside of the uterine cavity. It is estimated to affect 2–10% of women in the reproductive age group (1). The endometrium is unique among adult tissues because it undergoes intense proliferation, secretion, regression, and regeneration during each menstrual cycle. At menstruation, endothelial cells sprout out from the ruptured spiral arterioles and venules and

recruit other cells: Pericytes for vessels to become capillaries and smooth muscle cells for the larger blood vessels (2). It is now clearly evident that angiogenesis plays a key role in reproductive processes (3). Excessive endometrial angiogenesis is proposed as an important mechanism in the pathogenesis of endometriosis and cancer. Angiogenesis and endometrium proliferation are the fundamental steps in regeneration of normal endometrium and establishment of endometriosis (4). Aromatase expression was studied in greatest detail in establishment of endometriosis (5-7). Firstly, extremely high levels of aromatase mRNA were found in extraovarian endometriotic implants. Secondly, endometriosis-derived stromal cells in

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culture incubated with a cAMP analog displayed extraordinarily high levels of aromatase activity comparable to that in placental syncytiotrophoblast (5). These exciting findings led us to test a battery of growth factors, cytokines and other substances that might induce aromatase activity via a cAMP-dependent pathway in endometriosis (5). Aromatase is the key enzyme for estrogen production. There is an 'intracrine' effect of estrogen in uterine leiomyomas and endometriosis (5, 8, 9). In endometriosis-derived stromal cells, it appears that the stimulation of aromatase activity by PGE₂ may be mediated by a cAMP-dependent pathway (EP₂ receptor binding), as Bt₂cAMP also induces aromatase expression in these cells (5). In fact, this PGE₂ effect was found to be mediated via the cAMP-inducing EP₂ receptor subtype. Moreover, estrogen was reported to increase PGE₂ formation by stimulating cyclo-oxygenase type 2 (COX-2) enzymes in endometrial stromal cells in culture (10). Our group has reported the expression of glycodelin (Gd) and cyclooxygenase-2 (COX-2), two angiogenic factors which correlate with aromatase expression in endometrial diseases. The aim of the present study was to detect aromatase activity in in vitro cultured endometrial fragments from non-endometriosis patients.

Materials and methods

Patients

Endometrial tissue in premenopausal patient's women referred to T.C.A.R.T for infertility treatment. Exclusion criteria included any endometrial abnormality (endometriosis, polyps, hyperplasia, or cancer), and administration of any hormones, GnRH agonist therapy, or intrauterine device (IUD) within last 3 months. The Research Ethics Committee of T.C.A.R.T authorized the use of fragments of human endometrium as described. A written informed consent describing the procedures and aims of the study was obtained from each donor in compliance with regulations concerning the use of human tissues. Endometrial samples were collected from a total of ten normal ovulating women on cycle days 19-24 and every patient 10 wells (100 wells). The biopsies were obtained from the fundal region of the uterine cavity using an endometrial sampling device (Endocell; Wallach Surgical Devices Inc., Orange, CT). In all patients, accurate menstrual dating was carried out according to the last menstrual period in the early proliferative phase of the cycle and appropriate histological dating of each biopsy confirmed the endometrium as proliferative (11).

Materials

Medium 199 for cell culture was purchased from GIBCO (Burlington, ON, Canada); cell culture supplements and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Plastics for cell culture were supplied by Falcon (Becton Dickinson Labware, Franklin Lakes, NJ). monoclonal horse serum and red fluorescent stain (Chicago Sky Blue, Sigma, MO, USA, C-8679), mouse antihuman Gd A primary antibody (Antibodyshop, Denmark, Cat # BTE 001-13), polyclonal rabbit immunoglobulin G (IgG) antihuman Cox-2 primary antibody (Cedarlane Laboratories, Canada, Cat # PG 27 B), mouse anti human cytochrome P450 aromatase primary antibody (Serotec, UK, Cat # MCA2077S), donkey and mouse IgG, fluorescein isothiocyanate (FITC) conjugated (Chemicon International, CA, USA Cat # AP192F), mouse IgG1 as negative control (Dako Cytomation, Denmark, Cat # X 0931), 4, 6-diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Target retrieval solution (DakoCytomation, Denmark, Code S1700). Olympus IX-70 inverted fluorescence microscope with custom optical filters.

Human endometrial tissue in 3-D in vitro model

All endometrial biopsies collected were placed in cold sterile phosphate-buffered saline (PBS) solution containing 2.5 µg/mL of amphotericin B plus 50 µg/mL of gentamycin and immediately cut into approximately 1-mm fragments using fine dissecting forceps and a scalpel. These explants were cleared of residual clots and placed in PBS before their use. Ten tissue fragments were cultured for each patient. Cultures were performed in 24-well culture plates; 0.5 mL/well of a solution of fibrinogen 3 mg/mL in Medium 199 was added to each well and mixed with 15 µL of thrombin (50 NIH U/mL in 0.15 M NaCl). Endometrial fragments were quickly placed in the center of the wells after clot formation and covered by an additional 0.5 mL/well of the fibrinogen/thrombin solution, to hold them at the same level between the two clots. After gel formation, 1 mL/well of Medium 199, supplemented with 5% of heat-inactivated fetal bovine serum, 0.1% ϵ -aminocaproic acid, L-glutamine (2 mM) and antibiotics (streptomycin 50 µg/mL, penicillin 50 IU/mL, and amphotericin B 2.5 µg/mL). Explants were cultured at 37°C in 95% air/5% CO₂ in a humidified environment for 4 weeks, and the medium was changed every 3 days.

Histology and immunofluorescent

The tissues were fixed and block in the fibrin 3-dimensional matrix in 10% buffered formalin, processed into paraffin blocks, cut into 5 micrometer paraffin sections, and put on microscope slides. The sections on slides were deparaffinized in xylenes and rehydrated in graded ethanol series.

Immunofluorescent for human cytochrome 450 aromatase

After blocking nonspecific binding with 10% normal horse serum, and staining background with red fluorescent stain (Chicago Sky Blue, Sigma, MO, Cat # C-8679), mouse IgG2 anti human P450 aromatase primary antibody (Serotec, UK, Cat # MCA2077) was applied (dilution 1:50). Some sections were treated with mouse IgG1 (Dako Cytomation Denmark, Code-# X 0931) as negative control for the first antibody (dilution 1:50). Slides with sections of human placenta prepared in the same manner were used as positive control. Secondary antibody, donkey anti mouse IgG, FITC conjugated (Chemicon International, CA, Cat # AP192F) was applied at 1:200 dilution. On some sections the secondary antibody was omitted for negative control.

Slides were examined on Zeiss Axioplan Photomicroscope equipped with fluorescent ultraviolet light and corresponding excitation and barrier filters. Pictures were taken on a Nikon Coolpix4500 digital camera or on a Delta Vision wide-field, optical sectioning microscope workstation capable of recording three-dimensional images of fluorescently labeled specimens (Issaquah, Washington). The station includes: an Olympus IX-70 inverted fluorescence microscope with custom optical filters, and precision XYZ motorized stage, O2 silicon Graphics computer work station with image collection and deconvolution software. The final figures were assembled using the Photoshop 6.0 software.

Statistical analysis

Statistical analysis was performed by chi-square using SigmaStat Version 1.0 (SigmaStat Software HighEdit Professional Copyright 1993, MicroHelp Inc. and HeilerSoftware GmbH, San Rafael, CA, USA). A p-value of <0.05 was considered significant.

Results

Three-dimensional culture of human endometrial tissue in fibrin matrix resulted in angiogenesis, glandular epithelium and proliferated endometrial cells after 2–3 weeks (Figure 1). Cell proliferation was observed in 91% of the wells. Angiogenesis was observed in 51 wells that showed cell proliferation (56%) and endometrial glands were observed in 40 wells with cell proliferation (44%) (Figure 2). No positive aromatase activity was found in the wells after 4 weeks of culture (Figure 3).

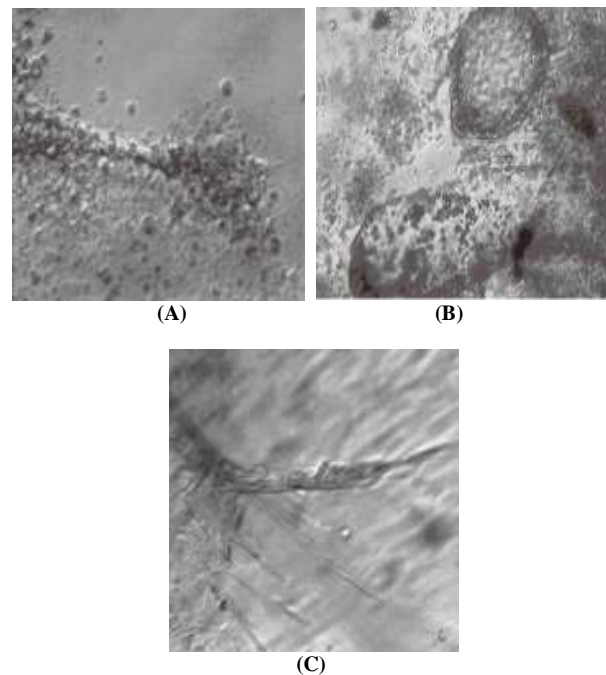


Figure 1. Microscopic phase contrast pictures of stromal cell invasion into the fibrin matrix during the first week of culture (A), microscopic phase contrast picture of typical endometrial glands in a control well (B) and appearance of rudimentary capillary-like structures consistent with angiogenesis after 2–3 weeks in culture (C).

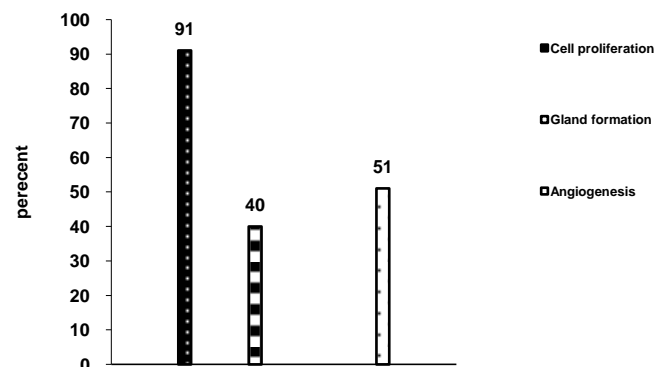


Figure 2. Cell proliferation, gland formation and angiogenesis in three-dimensional cultured endometrial fragments. Bar indicate percentage.

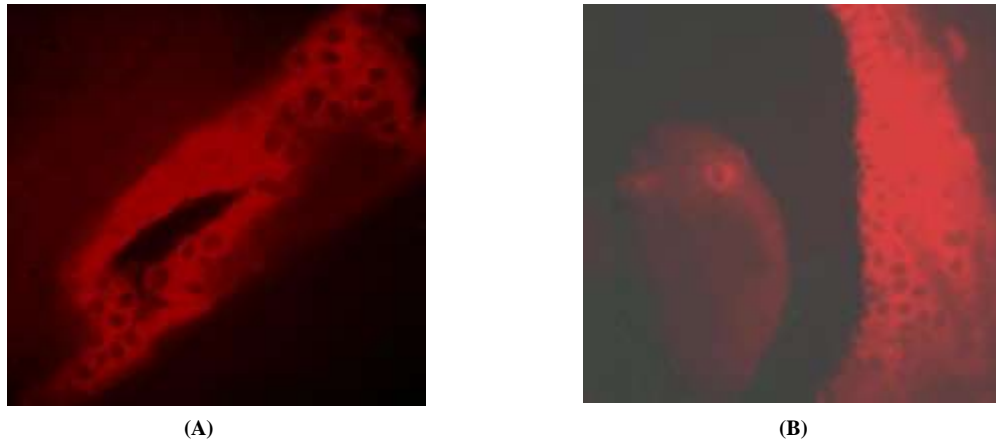


Figure 3. Positive immunofluorescence reaction for aromatase (A), without any reaction staining and negative control for aromatase (B).

Discussion

In this study, we sought to describe the expression of aromatase in human endometrial tissue in 3D culture. Considering that proliferation, invasion and angiogenesis are likely important mechanisms in the pathogenesis of endometriosis, this study may provide further validation of the 3D culture model of endometriosis. Aromatase mRNA was detected in the eutopic endometrial samples of women with moderate to severe endometriosis (8). When defective endometrium with low levels of aberrant aromatase expression reaches the pelvic peritoneum by retro-grade menstruation, it causes an inflammatory reaction that exponentially increases local aromatase activity, estrogen formation, induced directly or indirectly by PGs and cytokines. This may be a major contributing factor in the formation of endometriotic implants (5-10). The molecular mechanism by which aromatase cytochrome P450 is expressed in the diseased endometrium (7).

The clinical relevance of aromatase expression in endometriosis was shown recently by the successful treatment of an unusually aggressive case of postmenopausal endometriosis with use of an aromatase inhibitor (12). It was suggested that aromatase expression may be an endometrial marker for diagnosing endometriosis in late stage (13).

We were unable to detect any aromatase activity in our culture system using endometrial fragments from non-endometriosis patients and our finding indirectly supports the hypothesis on the contribution of aromatase in endometriosis. We aim to assess the aromatase expression in endometrial tissues from endometriosis patients as a next step of our study to have a better understanding of mechanisms by which

endometriosis is established. This will help us to find a reliable preventive or curative therapy for endometriosis.

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