Semi-invasive Diagnosis of Endometriosis
Doctoral Thesis

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Introduction

Endometriosis is a common, benign, oestrogen-dependent, gynaecological disorder associated with pelvic pain and infertility. While endometriosis has been described for more than one hundred years, our current knowledge of its pathogenesis remains unclear.

The ectopic transplantation of endometrial tissue, originally proposed by Sampson in 1924, is the most widely accepted theory on the pathogenesis of endometriosis. It claims that the disorder originates from retrograde menstruation of endometrial tissue sloughed through patent fallopian tubes into the peritoneal cavity. Retrograde menstruation occurs in 70% to 90% of women and may be more common in women with endometriosis than in those without the disease. The presence of endometrial cells in the peritoneal fluid, indicating retrograde menstruation, has been reported in 59% to 79% of women during menses or in the early follicular phase. Therefore the critical evaluation of the retrograde menstruation and transplantation theory is crucial in the understanding of the pathomechanism of endometriosis.

The current clinical opinion is that laparoscopy is required for definitive diagnosis of endometriosis.

The average delay between the onset of pain symptoms and surgically confirmed endometriosis is quite long: mean 8 years in the United Kingdom and 9 to 12 years in the United States.

It has been known for some time that the myometrium, the endometrial–myometrial interface and the deeper portion of the basal endometrium can be innervated by nerve

fibres, but that nerve fibres are absent from the superficial two-thirds of the endometrium (the functional layer) in the normal human uterus. The function of these nerve fibres in normal basal endometrium is not well understood. However, some acetylcholinesterase (AChE)-immunoreactive nerve fibres were detected in the basal layer of normal human endometrium. Neuropeptide -(NPY), substance P- (SP), vasoactive intestinal peptide-(VIP) and neurotensin (NT)- immunoreactive nerve fibres were also present in normal human endometrium. Yet little is known about the functions of these nerve fibres in human endometrium.

Due to the lack of a no–or semi–invasive diagnostic tool, the delay between onset of pain symptoms and surgically confirmed endometriosis is long. The current delay in diagnosis and treatment contributes to years of suffering and potential infertility if the disease is left untreated. Clearly, a simple noninvasive diagnostic method may greatly help to reduce this delay, especially for minimal–mild endometriosis which cannot be diagnosed by clinical examination or ultrasound.

Based on the fact that eutopic endometrium from women with endometriosis is biologically different from women with a normal pelvis a semi–invasive diagnostic test for endometriosis can potentially be developed in endometrium obtained after transcervical endometrial biopsy.

This demonstration of small nerve fibres in the functional layer of eutopic endometrium of women with endometriosis is so striking in the present study that we believe it could become a relatively simple surrogate marker of this condition using endometrial biopsies.

Aims

1. The aim of our study was to test the hypothesis that multiple sensory small diameter nerve fibers are present in a higher density in endometrium from patients with endometriosis when compared to women with a normal pelvis.
2. The assumed difference enables the development of a semi-invasive diagnostic test for minimal-mild endometriosis.
3. In our second study we have tested the hypothesis that menstruation is associated with a higher concentration of endometrial cells in peritoneal fluid (PF).
4. To prove that endometriosis is associated with an active immunologic process with increased white and red blood cell concentration in PF when compared to nonmenstrual phases of the cycle.

Material and Methods

Secretory phase endometrium samples (n=40), obtained from women with laparoscopically/histologically confirmed minimal-mild endometriosis (n=20) and from women with a normal pelvis (n=20) were selected from the biobank at the Leuven University Fertility Centre. Immunohistochemistry was performed to localise neural markers for sensory C, Aδ, adrenergic and cholinergic

nerve fibers in the functional layer of the endometrium. Sections were immunostained with antihuman protein gene product 9.5 (PGP9.5) anti-neurofilament protein (NF), anti-substance P (SP), anti-vasoactive intestinal peptide (VIP), anti-neuropeptide Y (NPY), and anti-calcitonine gene-related polypeptide (CGRP). We used normal human skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibers expressing PGP9.5, VIP, SP, CGRP NPY, and NF. Rabbit and mouse immunoglobulin fractions were used as respective negative controls, the concentrations were matched with the concentrations of the antibodies.

Assessment of nerve fibre density was performed using image analysis software KS400 3.0 (Zeiss, Göttingen, Germany) linked to a Zeiss microscope (Axioskop 50) fitted with a Zeiss color camera (Axiocam MRc5). The evaluation of all immunohistochemical stainings was done blindly by the evaluation of the whole surface of each section on high power images (objective 40x, optovar 1, resolution 860x644 Px) of adjacent non overlapping fields from left to right and from top to down. Patients and sample collection for the PF study:

PF was obtained at laparoscopy from 107 women with endometriosis (n= 59) and controls with a normal pelvis (n= 48) during the luteal (n=46), follicular (n=38) or menstrual (n=23) phase of the cycle. Endometriosis was classified according to the classification of the American Society for Reproductive Medicine (rAFS into minimal (n=25), mild (n=20), moderate(n=6) and severe(n=8) disease. Cell counts (leucocytes, erythrocytes, thrombocytes) were determined on a cell counter. In a subset of 32 patients (13 controls and 19 women with

endometriosis), PF was fixed, processed and thinlayers were prepared and stained with Papanicolaou method and with immunocytochemistry using monoclonal antibodies against cytokeratin 7(CK 7), CK 8/18, Ber-Ep4, vimentin, calretinin and CD68. Ber-Ep4 is a marker for cells with epithelial origin (in some cases for mesothelial cells as well). CD68 is specific for cells from monocyte/macrophage lineage; CK7 and CK8/18 are markers for both endometrial epithelial and mesothelial cells, whereas calretinin and vimentin are markers for both endometrial stromal and mesothelial cells.
Statistical analysis was done using Mann-Whitney test, Receiver Operator Characteristic (ROC) analysis, Stepwise Logistic Regression and Least Squares Support Vector Machines (LSSVMs).

Results

The density of small nerve fibres was about 14 times higher in endometrium from patients with minimal-mild endometriosis(1.96±2.73) when compared to women with a normal pelvis (0.14±0.46, p <.0001).
Using Leave–One–Out cross validation (LOO–CV) analysis with LS–SVM modelling, the best result was obtained when selecting the top 3 neural markers based on their p–value (Mann–Whitney U–test). A LS–SVM model, built on the complete data set with the top 3 neural markers VIP, PGP9.5 and SP had an AUC of 0.99, (SE 0.01). After choosing an operating point, this model allowed the diagnosis of endometriosis with a sensitivity of 95%, specificity of 100%, accuracy of 97.5%, PPV of 100%, and NPV of 95%, corresponding to one

endometriosis patient classified as control by the model (i.e. false negative).
In comparison with the nonmenstrual phase of the cycle, analysis of PF during menstruation showed an increased concentration of leucocytes ($3.3x10^9$/L vs $0.8x10^9$/L, P = 0.03), erythrocytes ($0.3x10^{12}$/L vs $0.02x10^{12}$/L, P = 0.006), hematocrit (0.03 L/L vs 0.003 L/L, P = 0.01) and hemoglobin (0.8 g/dL vs 0.1 g/dL, P = 0.01). Mesothelial cells stained positively with CK7, CK8/18, vimentin, and calretinin. Cells positive for Ber-Ep4 were not observed, except in 2
patients with endometriosis investigated during menstruation.

Conclusions:

1. The density of small nerve fibres is significantly higher in endometrium from patients with minimal-mild endometriosis when compared to women with a normal pelvis
2. The combined analysis of neural markers PGP9.5, VIP, SP could predict the presence of minimal-mild endometriosis with 95% sensitivity, 100% specificity, and 97.5 % accuracy.
3. The results of our second study demonstrate for the first time that menstruation in women is associated with an increased PF concentration of leucocytes, erythrocytes and hemoglobin when compared to nonmenstrual phases of the cycle, supporting the concept of retrograde menstruation.
4. An increased PF concentration of PF endometrial cells was not observed during menstruation when compared to nonmenstrual phases of the cycle.

Articles related to the thesis

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