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Promotion of angiogenesis and proliferation cytokines patterns in peritoneal fluid from women with endometriosis

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ABSTRACT

Studies have long sought specific cytokines that could characterize endometriosis. Either due to variations between study designs regarding the assessment criteria for the cytokine or to low power resulting from small sample size, no factor proved to be sufficiently specific to endometriosis. In other clinical fields, a combination of several markers proved to be more powerful than a single-molecule approach. As well, in the context of endometriosis, simultaneous assessment of several cytokines present in the peritoneal fluid might help in unveiling patho-physiological processes, thus contributing to a better understanding of the condition. Therefore, the objective of this study was to investigate peritoneal fluid cytokines-derived of endometriotic women. For this retrospective case-control study, peritoneal fluid samples were obtained at laparoscopy and assessed by multiplex. Our data showed distinct patterns of peritoneal fluid cytokine concentrations in endometriotic women most notably a marked increase in EGF, FGF-2, IL-1α, MIP-1β, TGFα, PDGF-AA, PDGF-BB, MCP-3, sCD40L, Gro Pan, IL-17α, MDC and Rantes. The overall effect of fertility status revealed a significant difference for only one cytokine, namely MDC. Furthermore, FLT-3L and IP-10 levels were decreased in endometriosis patients, the former in both menstrual cycle phases and the latter in the secretory phase. A significant inverse Pearson correlation ($p < 0.05$) was noted between pro-angiogenic cytokines EGF and FGF and the anti-angiogenic cytokine IP-10 in endometriosis patients at stages III–IV and in the secretory phase. These changes may exacerbate the local peritoneal angiogenic and proliferative reaction observed in women with endometriosis, and contributes to its pathophysiology.

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1. Introduction

Endometriosis is a common and sometimes debilitating condition for women of reproductive age (Giudice and Kao, 2004). It is defined as the presence of endometrial-like tissue outside the uterus also called endometriotic implants (Kennedy et al., 2005). Although multiple theories exist regarding the etiology of endometriosis, the implantation hypothesis is the most commonly accepted (Sampson, 1927). Shed endometrial fragments are believed to adhere to peritoneal surfaces, proliferate and invade deeply into the subperitoneal space. A variety of genetic (Lee et al., 2015; Sapkota et al., 2015; Zondervan et al., 2001), neuronal (Zevallos et al., 2015; Newman et al., 2013), hormonal (Melin et al., 2013; Budinetz and Sanfilippo, 2010), immune (Ahn et al., 2015) and inherent endometrial changes (Rakhila et al., 2015; Rakhila et al., 2013) may support the development of endometriotic implants within the peritoneum of affected women and the progression of the disease. Although a plethora of available literature, causes behind the development of endometriotic implants still remain unknown.

Studies have long sought specific biological markers that could characterize endometriosis. May and colleagues performed a systematic review to critically assess the clinical value of markers retrieved from endometrial tissue, menstrual or uterine fluids. All of the 182 studies included in the review had visual and/or histological confirmation of endometriosis from laparoscopy or laparotomy (May et al., 2011) while each study focused on individual, or small groups of molecules, such as glycoproteins (e.g., CA125) (Xavier et al., 2005), inflammatory (e.g., CCL8) (Barcz et al., 2012) or non-inflammatory (e.g., CXCL10) (Galleri et al., 2009)

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cytokines, adhesion molecules (e.g., E-Cadherin) (Darai et al., 1998), angiogenic (e.g., CCL5) (Fang et al., 2009) or growth factors with their receptors (e.g., EGF) (Matalliotakis et al., 2003). Either due to variations between study designs regarding the assessment criteria for the markers or to low power resulting from small sample size, none of the surveyed factors proved to be sufficiently specific to endometriosis. Thus, despite the need for a better understanding of the pathophysiology of endometriosis, a useful molecular outline of this condition remains elusive.

In several clinical research fields, including cardiovascular risk (Adamcova et al., 2013), cancer (Laxman et al., 2008), or pneumonia (Martin-Loeches et al., 2015), a combination of several markers proved to be more powerful than a single-molecule approach, for biomarker development. As well, in the context of endometriosis, simultaneous assessment of several cytokines present in the peritoneal fluid might help in unveiling patho-physiological processes, thus contributing to a better understanding of the condition.

In this study, we aimed to generate and compile the most comprehensive panel of cytokines to date, in peritoneal fluid of women with or without endometriosis. The results highlight a profile of cytokines tipping the balance toward tissue proliferation and angiogenesis.

2. Material and methods

2.1. Study design

The Institutional Review Board (IRB) of Université Laval approved this retrospective case-control study (project identification: 2016-2406). We collected peritoneal fluid (PF) samples from women with and without endometriosis for evaluation of cytokine concentrations in these samples, using a multiplex array. We included 74 endometriosis patients and 20 reproductive age women without endometriosis. Endometriosis patients were classified based on the stage of the disease as per the American Society for Reproductive Medicine (ASRM, 1996) classification (stages I–II, $n = 40$; stages III–IV, $n = 34$) according to the surgeon expertise during laparoscopy (URL: http://endometriosisinfo.ca/documents/11FORM_ClassificationForEndo_EN.pdf), as well as according to the cycle phase (follicular, $n = 37$; luteal, $n = 37$). Age-matched control patients were being surgically treated for tubal ligation ($n = 15$) or reanastomosis ($n = 5$) and having no visible evidence of endometriosis at the laparoscopy. All patients signed an informed consent form in accordance with the declaration of Helsinki. We excluded patients, from either group, who presented any kind of concomitant neoplasia, autoimmune disease, peritonitis for causes other than endometriosis and patients who had used hormonal medication during the previous three months, as well as patients whose sample amount was not sufficient.

2.2. Sample collection and processing

Peritoneal Fluid (PF) was collected from patients with and without endometriosis undergoing surgical treatment at the Centre Hospitalier Universitaire de Québec, between 1996 and May 2015. PF were obtained at the time of laparoscopy by aspiration using a laparoscopic cannula before any surgical intervention in order to minimize blood contamination. Peritoneal fluid samples were excluded if bleeding into the pelvic cavity occurred during puncture. The peritoneal fluid sample was placed in a sterile tube, kept on ice until arrival at the laboratory, and immediately centrifuged at 4 °C for 10 min at $2000 \times g$. Cell-free supernatant was then recovered, separated into small aliquots and stored at −80 °C until assayed.

2.3. Cytokine measurements

We used the Human Cytokine Array/Chemokine Array 41-Plex Discovery Assay, which include chemokines, growth factors, hematopoeitins, interferon, tumor necrosis factors and interleukins. This assay is based on a MILLIPLEX® MAP assay kit, according to the manufacturer's instructions (Millipore Canada Ltd., Etobicoke, ON). Briefly, Milliplex utilizes a multiplexing laser bead technology that is based on fluorescently labeled magnetic bead populations that are distinguishable from each other. Each sample is incubated with the multiplexed beads in a single microwell. A detection antibody cocktail containing biotinylated antibodies against each target analyte, is then introduced. The reaction mixture is then incubated with Streptavidin-Phycoerythrin conjugate, the fluorescent reporter molecule, to complete the reaction on the surface of each microsphere. The beads are then analyzed with a Bio-Plex 200® (Bio-Rad Laboratories Canada Ltd., Mississauga, ON). In the Bio-Plex® 200 the one laser activates the fluorescent label within each bead to identify the specific bead/target analyte. A second laser excites the reporter molecule that has been bound to the beads during the assay. The amount of reporter detected by the Bio-Plex 200 is in direct proportion to the amount of the target analyte. Results are quantified according to a standard curve. The exhaustive list of the 41 analytes is presented in Table 1.

2.4. Statistical analyses

Statistical computations were conducted with SAS version 9.3 (NC, USA), and statistical significance was assessed with an alpha level of $p < 0.05$.Women's age groups were compared using Student's *t*-test. The cycle phase was described as groups using absolute and relative frequencies and, to verify the existence of association, we used the Chi-squared test and the likelihood ratio respectively. The stage was also described among the women with endometriosis using absolute and relative frequencies. The chemokine levels were described according to groups, cycle phase, and stage of the disease using summary measures and compared among categories using the Mann–Whitney test. Cytokines levels were compared using the Wilcoxon-Mann-Whitney test, followed by Dunn's multiple comparisons test.

3. Results

3.1. Univariate analysis

3.1.1. Endometriosis versus normal

We measured the levels of 41 soluble factors in the peritoneal fluid from endometriosis and control patients. As can be appreciated from the 2D radar chart (Fig. 1A) endometriosis patients exhibited a larger pattern than that observed for control group. While comparable increases can be observed in the different classes of factors, PDGF-BB, PDGF-AA, EGF, sCD40L, IL-8, IL-17 and RANTES were particularly elevated in the endometriosis group. However, only the concentration levels of EGF ($p < 0.01$), FGF-2 ($p < 0.05$), IL-1α ($p < 0.01$) and MIP-1β ($p < 0.05$) were significantly higher in the PF of women with endometriosis compared to the control group. Also, it is worth mentioning that MCP-3, MDC, PDGF-AA and IL-6 also showed an interesting pattern even though no significant difference was reached, based on Wilcoxon Mann–Whitney test.

3.1.2. Analysis of early and late stages

We then performed a comparative analysis between normal, early, and late stages endometriosis patients. Cytokine expression's alterations appeared particularly important in late stages of endometriosis, as visualized by the heat map (Fig. 1B). Statistical analysis revealed significant increases in late stages for 15

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