Identification of local angiogenic and inflammatory markers in the menstrual blood of women with endometriosis

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1. Introduction

Endometriosis is a benign gynecological disorder characterized by the presence and growth of endometrium-like tissue in sites outside the uterine cavity, primarily on the pelvic peritoneum and ovaries [1–4]. In spite of affecting 5 to 10% of women in reproductive age, the exact etiology of the disease remains an intriguing enigma, since it is currently accepted that multiple factors are involved in the pathogenesis of endometriosis [2–8]. In addition to an invasive phenotype of endometrial cells, neoangiogenesis and sustained local inflammatory response are believed to be crucial points in the pathophysiology of endometriosis [9–15]. Neoangiogenesis is evident by the visualization of peripheral vascularization of ectopic endometriotic tissue at laparoscopy and increased angiogenic activity and vascular endothelial growth factor (VEGF) levels found in peritoneal fluid of patients with endometriosis [3,11,12,16–18].

Immune dysfunction has been identified in these patients either as a result or as a consequence of disease [7,19–21]. In recent years, alterations in both cell-mediated and humoral immunity have been observed in monkeys and in women with endometriosis [17,21–24].

Myeloperoxidase (MPO), an enzyme restricted to the azurophil granules of neutrophils, has been extensively used as a marker of polymorphonuclear leukocytes accumulation in tissue samples [25–30]. N-acetyl-β-D-glucosaminidase (NAG), present in lysosomes, has been employed to detect macrophage accumulation/activation in a variety of animal and human tissues [26–31]. Both NAG and MPO evaluated in infertile women with endometriosis undergoing in vitro fertilization, showed a distinct pattern of macrophage/neutrophil activation in the serum and follicular fluid. This suggests the possibility of an altered immunologic function in the follicular fluid of patients with endometriosis, which could contribute to infertility in these women [28].

Tumor necrosis factor-α (TNF-α) plays a pivotal role in the establishment and maintenance of endometrial deposits [32] and high concentrations in the peritoneal fluid of women with endometriosis suggest local release from activated peritoneal macrophages [32–34]. Published evidence shows that TNF-α,
directly or indirectly promotes the proliferation and adhesion of endometrial cells and associated angiogenesis seen in endometriosis [32,35]. The search for an innate or acquired survival advantage of eutopic endometrium favoring ectopic implantation has fueled a number of studies comparing eutopic endometrium from women with and without endometriosis [10,36–40]. Menstrual blood offers a unique minimally invasive diagnostic opportunity, as it has elements from endometrium and has already been used for the diagnosis of dysfunctional uterine bleeding [41]. The study of inflammatory markers in the endometrium and menstrual blood samples may help to elucidate the steps of the pathogenesis of endometriosis and to find a biomarker for the disease.

Our study sought to determine whether the menstrual blood of women with endometriosis expresses inflammatory and angiogenic markers differently from that of women without endometriosis, comparing the activity of NAG and MPO, and the expression of TNF-α and VEGF in menstrual and peripheral blood.

2. Materials and methods

2.1. Patients

The study protocol was approved by the local ethics committee. A written informed consent was obtained from all patients involved in the procedure. Our transversal study involved seventeen women undergoing infertility or chronic pelvic pain treatment at Hospital das Clínicas at Universidade Federal de Minas Gerais (HC-UFMG). These patients were recruited among those referred to our department from February 2011 to December 2012 and were divided into two groups: endometriosis (n = 10) and normally cycling women without endometriosis (n = 7), which served as a control group. Inclusion criteria were regular menstrual cycles in the six months preceding sample collection, no use of hormonal nor anti-inflammatory agents in the previous three months and surgical confirmation or exclusion of endometriosis in agreement with the ESHRE guidelines [42]. The control group has undergone surgery as part of infertility evaluation or tubal sterilization. None of the women had a significant past medical history. Endometriosis was staged according to the classification proposed by the American Society for Reproductive Medicine (ASRM, 1996) [43].

Menstrual and peripheral blood samples were collected in the follicular phase (1st to 4th day of menses). The activity of inflammatory markers (NAG and MPO) was evaluated by enzymatic methods whereas TNF-α and VEGF were measured by ELISA (Kit Duoset R&D Systems). NAG, MPO, TNF-α and VEGF were evaluated in serum and menstrual blood. Menstrual blood samples were collected at the external cervical os by gentle syringe aspiration, and peripheral blood was withdrawn from antecubital vein concomitantly. Blood was centrifuged (3600 rotations per minute for 20 minutes at 4 °C). Serum was withdrawn and stored at −25 °C until assayed for markers.

2.2. Assays

2.2.1. Determination of NAG activity

Accumulation of mononuclear cells in menstrual and peripheral blood was quantified by measuring the levels of the lysosomal enzyme NAG, which is present in high levels in activated macrophages [26–28]. An aliquot of the serum/period blood (50 µL) was homogenized in 150 µL NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega, São Paulo, Brazil) and then centrifuged (3000 rpm; 10 min at 4 °C). Samples of the resulting supernatant (50 µL) were incubated with 100 µL of p-nitrophenyl-N-acetyl-beta-D-glucosaminide (2.24 mM) prepared in 50 µL citrate/phosphate buffer (39 mM pH = 4.5). The reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer and the product was detected colorimetrically at 400 nm. NAG activity was expressed as change in optical density (OD).

2.2.2. Determination of MPO activity

The extent of neutrophil accumulation in menstrual and peripheral blood was measured by assaying MPO activity as previously described [44]. An aliquot of serum/period blood (50 µL) was homogenized in 150 µL sodium phosphate pH 5.4 buffer for 30 seconds. The supernatants (200 µL) were then re-suspended in 400 µL (hexadecyltrimethylammonium bromide) HTAB and centrifuged at 5000 g for 10 min at 4 °C. This solution (200 µL) was added to 100 µL of H2O2 and 100 µL of tetramethylbenzidine. The reaction was stopped by the addition of 100 µL of H2SO4 (4 M). MPO activity in the samples was obtained by measuring the change in absorbance (OD) at 450 nm.

2.2.3. Determination of TNF-α and VEGF

TNF-α and VEGF were evaluated using commercial specific enzyme-linked immunosorbent assays (ELISA). Kits for Human VEGF (Duoset R&D Systems DY293B range: 31.2–2000 pg/mL) and Human TNF-α (Duoset R&D Systems DY210, Minneapolis, MN – USA, range: 15.6–1000 pg/mL) were used to quantify the expression of each protein. Samples from each patient were tested in duplicate, according to the detailed protocol provided by the manufacturers. Briefly, samples and standards were added to a 96-well antibody-coated plate, which were shaken, sealed and stored overnight at 4 °C. The plate was then washed with wash buffer. After then, the biotin conjugate-labeled second antibody was added in plates and stored for a further one hour at room temperature. The plate was washed again and the estreptavidin conjugate was added, sealed, shaken and stored at room temperature for 20 minutes and after washed again. The OPD (o-phenylenediamine dihdrochloride) substrate was added in all plates and stored at room temperature out of light for 10 minutes and then the stop solution – H2SO4 4 M was added. The absorbance was measured by spectrophotometry.

2.3. Statistical analysis

Calculations were carried out using GraphPad Prism 5 and IBM SPSS Statistics 21 (SPSS Inc; Chicago, IL; EUA), and P < 0.05 was considered statistically significant for all analysis. Mann-Whitney test was used to compare of unpaired groups, the Wilcoxon test was used to compare paired groups, once normality could not be assumed after application of the Kolmogorov-Smirnov test. Correlations were calculated using Spearman’s rho coefficient.

Power calculations based on the expected or desired effect size showed that 10 patients in the case group and seven individuals in the control group showed a confidence level of 90%, statistical power of 80% and a minimal detectable difference of 4 and a standard deviation of 3.

3. Results

The age of the patients ranged from 31 to 48 years, and in both groups the median was 36. No significant difference was found between endometriosis and control group, neither for BMI (median 27.17 kg/cm² in control group versus 24.86 kg/cm² in endometriosis) or menstrual cycle length (median 28.5 in control group and 28 days in endometriosis) (Fig. 1). Endometriosis stage according to ASRM was stage II in the five patients (50%), IV in two (20%) and in three (30%) no surgical staging was available in spite of surgical confirmation. The majority of women in the endome-
Endometriosis group presented pelvic pain (n = 9; 90%), six patients (60%) had dyspareunia and three (30%) presented infertility.

No difference in NAG activity between control and endometriosis groups was detected, nor in peripheral neither in menstrual blood. NAG activity in menstrual blood was significantly higher than in peripheral blood from patients with endometriosis (P = 0.0391), but not in controls (P = 0.078) (Fig. 2). MPO activity showed no difference comparing menstrual and peripheral blood from women with endometriosis and controls. MPO activity in menstrual blood was significantly higher than in peripheral blood in the endometriosis group (P = 0.017), but not in control group (P = 0.078) (Fig. 3). There was a significant positively linear correlation between MPO activity and NAG in menstrual blood (Spearman's rho test, P = 0.01; r = 0.603).

When groups were compared, no statistically significant difference was found for TNF-α VEGF neither in the menstrual nor in peripheral blood (Figs. 4 and 5). Comparison between symptomatic patients (pelvic pain, dyspareunia and infertility) and asymptomatic for NAG, MPO activity as well as TNF-α and VEGF level revealed no statistically significant difference.

4. Discussion

This transversal study measured the activity of the inflammatory enzymes MPO and NAG as well as TNF-α and VEGF in menstrual and peripheral blood of patients with and without endometriosis. To the best of our knowledge, no previous studies have been published regarding the activity of these inflammatory and angiogenesis markers in menstrual blood of woman with endometriosis in comparison to serum levels. As for VEGF, we
found only two studies evaluating VEGF-A in menstrual blood in women with and without endometriosis [45,46].

In patients with endometriosis, NAG activity in menstrual blood was significantly higher than in peripheral blood, but this was not the case in the control group. As NAG is present in high levels in activated macrophages, this finding may reflect higher macrophage activation in menstrual blood from patients with endometriosis.

The same pattern was found for MPO activity in women with endometriosis, which certainly reflects increased local neutrophil accumulation in menstrual blood of these patients, thus reinforcing the presence of abnormal local inflammatory activity in the endometrial cavity of women with endometriosis. The correlation between MPO and NAG activities in menstrual blood from women with the disease provides a linkage between the activity of macrophages and neutrophils in menstrual blood.

Changes in the response of certain immune cells within the peritoneal cavity of women with endometriosis have been demonstrated by many studies and intrinsic alterations within the eutopic endometrium are beginning to be unravelled population [12,22,33,36,37,47] and activation in controversies eutopic exist about and macrophage fluids from endometrium endometriosis patients. One study evaluating endometrial samples showed a reduction in macrophage cell populations during early proliferative phase with no further differences during later stages of the cycle [48]. Berbic et al. (2009), on the other hand, demonstrated a significant increase in macrophage cell populations across all proliferative phase in women with endometriosis, as well as a significant increase in macrophage density in women without endometriosis compared with those with the disease during the mid-menstrual phase. There was a gradual decline in endometrial macrophage density in women with endometriosis throughout the menstrual phase [37].

Brandel and Passos (1998) found elevated NAG enzymatic activity in the peritoneal fluid of infertile women with endometriosis [49]. Lanaíta et al. (2012) also showed a significantly higher NAG activity in serum and in the follicular fluid of infertile patients with endometriosis, thus reinforcing the role of macrophages in endometriosis pathogenesis [28].

MPO activity has been reported to be lower in the peritoneal fluid from women with stage I endometriosis compared to women without endometriosis and to those with stage III/IV [25]. Others have found a decreased MPO activity in the follicular fluid of women with endometriosis [28]. Data from these studies cannot be directly compared to our findings because different body fluids were tested in different phases of the menstrual cycle. Taken together, these data provide solid evidence for a dysregulation of the secretion of inflammatory mediators in different body environments in women with endometriosis.

It is important to acknowledge that a great degree of macrophage density appears to exist in macrocytosis content in specific microenvironments in the eutopic endometrium. Discrepancies in cells densities reported between women with and without endometriosis may purely reflect differences in the day of the sample collection [37]. Endometriosis is a heterogeneous disease and different stages can represent molecular differences between patients and thus produce different results.

A limitation of the current study was the small number of patients. Our inclusion and exclusion criteria were quite strict, which did not allow a large number of subjects to be recruited. Studies obtaining large enough number of specimens in different stages of endometriosis during several sub-phases of the menstrual cycle for comparative analysis would elucidate the behavior of many endometrial markers of inflammation, angiogenesis and other altered processes.

Changes in eutopic endometrium of women with endometriosis in terms of microscopic structure, immune components and cytokine production, angiogenesis, aromatase enzyme expression, and the presence of nerve fibers and adhesion molecules, as well as altered gene expression, have been widely reported [36,50,51]. A large body of data points to specific intrauterine changes occurring in women with endometriosis. The eutopic endometrial glandular and stromal cells may be functioning differently in these women. The endometrial cells of women with endometriosis may therefore have intrinsic abilities to escape immune surveillance, implant, proliferate and respond differently to stimuli present in their new environment. These cells may hold constituents, or acquired characteristics, that favor their survival outside the uterine cavity. The secretion of macrophage inflammatory proteins (MIP)-1α and MIP-2, IL-6, TNF-α and VEGF by neutrophils and macrophages can create a positive loop to amplify the signal for the establishment and growth of endometriotic tissue in ectopic sites [18], and our study corroborated this hypothesis, showing higher activity of NAG and MPO in menstrual blood from endometriosis patients.

No differences were found when comparing TNF-α and VEGF levels from patients with and without endometriosis. Despite not reaching statistical significance, the VEGF expression was higher in the peripheral blood from patients with endometriosis in comparison to the peripheral blood from controls. Small sample sizes could have compromised our data in this regard. Controversies exist in the literature regarding TNF-α and VEGF levels in different body fluids from patients with and without endometriosis [11,39,45,46,52–55]. Again, differences in menstrual cycle
phases make it difficult to compare the results. Furthermore, this was the first report of TNF-α measurement in menstrual blood. Two studies evaluating VEGF in menstrual blood from patients with and without endometriosis also did not report any difference between groups [45,46]. As angiogenesis takes place outside the uterine environment, after the invasion of shed endometrial cells, it is likely that differences in angiogenesis and its surrogates may be better evaluated in the peritoneal fluid or tissue samples of endometriotic lesions.

Many cytokines and inflammatory markers present in the peritoneal fluid have been studied in the context of endometriosis, but for most of them no correlation between their concentration and the extent of symptoms is present [56,57]. Although it wasn’t our objective, this study also designed to identify the relationship between inflammation and angiogenesis markers with pelvic pain, dyspareunia and infertility, the comparison of NAG and MPO activity and TNF-α and VEGF expression between symptomatic and asymptomatic patients revealed no statistical difference probably due to the small number of women studied. One has to bear in mind, however, that there is a poor correlation between the presence of symptoms and endometriosis staging and this may be true for these markers.

Menstrual blood proved to be an important biologic fluid, not yet adequately explored in scientific research focusing on the pathogenesis of endometriosis. It is possible to suggest, based on the existing literature and our study results that there is a local contribution of endometrial environment for the development of endometriosis, a permissive environment in the peritoneal cavity has also been shown to exist, allowing detached endometrial cells to implant and grow.

5. Conclusion

We confirmed the presence of inflammation and angiogenesis markers in the menstrual blood of women with endometriosis and controls. Macrophage and leukocyte activities were significantly greater in menstrual blood of women with endometriosis than in their peripheral blood, reflecting increased local inflammatory activity. However, more studies are warranted, with a greater number of patients stratified by disease stage and severity. This would help to establish the real role of these markers in the pathogenesis of endometriosis.

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References