Introduction

Endometriosis is defined as growth of endometrial glands and stroma outside the uterine cavity, and it affects approximately 10% of reproductive-aged women [1]. Symptoms of endometriosis vary greatly but can include menorrhagia [2], debilitating pain, and infertility [3, 4]. The most accepted etiology of peritoneal endometriosis is Sampson’s hypothesis, which proposed that retrograde menstruation allows endometrial cell fragments to enter the peritoneal cavity via the fallopian tubes where they can attach to and invade the peritoneal tissues [5]. While evidence of retrograde menstruation of viable endometrial cells has been documented [6, 7], almost all women experience menstrual reflux, suggesting other factors are involved in the etiology of endometriosis.

Endometriosis has been hypothesized to be both a disease of the macrophage and a disease of the peritoneal fluid [8-11]. The immune response to the invading tissue is hypothesized to impair fertility by causing adhesions and altering pelvic anatomy [12]. Throughout the menstrual cycle, monocytes/macrophages are the most abundant cell types in the peritoneal cavity [13, 14], and women with endometriosis have increased numbers of these cells in their peritoneum. These peritoneal macrophages are predominately alternatively activated rather than classically activated, but demonstrate more inflammatory but less phagocytic activity than in unaffected women [15-20]. In a normal menstrual cycle, peritoneal macrophages may aid in vasculogenesis in response to endometrial ischemia, conferring a survival advantage to ectopic endometrium [8]. The presence of large numbers of peritoneal macrophages may also contribute to the inflammatory response due to the release of significant amounts of cytokines, providing a positive feedback loop which increases tissue damage and patient symptomatology [6, 8]. Women with endometriosis have altered peritoneal fluid cytokine profiles compared with normal women [21-29]; one hypothesis to explain this difference is that a dysfunctional immune system may not properly remove the foreign tissue from the peritoneal cavity, leading to endometriosis.

The composition of peritoneal fluid from patients with and without endometriosis have been well-characterized and many known differences in the cytokine profile of peritoneal fluid from women with endometriosis versus controls have been reported [30-33]. These differences are thought to contribute to the increased number and altered function of peritoneal macrophages from endometrial patients which can subsequently impact the pathogenesis of this disease. It is presently unknown if the aberrant macrophage number and activity in endometriosis is due entirely to the known differences in the peritoneal fluid cytokine profile or if inherent differences in the macrophage response plays a role. To address this question the authors chose to study peripheral blood monocytes (PBM). PBM from endometriosis patients have been noted to secrete dif-

C.E. Bedient¹, D. Rodriguez¹, N. Sidell¹, C. Roberts², S.C. Schutte¹

¹Department of Gynecology and Obstetrics, Emory University School of Medicine, Atlanta, GA
²Reproductive Surgical Specialists, Cumming, GA (USA)

Summary

Purpose of this Investigation: The primary aim of this pilot study was to determine if the increase in monocyte/macrophage number in the peritoneal cavity of women with endometriosis is due to differences in peritoneal fluid signaling content (e.g. cytokine levels) or inherent differences in monocyte migration in response to those signals. Materials and Methods: Peripheral blood and peritoneal fluid samples were collected from endometriosis and surgical control patients at the time of surgery. Peripheral blood was also collected from a group of healthy ‘non-surgical control’ patients in an internal medicine clinic. Monocytes were isolated from blood and invasion was assessed using peritoneal fluid from endometriosis patients and controls as a chemoattractant. Results: Regardless of peritoneal fluid source a > 10-fold change was seen in monocyte invasion with endometriotic monocytes. Conclusion: Peripheral blood monocytes from women with endometriosis are more invasive than those from other women. This is true when compared to both surgical and non-surgical controls.

Key words: Monocyte invasion; Peritoneal fluid; Endometriosis; Peripheral blood monocytes.
ferring cytokine profiles, including increased levels of TNFα, IL-6, IL-8, and plasma from endometriosis patients have shown altered levels of MCP-1, IFN-γ, and M-CSF [34-37]. However, alterations in the migration of PBM from endometriosis patients have not, to the present authors' knowledge, been reported. In this study, it was their goal to determine if there are inherent differences in the invasive properties of PBM from patients with endometriosis versus controls, as reflected by an in vitro invasion assay.

Materials and Methods

This is a prospective controlled cohort study. Emory University IRB granted approval for this study and informed consent was obtained from patients that fit into one of three experimental groups. Group 1 consisted of women between 21- to 45-years-old undergoing reproductive surgery for known or suspected endometriosis. Patients with endometriosis were diagnosed via pathological examination of routinely obtained peritoneal biopsies taken during surgery. Peripheral blood was collected prior to surgery, the invasion results from women whose pathology reports did not confirm endometriosis were excluded from the analysis. A total of seven women with confirmed endometriosis were included in the study results. Group 2 consisted of control patients undergoing surgery for benign reproductive pathology (including fibroids, tubal ligation, hydrosalpinges, and menorrhagia) between the ages of 21 and 45 years. Patients undergoing surgery were confirmed to have no signs of endometriosis during laparoscopic inspection by surgeons experienced in the treatment of endometriosis. A total of five women were included. Group 3 consisted of control patients between 21- and 45-years-old presenting to an internal medicine clinic for routine primary care with no history of endometriosis, dysmenorrhea, dyspareunia or malignancy (non-surgical controls). A total of six women were included in this group. The authors collected information regarding gravidity, parity, medical history, medication use, and symptoms for all patients. Exclusion criteria included post-menopausal status, current or historical malignancy, or immunosuppression.

After obtaining written informed consent, approximately 8 ml of blood was collected in K3-EDTA coated blood collection tubes at the time of medically indicated venipuncture. This occurred at the time of intravenous line placement for surgery in the case of groups 1 and 2, and at the time of office visit for previously scheduled routine blood work in the case of group 3. Samples were placed on ice prior to arrival at the laboratory for isolation. Cells were isolated within four hours of collection.

Monocytes were isolated from patient serum by gradient centrifugation, followed by magnet-assisted cell sorting (MACS) with CD14+ beads. Efficacy of monocyte isolation via CD14+ microbead separation has been shown to be effective by Zhou et al. [38], with purity of over 98%. The present authors had similar results in cell purity in their isolations. After isolation, monocyte numbers were quantified via hemocytometer.

Peritoneal fluid from endometriosis and controls subjects had been collected as part of a previously published study and were stored at -80°C in 1.0-mL aliquots [39]. The samples were centrifuged to remove cells and debris prior to storage. After thawing, protein levels were quantified using the bicinchoninic acid (BCA) method. This was done to exclude any potentially diluted samples and so that equal protein concentrations could be used in each assay. In addition to the individual peritoneal fluid samples, a large pooled peritoneal fluid stock was created that included both endometriotic and control peritoneal fluid.

Macrophage invasion was determined by utilizing Matrigel-coated Transwell inserts using peritoneal fluid as the chemoattractant. Transwell inserts with 0.8 µm pores were coated with 50 µl of 11% Matrigel and the Matrigel was allowed to gel overnight at 37°C prior to the addition of the cells. Phenol-red free RPMI containing 49.5 mg protein/ml peritoneal fluid was added to the bottom well. This corresponded to approximately 10% peritoneal fluid per well. The upper well was filled with 50,000 monocytes suspended in 100 µl RPMI. All monocytes (groups 1-3) were placed in multiple Transwells so that each sample was exposed to both endometriotic, control, and pooled chemoattractant (Figure 1). Transwells were incubated for 48 hours at 37°C. After 48 hours, images of three representative fields of view were taken of the bottom of the wells where monocytes that had invaded through the Matrigel-coated insert had come to rest. These migrated cells were counted and the average cell number per frame was determined. The counts for all of the different peritoneal fluid sources within each group were averaged together. Monocytes from each patient were exposed to at least two different control and two different endometriotic peritoneal fluid samples along with the pooled sample. Peritoneal fluid was used in invasion assays with monocytes from at least one endo and one control patient.

After monocyte isolation, a portion of the cells not utilized in invasion assays was assayed for gelatinase activity using gelatin zymography. The isolated cells were lysed in RIPA buffer with supplemental protease inhibitors and stored at 80°C until analysis. Protein content was determined via BCA analysis and equal protein content was loaded into a gelatin zymogram gel. SDS-PAGE was run under non-reducing conditions. After electrophoresis, the matrix metalloproteinas (MMPs) were reanimated and allowed to develop overnight at 37°C using commercially available buffers. The gels were then stained with colloidal blue stain and imaged. The image was converted to grayscale and colors were inverted so that densitometry could be completed using ImageJ. These semiquantitative results are reported in arbitrary units (AU).

Analysis of age was done using a one-way ANOVA with a post-hoc t-test; comparisons of the racial makeup were done using chi-square analysis. A two-way ANOVA with a post hoc Tukey’s t-test was performed to assess monocyte invasion. The densitometry data was analyzed using the Student’s t-test. A 95% confidence interval was used for all statistical analyses unless noted.

Results

For the invasion studies, seven endometriosis patients, five surgical controls, and six non-surgical controls were obtained for these studies. There were significant differences in race between the endometriosis patients, and surgical and non-surgical controls, as shown in Table 1. The non-surgical controls were chosen based on the available patient population at that time and lacked Afro-American patients. The peritoneal fluid was obtained from a frozen stock. The protein content was determined for each sample to ensure equal protein content was used in the Transwell assays and also to assure that no diluted peritoneal fluid from peritoneal cavity washings was used. The average protein content of the endometriotic peritoneal fluid was 43.7 ± 5.5 mg/mL and the content of the control peritoneal fluid was 24.5 ± 6.9 mg/mL; these values were not statistically different (p = 0.588).
Monocyte invasion was determined by taking representative images of the bottom well and counting the cells at the end of the Matrigel invasion assay. Representative images are shown in Figure 1. The average cell count per frame was determined and compared between the nine conditions (Figure 2). Regardless of the peritoneal fluid source, a greater than ten-fold change was seen in macrophage migration using monocytes from endometriosis patients compared to either group of controls with a p-value of < 0.001.

Invasion of PBM, regardless of group, was not significantly altered due to peritoneal fluid source (p-values of 0.823 for peritoneal fluid source and 0.924 for the interaction between PBM and peritoneal fluid).

Gelatin zymography was performed to determine if there were differences in MMP-9 expression in the different monocyte sources. Cell lysates from monocytes after CD14 isolation, but prior to peritoneal fluid exposure were assayed. Five samples from controls and five samples from endometriosis patients were used; two of the endometriosis samples were excluded due to insufficient amounts and the controls were matched for number. As no differences were seen in invasion between the surgical and non-surgical controls, both were used and were grouped together as a generic ‘control’ group. All cells produced MMP-9; however, most cells from women with endometriosis expressed MMP-9.

### Table 1. Patient demographics.

<table>
<thead>
<tr>
<th></th>
<th>Endometriosis</th>
<th>Surgical controls</th>
<th>Non-surgical controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>34 ± 2</td>
<td>37 ± 2</td>
<td>33 ± 2</td>
<td>0.629</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caucasian</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. — Experimental set-up. The figure shows the basic Transwell setup. A thin layer of Matrigel was added to the membrane in the upper chamber. The monocytes were added to the upper chamber in serum-free medium. The lower chamber was filled with RPMI supplemented with 49.5 mg/ml peritoneal fluid as a chemoattractant. The three monocyte groups, endometriotic, and surgical controls and non-surgical controls, were exposed to control, endometriotic, and pooled peritoneal fluid creating nine separate groups. All monocytes were exposed to at least one source of endometriotic and control peritoneal fluid. All peritoneal fluid was used in at least two experiments, with at least one control and one endometriotic monocyte source.

Figure 2. — Monocyte invasion is depicted as average cell count per frame. There is a significant difference in the invasion in monocytes from patients with and without diagnosed endometriosis; however, no significant differences are seen based on the source of the peritoneal fluid. No difference in invasion are seen between the surgical and non-surgical controls. *depicts p < 0.001 as compared to the other monocyte sources.

Figure 3. — Gelatin zymography. Cell homogenates from monocytes after CD14 isolation and prior to exposure to peritoneal fluid were assayed for gelatinase activity (n=5). A representative image from three endometriosis and three controls is shown. MMP-9 is present in all samples and is found in larger amounts in the endometriotic samples. Densitometry confirms these differences are significant at a 90% confidence interval.

Gelatin zymography was performed to determine if there were differences in MMP-9 expression in the different monocyte sources. Cell lysates from monocytes after CD14 isolation, but prior to peritoneal fluid exposure were assayed. Five samples from controls and five samples from endometriosis patients were used; two of the endometriosis samples were excluded due to insufficient amounts and the controls were matched for number. As no differences were seen in invasion between the surgical and non-surgical controls, both were used and were grouped together as a generic ‘control’ group. All cells produced MMP-9; however, most cells from women with endometriosis expressed MMP-9.

---

**Alterations in the invasive properties of peripheral blood monocytes from patients with endometriosis**

---

Monocyte invasion was determined by taking representative images of the bottom well and counting the cells at the end of the Matrigel invasion assay. Representative images are shown in Figure 1. The average cell count per frame was determined and compared between the nine conditions (Figure 2). Regardless of the peritoneal fluid source, a greater than ten-fold change was seen in macrophage migration using monocytes from endometriosis patients compared to either group of controls with a p-value of < 0.001. Invasion of PBM, regardless of group, was not significantly altered due to peritoneal fluid source (p-values of 0.823 for peritoneal fluid source and 0.924 for the interaction between PBM and peritoneal fluid).

Gelatin zymography was performed to determine if there were differences in MMP-9 expression in the different monocyte sources. Cell lysates from monocytes after CD14 isolation, but prior to peritoneal fluid exposure were assayed. Five samples from controls and five samples from endometriosis patients were used; two of the endometriosis samples were excluded due to insufficient amounts and the controls were matched for number. As no differences were seen in invasion between the surgical and non-surgical controls, both were used and were grouped together as a generic ‘control’ group. All cells produced MMP-9; however, most cells from women with endometriosis expressed MMP-9.
higher levels of the protease than controls (Figure 3). Densitometry confirmed these results with controls having MMP-9 levels of 0.58 ± 0.23 AU and endometriosis patients having levels of 1.09 ± 0.21 AU. These differences were significant when a 90% confidence interval was used.

Discussion

When comparing PBMs from endometriosis and control patients, the authors have shown that patient source has a significant impact on monocyte invasion, while no significant differences were seen with respect to the peritoneal fluid source. Limitations of this study include the small number of patients for both the peritoneal fluid source and monocyte source. Although there was a relatively large inter-patient variability, the authors were able to discern significant differences in migration between the PBM from endometriosis versus control patients. Due to the limitations in peritoneal fluid volumes available, not all monocytes were exposed to the exact same peritoneal fluid samples. To minimize this impact, each peritoneal fluid source was used with monocytes from at least one control and one endometriosis patient, as well as a pooled peritoneal fluid source. The source of control monocytes (surgical vs. non-surgical patients) did not result in significant differences of invasion. This finding gives credence to using asymptomatic, non-surgical patients in future studies to increase the applicability of findings, rather than turning to a control patient population with additional underlying benign pathology requiring surgery. Given the lack of dysmenorrhea, the authors believe that they are the ideal control for understanding differences between women with severe endometriosis symptoms versus women without these symptoms. While there may be doubt over the presence of endometriosis in these patients, it does highlight the differences in women with symptomatic endometriosis and those without clinical symptoms. The advantage of laying the preliminary groundwork for a diagnostic test for endometriosis in the general population led us to keep the non-surgical control group as a comparison.

The present findings do not support prior studies that demonstrated peritoneal fluid has a significant impact on macrophage invasion [34]. The authors’ use of primary PBM from different donor groups coupled with the relatively small number of patient samples may have “masked” this finding in this study. They expect that a larger sample size utilizing primary cells may show the additional impact of peritoneal fluid along with monocyte source. Regardless, it is clear from this study that the impact of PBM source is greater than that of peritoneal fluid on monocyte invasion in the present assay.

The exact mechanism(s) of patient-related differences in PBM invasive properties is not yet known. The present authors found that MMP-9 levels were elevated in the PBMs prior to exposure to the peritoneal fluid, suggesting a more invasive phenotype. Preliminary studies to investigate the expression of several receptors that are known to influence monocyte invasion including CCR2 and CX3CR1 did not show differences between the subgroup donors (data not shown).

The significance of the present findings is three-fold. Firstly, this brings into question the commonly considered mechanism of injury in endometriosis that relates solely to ectopic endometrial cells and associated cytokines with exuberant macrophage responses being a direct result of pathological signals [40]. The present findings suggest PBMs are inherently different in endometriosis patients in terms of an invasive response to stimuli. This response is important as the macrophage has been shown to play an important role in early lesion formation [41, 42] Secondly, the clinical implications of these findings directly relate to treatment options for patients suffering the sequela of ectopic endometrial implants. Currently, the primary modalities of treatment focus on hormonal suppression of already established ectopic tissue or surgical removal of such tissue. Neither of these treatment options addresses abnormal immunologic mechanisms associated with endometriosis, nor do they consistently work in all patients. While pain management with non-steroidal anti-inflammatories is common, NSAIDs do not directly inhibit monocyte recruitment, instead focusing on the cyclooxygenase pathways [43]. Management of endometriosis using immunomodulators is an area of increasing interest [44, 45] and the present findings suggest that targeting immune cells as a treatment for endometriosis remains a worthwhile pursuit. Lastly, there is a quest to find a screening method for endometriosis, preferably utilizing an easily performed peripheral blood sample.

Conclusions

The present work shows significant differences in the migration properties of peripheral blood monocytes of women with endometriosis compared to both surgical controls as well as an asymptomatic, non-surgical population. Determining the mechanism behind these differences may allow for a simple, non-invasive method of screening for endometriosis.

Acknowledgements

Many thanks to Dr. Nicholas Fogelson, Dr. Steven Weiss, Dr. Heather Hipp, Dr. Hanh Cottrell, the staff of the Emory Reproductive Center, and to the residents of the Emory University Department of Gynecology and Obstetrics for helping collect patient samples. The authors have much appreciation for Dr. Jennifer Zreloff and the staff at Patient Centered Primary Care at Emory University for assistance in collection of non-surgical control samples. Thank you to Dr. Juanjuan Wu for help with flow cytometry. This re-
search was supported in part by a Eunice Kennedy Shriver NICHD grant as part of the Cooperative Research Partnership to Promote Workforce Diversity in the Reproductive Sciences (U01 HD66439).

References


Corresponding Author:
S.C. SCHUTTE, Ph D
Shriners Hospitals for Children
3229 Burnet Ave
Cincinnati, OH, 45229 (USA)
e-mail: sschutte@shrinenet.org