Clinical Study

Enhanced Inflammatory Activity of Endometriotic Lesions from the Rectovaginal Septum

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Endometriosis is characterised by the growth of ectopic lesions at multiple locations outside the uterine cavity and may be considered a collection of distinct but related conditions. The exact aetiology of endometriosis is still not clear although a role for inflammation is increasingly accepted. We therefore investigated the inflammatory activity of eutopic tissue and that of the matching ectopic lesions from different locations by measuring the genetic expression of inflammatory chemokines and cytokines.

The gene expression in matching eutopic and ectopic tissue was compared, as was the gene expression in lesions from different locations. A significantly higher mRNA expression of the chemokines ENA-78 and RANTES and the cytokines IL-6 and TNFα was observed in endometriotic lesions of the rectovaginal septum (RVS) compared to that of matching eutopic tissue. Comparisons across lesion location showed a significantly higher expression of IL-6 and TNFα in the RVS compared to lesions from either the ovaries or the peritoneum. These results show that the production of some inflammatory chemokines and cytokines is significantly increased in the ectopic endometrial tissue compared to matching eutopic tissue. Furthermore, IL-6 and TNFα are produced in significantly higher quantities in RVS lesions compared to other lesions.

1. Introduction

Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity. The most common symptoms leading to a diagnosis are dysmenorrhoea, pelvic pain, and reduced fertility [1]. It is a very prevalent disease affecting up to 10% of the reproductive-aged female population [2].

The precise aetiology of endometriosis is not yet clear. Currently, the most widely accepted theory is the implantation theory: retrograde menstruation can result in viable endometrial cells and fragments entering the peritoneal cavity [3] and once attached [4], they promote a chronic pelvic inflammatory response [5]. Retrograde menstruation however cannot explain all cases, as endometriotic lesions have been identified in diverse locations such as the brain [6]. It is broadly accepted however that most of the ectopic lesions can be separated into three main regions: (i) ovarian, (ii) rectovaginal septum (RVS), and (iii) peritoneum. Biochemical and pathological differences between the lesions found in these locations have led to suggestions that endometriosis may represent a collection of related but distinct conditions [7]. It is possible that the variability between these distinct but related lesions is what contributes to the enigmatic nature of the disease.

The contribution of inflammation to the progression of endometriosis is increasingly being recognised. Endometriotics lesions that are established at ectopic sites secrete chemokines which attract macrophages into the peritoneal cavity, further stimulating the inflammatory response and release of cytokines [8]. Significantly increased numbers of activated macrophages have been identified in the peritoneal fluid of women with endometriosis [9], as has an increased concentration of various chemokines and cytokines. Significantly elevated levels of epithelial neutrophil-activating
peptide (ENA-78) [10], monocyte chemotactic protein (MCP-1) [11], interleukin (IL)-8 [12], tumor necrosis factor (TNF)-α [13], IL-6 [14, 15], and regulated on activation normal T cell expressed and secreted (RANTES) [12] have all been found in the peritoneal fluid of patients with endometriosis. Underlying the inflammatory nature of the condition is the fact that TNFα [16], ENA-78 [17], and IL-6 [18, 19] are also elevated in the serum of women with endometriosis. Less data is however available on the inflammatory response of the lesion itself and whether there is variability based on the type or lesion location. A difference in the production of specific cytokines may provide an insight into the inflammatory activity of lesions that grow in different locations.

In order to gain a better understanding of this complex disease and the differences that can occur between various lesions, this study investigated the production of several chemokines and cytokines in matching eutopic endometrial and ectopic endometriotic tissue and compared their gene expression levels in the three most common presentations of the disease.

2. Patients and Methods

2.1. Sample Collection and Patient Data. Informed consent was collected prior to surgery from all women included in the study. Laparoscopic surgery was performed for the investigation of pelvic pain or infertility, and any endometriotic lesions identified were removed and their location was noted. Where possible, an endometrial biopsy was also collected using a soft curette (Pipelle-de-cornier, Laboratoire CCD, France). All tissue collected during the surgery was stored in RNAlater (Invitrogen Life Technologies, Zug, Switzerland) at −80°C until further use. Exclusion criteria for the study included prior or current infections, liver dysfunction, or the use of hormonal treatments, including any hormonal contraceptive or gonadotropin releasing hormone analogues (GnRHa) within the past 3 months. All laparoscopies were performed in the proliferative phase of the menstrual cycle. Institutional review board approval was obtained from the ethical committee prior to the commencement of the study.

After the informed consent was obtained and exclusion criteria were satisfied, we collected eutopic endometrial biopsies from 17 patients. A single matching ectopic lesion was collected from 15 women, two lesions were collected from another, and three lesions in the final case, resulting in 20 ectopic lesions with matching eutopic samples. The primary indication for surgery was dysmenorrhea for ten of these women, pelvic pain for four women, and infertility for the remaining three. The average age of the patients was 32.94 ± 1.454, range 24–41, and the body mass index (BMI) was 23.39 ± 0.914, range 18.90–33.10.

For the further comparison of the mRNA expression across ectopic sites additional lesions were collected from another 23 patients to make a total of 40 patients. A single lesion was collected from 34 patients, two lesions were collected from five patients, and three lesions were collected from one patient, resulting in a total of 47 ectopic endometriotic lesions. In some cases the isolated mRNA was insufficient to determine the concentration of all genes of interest and as such n values are included with each mean and SEM. The primary indication for surgery was dysmenorrhea for 17 women, pelvic pain for another 14, and idiopathic infertility for the remaining nine. The average age was 35.58 ± 1.265, range 22–58, and the BMI was 23.79 ± 0.811, range 18.00–47.30. No significant difference in either age or BMI was observed in the three groups based on lesion location.

2.2. Determination of Gene Expression in Eutopic Endometrium and Ectopic Endometriotic Tissue. Approximately 30 mg of tissue from both the eutopic endometrial biopsies and ectopic endometriotic lesions was excised and homogenized in the FastPrep 120 tissue homogenizer (30 seconds at 4.0 m/sec) in cell lysis buffer (Qiagen, Düsseldorf, Germany). RNA isolation was performed with the RNeasy minikit (Qiagen) and after isolation the TurboDNase kit (Ambion, Life Technologies, Zug, Switzerland) was used for genomic DNase digestion. One microgram of the total RNA was reverse transcribed in a 25 μL reaction volume with the Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Dübendorf, Switzerland) and random primers. The resulting cDNA was diluted 1:20 and the absence of genomic DNA was confirmed with a reverse transcriptase control.

The quantitative real time polymerase chain reaction (qPCR) was performed with the SYBR green Fast Advance Master Mix (Qiagen) and a Rotor-Gene RG 2000 (Corbett Research, NSW, Australia), under the following conditions, 95°C for 5 min, followed by 40 cycles of 95°C for 5 second, and 60°C for 10 seconds. Specificity of the reaction was confirmed via melt curve analysis and the product size was confirmed on a 4% agarose gel.

The Genbank accession number and the primer sequences for all genes examined by qPCR are shown in Table 1.

2.3. Statistical Analysis. The most stable reference genes and the optimal combination to provide minimal variability were selected via the geNORM software program and a geometric mean of the four reference genes selected was used to normalise the expression of the genes of interest for both the eutopic and ectopic tissue [20]. The reaction efficiency of each assay was determined via linear regression [21] and the fold change calculated with the qBASEplus software (Biogazelle, Zwijnaarde, Belgium).

The difference between the matched eutopic and ectopic mRNA expression at different locations and the difference between mRNA in different ectopic locations were determined by a one-way Analysis of Variance (ANOVA) test with a post hoc Bonferroni’s multiple comparisons test between selected groups. All values are presented as mean ± SEM and all statistical analysis was performed with Graphpad Prism 5.0 and significance was set at a value of P < 0.05.

3. Results

3.1. Cytokine mRNA Concentrations in Matching Eutopic and Ectopic Endometrial Tissue. For the chemokines a one-way ANOVA test confirmed a significant variation between
the mRNA concentrations of the ectopic endometriotic tissue with eutopic endometrial tissue for ENA-78 ($P = 0.0039$) and RANTES ($P = 0.0490$), but not for MCP-1 ($P = 0.1251$) or IL-8 ($P = 0.7991$) (Figure 1). A Bonferroni’s multiple comparisons test was performed to compare the mean of each location against the eutopic mean. No significant difference was observed for MCP-1 mRNA expression between the eutopic tissue ($0.107 \pm 0.015, n = 7$) and the ovarian lesions ($2.751 \pm 1.943, n = 8, P < 0.05$), the peritoneal ($0.590 \pm 0.167, n = 7, P < 0.01$) or the RVS ($1.865 \pm 0.712, n = 4, P < 0.01$) lesions (Figure 1(a)). For ENA-78 there was a significantly stronger expression in the RVS lesions ($5.905 \pm 3.569, n = 4, P < 0.01$) compared to the eutopic tissue ($0.613 \pm 0.250, n = 17$), but no difference was observed in lesions from either the ovaries ($0.811 \pm 0.290, n = 8$), or the peritoneum ($1.444 \pm 0.504, n = 7$) (Figure 1(b)). For IL-8 there was no significant variation in the mRNA expression in either the peritoneum ($0.396 \pm 0.114, n = 8$), the ovarian ($0.409 \pm 0.084, n = 8$), or the RVS ($1.574 \pm 0.385, n = 5$) compared to the eutopic tissue ($3.979 \pm 3.337, n = 20$) (Figure 1(c)). A significantly higher expression of RANTES mRNA was observed in the RVS ($0.582 \pm 0.264, n = 5, P < 0.05$) compared to the eutopic tissue ($0.239 \pm 0.0432, n = 17$), but not in either the peritoneum ($0.220 \pm 0.030, n = 5$) or the ovarian tissue ($0.190 \pm 0.045, n = 8$) (Figure 1(d)).

For the inflammatory cytokines a one-way ANOVA test confirmed a significant variation between the mRNA concentrations in the eutopic tissue with the mRNA concentration in the ectopic tissue for TNFα ($P = 0.0014$) and IL-6 ($P < 0.0001$) (Figure 2). A post hoc Bonferroni’s multiple comparisons test indicated that TNFα mRNA expression in both the peritoneal (1.939 ± 0.667, $n = 8, P < 0.05$) and the RVS (3.128 ± 1.608, $n = 4, P < 0.01$) samples was significantly higher than that observed for their matching eutopic tissue ($0.444 \pm 0.106, n = 17$), although no difference was observed with the ovarian lesions ($0.291 \pm 0.034, n = 8$) (Figure 2(a)). For IL-6 there was a significantly higher expression in the RVS region ($9.308 \pm 3.714, n = 5, P < 0.0001$), but not the ovaries ($0.689 \pm 0.237, n = 7$) or the peritoneal region ($0.667 \pm 0.237, n = 7$) compared to the eutopic tissue ($0.152 \pm 0.091, n = 17$) (Figure 2(b)).

3.2. Cytokine mRNA Concentrations of Ectopic Endometriotic Lesions from Different Locations. A significant variation was observed between the mRNA expression of TNFα ($P = 0.0265$) and IL-6 ($P < 0.0001$), amongst the endometriotic lesions from different locations. A post-hoc Bonferroni’s multiple comparisons test indicated that the TNFα mRNA expression in the RVS ($2.590 \pm 1.357, n = 5$) was significantly higher than in the ovarian lesions ($0.813 \pm 0.144, n = 24, P < 0.05$), but not in the peritoneal lesions ($1.711 \pm 0.460, n = 12$). For IL-6 the mRNA expression in the RVS lesions ($10.150 \pm 3.148, n = 6$) was significantly higher than the expression in both the ovaries ($1.260 \pm 0.323, n = 24, P < 0.0001$) and the peritoneum ($1.211 \pm 0.400, n = 13, P < 0.0001$) (Figure 3).

In contrast no significant difference in mRNA expression was observed for any of the four chemokines examined in this study. MCP-1 expression in the RVS ($1.700 \pm 0.576, n = 5$) was not significantly higher than either the ovarian (1.393 ± 0.632, $n = 25$) or the peritoneum samples ($0.814 \pm 0.215, n = 13$), which was also the case for ENA-78 (Peritoneum; 1.497 ± 0.465, $n = 13$, ovarian; 2.988 ± 1.429, $n = 25$, RVS; 4.822 ± 2.969, $n = 5$), IL-8 (peritoneum; 1.548 ± 1.188, $n = 13$, ovaries; 1.352 ± 0.471, $n = 25$, RVS; 2.017 ± 0.543, $n = 6$), and RANTES (peritoneal; 0.288 ± 0.064, $n = 11$, ovarian; 0.364 ± 0.054, $n = 22$, RVS; 0.528 ± 0.222, $n = 6$) (Figure 3).

4. Discussion

The study showed that the mRNA expression of the chemokines ENA-78 and RANTES, as well as the inflammatory cytokines TNFα and IL-6, was significantly increased in the ectopic lesion compared to those in the matched eutopic tissue in women with endometriosis. For IL-6, ENA-78, and RANTES this increase was more significant in the RVS region, whereas for TNFα, it was in both the peritoneal lesions and the RVS lesions. In addition, when compared across lesion

### Table 1: Primer sequences of the reference genes and genes of interest.

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<tr>
<th>Cytokine</th>
<th>Genbank accession no.</th>
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<td>NM_006600</td>
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locations IL-6 was the most highly expressed in the RVS region compared to either the ovaries or the peritoneum. The results suggest therefore that different inflammatory proteins have separate roles in different lesions and understanding these roles may help to specifically target certain presentations of endometriosis. In addition, the increased production of many of these proteins by the RVS lesions provides some molecular evidence towards the notion that lesions developing in the RVS are strongly inflammatory.

Increased expression of chemokines by ectopic endometrial implants is an important early stage in the pathogenesis of endometriosis. Chemokines secreted by the ectopic lesions stimulate the infiltration of macrophages that further contribute to the development of the disease. In this
In conclusion, this study gives new insights in the production of chemokines and cytokines in endometriotic lesions from different locations and our results support the supposition that the RVS lesions are an intensely inflammatory form of endometriosis. Assessing lesions from different locations uniquely may be vital in understanding the pathological changes of the disease and potentially for their mode of treatment.
Conflict of Interests

None of the authors have a conflict of interests.

References


