Decreased levels of the potent regulator of monocyte/macrophage activation, interleukin-13, in the peritoneal fluid of patients with endometriosis

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Endometriosis is characterized by an increase in the number, activation and secretory activity of peritoneal fluid macrophages. Factors regulating the activation of these cells may be important in the pathophysiology of this disease. In this study we measured by enzyme-linked immunosorbent assay the concentrations of the macrophage inhibitory factor interleukin (IL)-13 in the peritoneal fluid of women with and without endometriosis. It was found that women with endometriosis had significantly lower amounts of IL-13 (95 ± 9.8 pg/ml) in peritoneal fluid, compared with women without endometriosis (115 ± 30 pg/ml) (P < 0.01). No cycle-specific variation was evident for either group. Another macrophage inhibitory interleukin (IL-10) was also measured, but no differences between women with (16.1 ± 13.2 pg/ml) or without (10.3 ± 5.6 pg/ml) endometriosis were seen. The immunolocalization of IL-13 was assessed in eutopic and ectopic endometrium and in isolated peritoneal fluid cells. Glandular epithelial cells and stromal cells in both eutopic and ectopic endometrium were immunopositive for IL-13. No cycle-specific differences in the immunolocalization of IL-13 were seen. In conclusion, the reduced amounts of IL-13 in the peritoneal fluid of women with endometriosis may lead to a lack of suppression of macrophage activation, thereby contributing to the overall pathogenesis of this disease.

Key words: endometriosis/IL-10/IL-13/interleukins/macrophages/peritoneal fluid

Introduction

Endometriosis is one of the commonest benign gynaecological conditions and is present in ~10% of women of reproductive age in the UK and USA (Strathy et al., 1982). Although the basic aetiology of the disease is unknown it is generally accepted that endometriosis results from the implantation of endometrium deposited in the peritoneal cavity following retrograde menstruation. Patients with endometriosis are characterized by the ability of the endometrium to implant and by the peritoneal response to this tissue. The peritoneal environment, most notably the contribution of the peritoneal fluid macrophages, is of critical importance in the aetiology and pathogenesis of endometriosis (Halme et al., 1987; Hellema, 1994; McLaren et al., 1996). Peritoneal fluid contains soluble growth and angiogenic factors (Ramey and Archer, 1993), some of which are elevated in patients with endometriosis (Oosterlynck et al., 1993; Ryan et al., 1995). The number, size, activity and secretory capacity of these peritoneal fluid macrophages is elevated in endometriosis (Halme et al., 1983, 1987; McLaren et al., 1995) and, given the increasing acceptance of the importance of these cells in endometriosis, any factors that can control their activity will be of obvious importance in the pathophysiology of this disease.

Cytokines are diverse proteins that play a central role in regulating cell proliferation, activation, motility, adhesion, chemotaxis and morphogenesis. Human interleukin (IL)-13 is a recently cloned interleukin (Morgan et al., 1992) with pleiotropic functions, many of which are shared with IL-4 (Zurawski and de Vries, 1994). Responsive cells include mononuclear phagocytes, B cells, large granular lymphocytes and endothelial cells (Minty et al., 1993; McKenzie et al., 1993; Sironi et al., 1994). In particular, IL-13 has the capacity to inhibit pro-inflammatory cytokine synthesis by activated monocyte/macrophages (McKenzie et al., 1993; de Waal Malefyt et al., 1993). IL-13 also down-regulates prostaglandin oestradiol production by activated monocytes and macrophages, via down-regulation of the inducible enzyme cyclooxygenase-2 (Endo et al., 1996). Thus IL-13 has a potent capacity to regulate monocyte/macrophage activation (Zurawski and de Vries, 1994), and since activation of these cells is a characteristic feature of endometriosis the local IL-13 status may be of importance in the pathogenesis of this disease. In this study we compare the concentration of IL-13 in the peritoneal fluid of women with and without endometriosis, and the implications are discussed.

Materials and methods

Patient details

Women between 24 and 44 years of age undergoing either diagnostic laparoscopy for pain or for infertility were included in this study. Endometriosis was diagnosed laparoscopically and confirmed histologically. Endometriotic tissue samples were biopsied from the surface of the peritoneal cavity. No ovarian or adenomyosis samples were used in this study. Peritoneal fluid samples were collected from women with (n = 16) and without (n = 16) endometriosis, throughout
the menstrual cycle. Each group contained eight patients from the proliferative phase and eight patients from the secretory phase of the menstrual cycle. The extent of endometriosis was determined according to the revised American Fertility Society (1985) scoring system. All patients included in this study had minimal to moderate endometriosis (scoring 1–12). This study was approved by the ethics committee of Addenbrookes NHS and written informed consent was obtained from each patient.

Preparation of peritoneal fluid
Peritoneal fluid was aspirated from the posterior cul-de-sac using a Verres needle during the laparoscopy. The cellular constituents of the peritoneal fluid were removed by centrifugation at 19 000 g for 30 min at 4°C. The clarified peritoneal fluid supernatants were then collected and stored in aliquots at −70°C until the cytokine concentrations were determined.

Isolation of peritoneal fluid cells
Peritoneal fluid was aspirated from the posterior cul-de-sac using a Verres needle. Peritoneal fluid cells were isolated by centrifugation through Ficoll-Hypaque 1077 (Sigma, Poole, UK) at room temperature for 10 min at 400 g. Cells were washed in phosphate-buffered saline (PBS) and counted before being resuspended at a concentration of 1×10⁶ cells/ml in PBS/1% bovine serum albumin (BSA). This method of isolation results in an enriched population of peritoneal fluid cell macrophages (80–90%) and T-lymphocytes (10–20%). These cells were then transferred onto microscope slides by cytopinning.

Collection of ectopic and eutopic endometrium
If the diagnosis of endometriosis was made, a second portal was inserted in the right iliac fossa through the rectus abdominus muscle lateral to the inferior epigastric vessels. A punch biopsy was obtained under direct vision on the video monitor. Eutopic tissue was obtained from the uterine cavity after gentle dilatation of the cervix to Heger 8. Strips of endometrium were obtained by sharp curettage. Eutopic and ectopic tissue from eight patients, four from each phase of the cycle, were assessed for the immunolocalization of IL-13.

Detection of IL-10 and IL-13 in peritoneal fluid
IL-10 and IL-13 in peritoneal fluids were determined using commercially available cytokine-specific enzyme-linked immunosorbent assays (ELISA) (R & D Systems, Abingdon, UK). Samples of peritoneal fluid from women with and without endometriosis were centrifuged at 19 000 g for 30 min and the resultant supernatant was analysed. Samples from each patient group were always measured in parallel and in triplicate to avoid interassay variance. The sensitivity of the IL-13 ELISA was <32 pg/ml, and the standard curve range was 62–4000 pg/ml. IL-10 ELISA had a sensitivity of 2 pg/ml with a standard curve range of 7.8–500 pg/ml.

Immunohistochemical staining for IL-13
Formalin-fixed paraffin-embedded sections of endometrium from women with and without endometriosis were de-paraffinized in xylene and hydrated gradually through graded alcohols. Antigenic unmasking was achieved by pressure cooking the slides for 1 min in 0.01 M sodium citrate. Frozen cytoospin slides of isolated peritoneal fluid cells from both patient groups were thawed and washed in PBS for 5 min. Both sets of slides were then incubated for 20 min in 10% rabbit serum to block non-specific binding, before incubation for 1 h with a 10-fold diluted anti-human IL-13 goat polyclonal antibody (R & D Systems). Endogenous peroxidase activity was inactivated by a 10 min incubation with 1% H₂O₂ in PBS. Binding was visualized following incubation with a biotinylated rabbit antigoat antibody (1:200) for 1 h and subsequent complexing with avidin–biotin peroxidase (Vector, Peterborough, UK). The complex was detected with diaminobenzidine and H₂O₂ in 0.1 M Tris–HCl (pH 7.5). The identity of the cells was confirmed using specific T-lymphocyte (CD3) (Dako, Cambridge, UK) and macrophage (Leu M3) antibodies (Dako).

Statistical analysis
Statistical analysis was performed on the original data. The data were not normally distributed and significance was assessed using the non-parametric Wilcoxon’s rank test for unpaired data. Statistical significance was accepted at P < 0.05.

Results
Peritoneal fluid concentrations of IL-13
Peritoneal fluids from women with and without endometriosis, throughout the cycle, were measured for IL-13, which was detectable in all samples analysed. The amount of IL-13 in peritoneal fluid for both patient groups did not show a cycle-specific pattern (Figure 1). However, fluid from women with endometriosis contained significantly lower (P < 0.01) amounts of IL-13 (95 ± 9.8 pg/ml) than that obtained from women without endometriosis (115 ± 30 pg/ml). The majority of the patients were scored (American Fertility Society, 1985) as having minimal to mild endometriosis and we were unable to determine whether any correlation existed between the severity of the disease and the concentration of IL-13 in the peritoneal fluid.

Peritoneal fluid concentrations of IL-10
IL-10 was measured in the peritoneal fluid from women with and without endometriosis. IL-10 was detected in all samples analysed. No significant differences were detected between women with (16.1 ± 13.2 pg/ml) and without (10.3 ± 5.6 pg/ml) endometriosis. No cycle specific differences were seen in either group.
Endometriosis and macrophages

Figure 2. Immunohistochemical localization of IL-13 in both eutopic and ectopic endometrium in representative tissue sections. (A) Proliferative phase eutopic endometrium, (B) secretory phase eutopic endometrium, (C) proliferative phase ectopic endometrium, (D) secretory phase ectopic endometrium. G = gland, S = stroma. Original magnification ×250.

**Immunohistochemical staining of peritoneal fluid cells for IL-13**

The cellular constituents of the peritoneal fluid were assessed for protein expression of IL-13 by immunohistochemical staining. Following staining it was seen that neither the peritoneal fluid macrophages nor T-lymphocytes were immunopositive for IL-13. This was the case for both patient groups and for both phases of the cycle.

**Immunohistochemical staining for IL-13 in eutopic and ectopic endometrium**

Eight samples of eutopic and ectopic endometrium, four from each phase of the cycle, were assessed for protein expression of IL-13 by immunohistochemical staining (Figure 2). In both eutopic and ectopic tissue the glandular epithelium cells were immunopositive for IL-13, as were stromal cells. However, not all the stromal cells were immunopositive. No cycle-dependent differences were noted in the localization of IL-13 in either eutopic or ectopic tissue.

**Discussion**

There are numerous theories proposed for the histogenesis of endometriosis, but the implantation of exfoliated endometrium due to the combined effects of a rich cytokine environment and reduced immunosurveillance is the one with the strongest supporting evidence (for review, see McLaren and Prentice, 1996). Central to this theory is the role of the peritoneal fluid macrophages. We and others have shown the increased contribution of these cells to the pathophysiology of the disease, in particular their increased number, activation status and secretion of pro-inflammatory cytokines and growth factors (Halme et al., 1983, 1987; McLaren and Prentice, 1996; McLaren et al., 1996). Most of this work has focused on the role of activated macrophages and the consequences of this activation in endometriosis. This study introduces the concept of the role of an inhibitor of macrophage function (IL-13) in macrophage activation, and the consequences this has for the pathogenesis of this disease.

IL-13 has the capacity to inhibit pro-inflammatory cytokine synthesis by activated monocyte/macrophages (McKenzie et al., 1993). Endometriosis is often associated with the signs of chronic inflammation, such as increased numbers of macrophages (Olive et al., 1985), higher activation status of these cells (Hill et al., 1988) and increased presence of pro-inflammatory cytokines (Ramey and Archer, 1993). The peritoneal environment contains a rich cocktail of cytokines, including interleukins such as IL-1β (Koyama et al., 1993), -4, -5, -6 (Punnonen et al., 1996) and -8 (Arici et al., 1996). These cytokines are pleiotropic in function; however, apart from the angiogenic properties of IL-8 (Ryan et al., 1995) their role in the pathogenesis of endometriosis remains unclear. So far there has been little indication of the presence of cytokines or of their possible contribution to the down-regulation of peritoneal fluid macrophage function. However, a recent paper by Punnonen et al. (1996) showed an increase in IL-10, a cytokine with known macrophage inhibitory function, in peritoneal fluid of women with endometriosis. They suggested that this supported the conclusion that macrophage activity.
activity is increased in these patients, but they failed to discuss the significance of such an increase or of the co-existence of activated macrophages. Furthermore, we (see Results) and others (Rana et al., 1996) have also measured IL-10 in the peritoneal fluid of women with and without endometriosis and failed to show any difference in the value of this cytokine between the patient groups. In this study, for the first time, we show significant reduction in the monocyte/macrophage inhibitor IL-13 in the peritoneal fluid of women with endometriosis. This fluid is in direct contact with the peritoneal fluid macrophages. Thus cells with the appropriate receptors could respond to this cytokine. Reduced amounts of IL-13 would suggest a reduction in effect of this cytokine on the peritoneal fluid macrophages in cases of endometriosis. This possible reduction in macrophage inhibition may play a critical role in the overall increased activation of the macrophages, which is characteristic of endometriosis.

IL-13 was not expressed by the peritoneal fluid macrophages or lymphocytes, indicating that the function of the macrophages may not be under paracrine control from the peritoneal fluid lymphocytes. However, as mentioned previously, IL-13 was found in the peritoneal fluid, thus other sources need to be identified. Such sources of cytokines in the peritoneal fluid may include refluxed endometrial tissue and/or follicular fluid as well as the ectopic endometrium itself. IL-13 protein was found to be localized in glandular epithelial and stromal cells in both eutopic and ectopic endometrium. Therefore, the eutopic and ectopic endometrium may be an important source of this factor. Another macrophage inhibitory interleukin, IL-10, has also been immunolocalized in cycling endometrium to both the glandular epithelium and stroma (Krasnow et al., 1996). IL-13 has not yet been detected in follicular fluid, but the latter as a potential source of IL-13 found in peritoneal fluid cannot be ruled out. We found no cycle-dependent variations in the immunolocalization of IL-13, suggesting that its expression is not controlled by ovarian steroids and that its effect on the resident macrophage population within the endometrium may be constant throughout the cycle.

In summary, IL-13 has the capacity to regulate monocyte/macrophage activation and, given the increasing evidence of involvement of these cells in endometriosis, this factor may be of great importance in the pathogenesis of this disease. We report a significantly lower concentration of IL-13 in the peritoneal fluid of women with endometriosis compared to normal women. We speculate that a reduction in concentrations of this factor may lead to increased activation of peritoneal fluid macrophages, which is characteristic of endometriosis and contributes to the overall pathogenesis of the disease.

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References