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Role of IL-18 in pathogenesis of endometriosis

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BACKGROUND: Endometriosis is a complex disease associated with a wide range of immune responses, including pain, adhesion, exudation of peritoneal fluid, elevation of cytokine levels and generation of autoantibodies. Interleukin (IL)-18 is a strong pleiotropic cytokine known to be involved in various immune diseases. The aim of this study is to elucidate the role of IL-18 in the pathogenesis of endometriosis. METHODS: IL-18 and IL-1β concentrations were measured in the peritoneal fluid and sera of 39 endometriosis patients and 15 control women. Expression of IL-18 and IL-18 receptor α-chain (IL-18Rα) was analysed in endometriotic tissues immunohistochemically. The effects of IL-18 on cyclooxygenase (COX)-II gene expression were analysed in peritoneal fluid monocytes and endometriotic cells of endometriosis patients. RESULTS: IL-18 concentrations in the peritoneal fluid of endometriosis patients averaged 592.57 ± 108.27 pg/ml, significantly higher than 260.50 ± 55.88 pg/ml in non-endometriotic samples. IL-18 concentrations in the serum did not differ significantly between endometriosis and control patients. Similarly, no significant differences were observed in IL-1β concentrations in either the peritoneal fluid or the serum. IL-18 and IL-18Rα were expressed in endometriotic tissues. IL-18Rα expression was also observed in cells infiltrating into the inflammatory area of the endometriosis patients. COX-II was induced in peritoneal fluid monocytes and in endometriotic cells in response to IL-18 stimulation. CONCLUSIONS: The elevation of IL-18 in the peritoneal fluid of endometriosis patients and the induction of COX-II in peritoneal monocytes by IL-18 suggest that IL-18 plays a pathogenic role in endometriosis.

Key words: COX-II/cytokine/endometriosis/IL-18/IL-1β

Introduction

Endometriosis is one of the common gynaecological diseases, affecting ~10% of women of reproductive age. Endometriosis is the presence of tissue with epithelial and stromal characteristics of endometrium outside the uterine cavity. It is a benign disease, but can cause adhesion, inflammation and pain during menstruation, and even infertility. The aetiology of endometriosis is not clear, but various factors have been suggested to be involved in its pathogenesis. In endometriosis, macrophages are seen to infiltrate ectopic endometrium, resulting in accumulation of peritoneal fluid with elevated levels of Th1 pro-inflammatory cytokines, such as interleukin (IL)-6 (Koyama *et al*., 1993; Rier *et al*., 1994; Punnonen *et al*., 1996), IL-1β (Anderson and Hill, 1987) and tumour necrosis factor-α (TNF-α) (Cheong *et al*., 2002). However, the elevation of these Th1 cytokines could not fully account for complex clinical features of endometriosis. For example, various autoantibodies have been found in endometriosis patients (Fernandez-Shaw *et al*., 1993), suggesting that the Th2 immune status is also activated in endometriosis.

IL-18 was initially conceived to be a Th1 cytokine which, together with IL-12, releases interferon-γ (IFN-γ) and TNF-α from T cells and natural killer cells (Okamura *et al*., 1995; Tsutsui *et al*., 1996). However, IL-18 was later found to play a role in stimulating the Th2 status, releasing IL-4, IL-5 and IL-13 (Hoshino *et al*., 1999; Nakanishi *et al*., 2001). IL-18 also stimulates a subset of macrophages to induce cyclooxygenase (COX)-II and, subsequently, prostaglandins (PGs) (Kashiwamura *et al*., 2002), which mediate various biological responses, including pain (Olee *et al*., 1999; Futani *et al*., 2002). It has been reported recently that the peritoneal fluid of endometriosis patients contains elevated levels of PGs (Wu *et al*., 2002). This, together with the complex immune status in endometriosis patients, prompted us to examine the role of IL-18 in the pathogenesis of endometriosis.

Materials and methods

Patients

Thirty-nine patients with endometriosis, 24–48 years of age, average 33.8 ± 6.8 years, participated in this study with informed consent. They did not take hormonal medication for at least 3 months before surgery. Pathological staging was determined at surgery according to the revised American Society for Reproductive Medicine's classifi-

cation (r-ASRM). Nineteen non-endometriosis patients, 20–46 years of age, average 31.7 ± 6.7 years, served as controls, with informed consent. They included four patients with serous cyst adenoma, two patients with mucinous cyst adenoma, six patients with a dermoid cyst, one patient with a paraovarian cyst, two patients with myoma, and four patients with infertility. All the women had normal ovulatory cycles, and specimens were obtained during the follicular phase of their menstrual cycle.

Preparation of sera, peritoneal fluids and tissue specimens

The peritoneal fluid was collected using laparoscopy, mixed with heparin and centrifuged, and the supernatant was stored at –80°C in 500 µl aliquots until use. Blood samples were processed and stored in a similar manner. Tissue specimens were obtained from peritoneal lesions and endometriomas. They were fixed in 10% buffered formalin and embedded in paraffin.

Measurement of cytokines

IL-18 in peritoneal fluids and sera was measured by enzyme-linked immunosorbent assay (ELISA) using the Human IL-18 Immuno Assay Kit (MBL Co. Ltd, Nagoya, Aichi, Japan). IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IFN-γ were determined by Bio-Plex Protein Array System (Bio-Rad Laboratories, Hercules, CA) using Human Cytokine Assay reagents (Bio-Rad).

Cytokines

Recombinant human IL-18 was purchased from MBL Co. Ltd and recombinant human IL-1β from R&D systems (Minneapolis, MN).

Preparation of CD14-positive cells from the peritoneal fluid and treatment with IL-18

Cells in peritoneal fluids were collected by centrifugation, treated with ACK solution to deplete red blood cells, and then incubated with micro bead-conjugated mouse anti-human CD14 monoclonal antibody (mAb) (Miltenyi Biotec, Gladbach, Germany). CD14-positive cells were separated using a magnetic cell sorter and a biomolecules (MACS) column (Miltenyi Biotec). The purity of cells was analysed by flow cytometry using fluorescein isothiocyanate (FITC)-labelled mouse anti-human CD14 mAb (BD Biosciences, San Jose, CA). CD14-positive cells (2×10^5 cells/ml) were cultured with IL-18 (20 ng/ml) or IL-1β (20 ng/ml) in phenol red-free RPMI-1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum at 37°C for 16 h under 5% CO_2.

Preparation of endometriotic cells and treatment with IL-18

Endometriotic tissues were incubated in RPMI-1640 (Invitrogen) containing 15 µg/ml DNase (Sigma Chemical Co., St Louis, MO) and 400 U/ml collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 37°C for 1 h. Cells released were collected by centrifugation at 150 *g* for 10 min and used for experiments without separation of inner and interstitial cells. Treatment of these cells with IL-18 or IL-1β was carried out in the same way as described above for CD14-positive cells.

RT–PCR analysis

Total RNA was extracted using Isogen (NIPPON GENE Co. Ltd, Toyama, Japan) and reverse transcribed by MuLV reverse transcriptase. The resultant cDNA was used as the template for PCR using primers specific to human COX-I, COX-II, IL-18, IL-18Rα and IL-18Rβ as follows: COX-I sense, 5′-GAGTCTTTCTCCAACGTGAGC-3′; antisense, 5′-ACCTGGTACTTGAGTTTCCCA-3′; COX-II sense, 5′-TGAAACCCACTCCAAACACAG-3′; antisense, 5′-TCATCAGGCACAGGAGGAAG-3′; IL-18 sense, 5′-GCTTGAATCTAAATTATCAGTC-3′; antisense, 5′-GAAGATTCAAATTGCATCTTAT-3′; IL-18Rα sense, 5′-GTTGAGTTGAATGACACAGG-3′; antisense, 5′-TCCACTGCAACATGGTTAAG-3′; and IL-18Rβ sense, 5′-ATGCTCTGTTTGGGCTGGATA-3′; antisense, 5′-CATCTTGACACAACAGGCTAC-3′. PCRs were denaturation at 94°C for 5 min, then 32 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 1 min, and extension at 72°C for 7 min.

PCR of β-actin cDNA was conducted using a sense primer, 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, and an antisense primer, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′, at 94°C for 5 min, then 35 cycles at 94°C for 30 s, at 66°C for 30 s, and at 72°C for 1 min, and then at 72°C for 7 min.

PCR products were electrophoresed in agarose gels, stained with ethidium bromide and visualized under UV illumination.

Immunohistochemical staining

Paraffin-embedded tissue specimens were cut into 4 µm sections, deparaffinized and incubated with mouse anti-human IL-18 mAb (a kind gift from Dr Torigoe, Institute of Bio-Research, Hayashibara, Okayama, Japan), mouse anti-human IL-18Rα mAb (a kind gift from Dr Torigoe), mouse anti-human COX-II mb (IBL, Fujioka, Gunma, Japan) or mouse anti-human CD68 mAb (DAKO, Glostrup, Denmark), and then with peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (Vector, CA). Specific bindings of antibodies were detected using a DAB reagent kit (DAKO).

Western blot analysis

Endometriotic cells were lysed in ice-cold NP-40 lysis buffer consisting of 20 mM Tris–HCl pH 8.8, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 50 mM phenylmethysulphonyl fluoride, supplemented with proteinase inhibitor cocktail (one tablet/10 ml of lysis buffer) (Roche Molecular Biochemicals, Mannheim, Germany). Each proteinase inhibitor cocktail tablet contains 3.0 mg of antipapain, 0.5 mg of bestatin, 1.0 mg of chymostatin, 3.0 mg of E-64, 0.5 mg of leupeptin, 0.5 mg of pepstatin, 3.0 mg of phosphramidon, 20.0 mg of pefabloc SC and 0.5 mg of aprotinin. The lysate (20 µg of protein) was subjected to SDS–PAGE under reduced conditions. Separated proteins were transferred onto PDFV membranes (Hybond-P, Amersham Biosciences Co., Piscataway, NJ). The membrane was soaked in phosphate-buffered saline (PBS) containing 2% non-fat milk, and incubated with 1 µg/ml of mouse anti-human COX-II mAb (IBL Co. Ltd, Fujioka, Gunma, Japan) and affinity-purified rabbit anti-human β-actin antiboby (Sigma Chemical Co.). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (1:1000 diluted) and the F(ab′)2 fragment of HRP-conjugated donkey anti-rabbit IgG antibody were used as the secondary antibodies (Amersham Biosciences). Specific bindings of antibodies were detected by the luminescent image analyser, LAS-1000 plus (Fuji Photo Film Co. Ltd, Tokyo, Japan), using ECL plus western blotting detection reagents (Amersham Biosciences).

Statistical analysis

Data were analysed by Mann–Whitney U-test for multiple comparisons. A P-value <0.05 was considered to be statistically significant. Results were expressed as the means ± SEs of repeated experiments. The Pearson correlation coefficient (r) was determined using Stat Mate III software (Atms K.K., Tokyo, Japan).

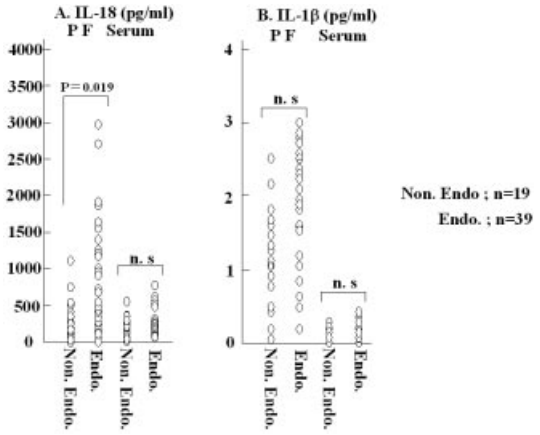

Figure 1. IL-18 and IL-1β concentrations in the peritoneal fluid and the serum in endometriosis and non-endometriosis patients. Concentrations of IL-18 (**A**) and IL-1β (**B**) in the peritoneal fluid (P.F.) and the serum of endometriosis patients (n = 39) and non-endometriosis patients (n = 19) were measured by ELISA.

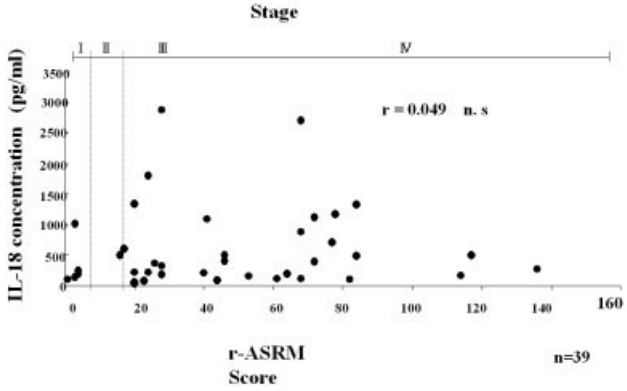

Figure 2. Relationship between IL-18 concentrations in the peritoneal fluid and r-ASRM scores of endometriosis patients. IL-18 concentrations in peritoneal fluids of endometriotic patients (n = 39) and their r-ASRM scores were plotted on the y- and x-axis, respectively. The pathological stages of the patients, classified from the r-ASRM score, are indicated on the top.

Results

IL-18 levels in the serum and the peritoneal fluid of endometriosis patients

IL-18 concentrations in the peritoneal fluid of endometriosis patients averaged 592.57 ± 108.27 pg/ml (mean ± SE), which was significantly higher than 260.50 ± 55.88 pg/ml in non-endometriosis patients ($P < 0.019$) (Figure 1A). IL-18 levels in the serum were 177.17 ± 28.37 and 174.14 ± 27.48 pg/ml in endometriosis and control patients, respectively, showing no significant difference ($P = 0.945$). IL-1β concentrations in the peritoneal fluid were 2.37 ± 0.50 and 1.23 ± 0.34 pg/ml in endometriosis and control patients, respectively, and those in the sera were 0.12 ± 0.09 and 0.17 ± 0.11 pg/ml, respectively (Figure 1B). In both cases, there were no statistically significant differences. Similarly, no significant differences were observed in concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, GM-CSF and IFN-γ in the serum and the peritoneal fluid between endometriotic and control patients (data not shown).

Comparison of IL-18 levels in the peritoneal fluid and r-ASRM scores

Analysis of the relationship between IL-18 levels in the peritoneal fluid and r-ASRM scores of the endometriosis patients showed a coefficient of correlation (r) of 0.049 (Figure 2), indicating that IL-18 levels in the peritoneal fluid were not correlated with r-ASRM scores.

Induction of COX-II by IL-18 in peritoneal fluid cells of endometriosis patients

CD14-positive cells were isolated from the peritoneal fluid of endometriosis patients, incubated with IL-18 or IL-1β, and analysed for the expression of COX-I and COX-II. The results showed that IL-18 induced COX-II mRNA but not COX-I mRNA (Figure 3), while IL-1β induced neither COX-I nor COX-II mRNA. These results suggested that peritoneal cells of endometriosis patients were at a pre-priming stage where COX-II expression can be induced by IL-18.

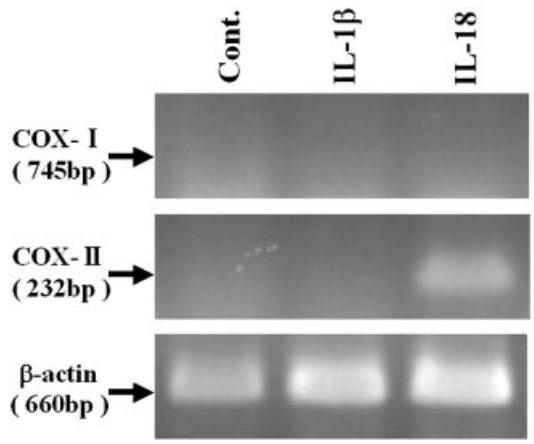

Figure 3. Induction of COX-II gene expression by IL-18 in peritoneal CD14-positive cells from endometriosis patients. CD14-positive cells were fractionated and stimulated with IL-1β (20 ng/ml) or IL-18 (20 ng/ml) for 16 h. RT–PCR analysis was performed with primers specific to the COX-I (upper panel), COX-II (middle panel) and β-actin (bottom panel) genes. Significant induction of the COX-II gene was observed with IL-18, but not with IL-1β. Arrows indicate bands specific to COX-II and β-actin. A representative of ten experiments is shown.

Immunohistochemical analysis of IL-18, IL-18Rα and COX-II in endometriotic tissues

Immunohistochemical analysis showed that IL-18 was expressed in the epithelium and stroma cells of endometriotic and adenomyotic tissues, but not in cells infiltrating into surrounding tissues (Figure 4A). On the other hand, IL-18Rα (Figure 4B) and COX-II (Figure 4C) were found to be expressed in infiltrating cells as well as in epithelium and stroma cells of the ectopic endometrium. The normal endometrium also expressed IL-18, IL-18Rα and COX-II, but to a much lesser extent (data not shown).

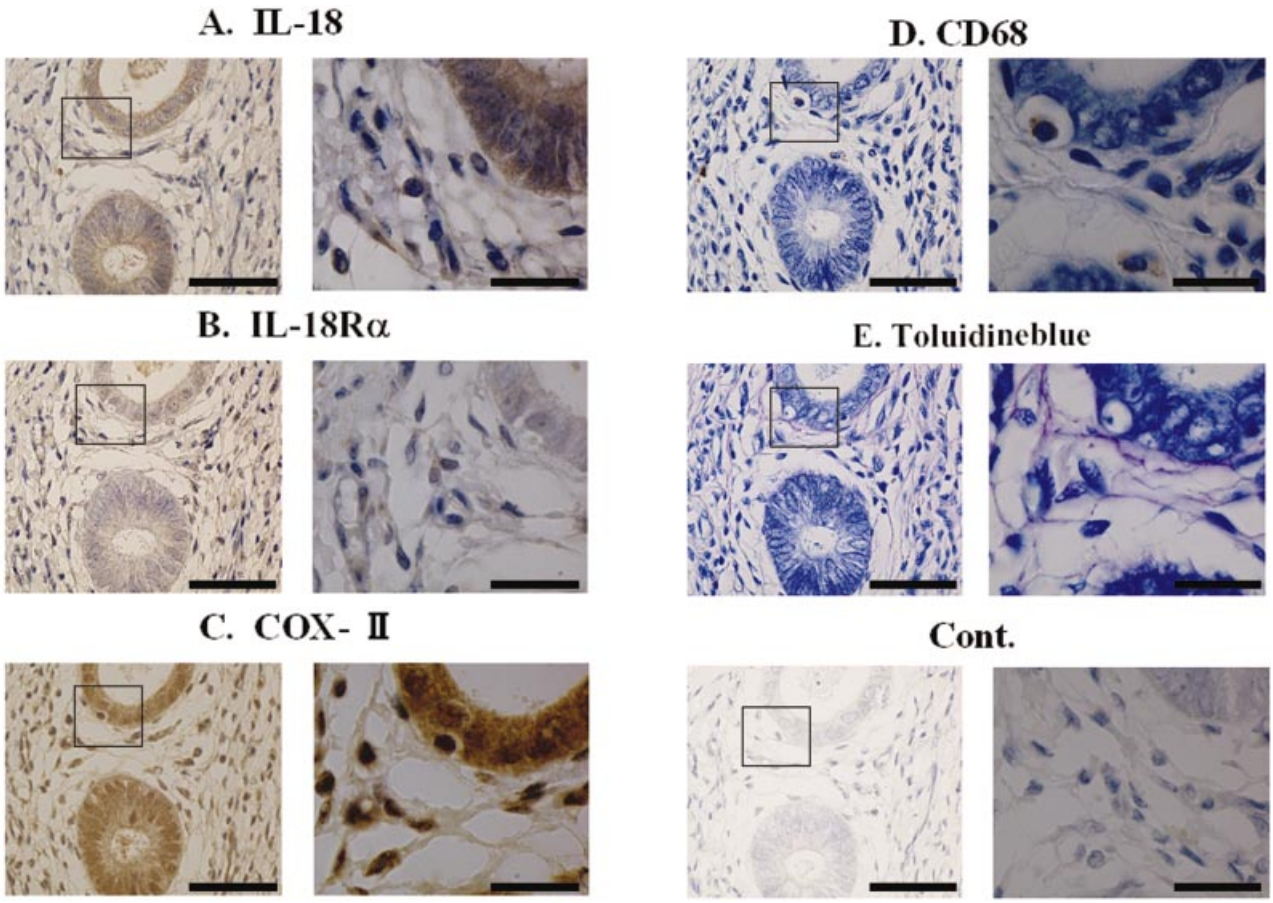

Figure 4. Immunohistochemical analysis of IL-18, IL-18Rα, COX-II and CD68 in endometriotic tissues. Endometriotic tissue, infiltrated cells and parenchymal cells were stained with antibodies to IL-18 (**A**) IL-18Rα (**B**), COX-II (**C**) and CD68 (**D**), and with toluidine blue (**E**). (Right columns, bar = 40 μm; left columns, bar = 100 μm). A representative of twenty-five samples is shown.

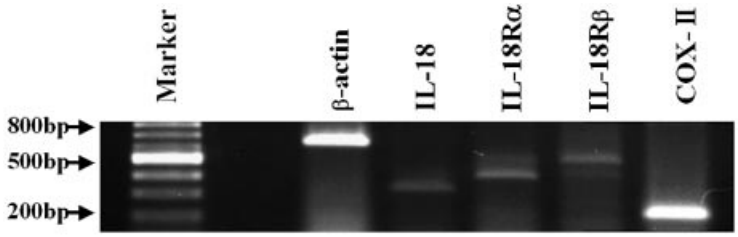

Figure 5. IL-18, IL-18Rα and IL-18Rβ gene expression in endometriotic tissues from endometriosis patients. Expression of IL-18, IL-18Rα, IL-18Rβ and COX-II was analysed by RT–PCR. β-Actin was used as an internal control. Arrows indicate bands specific to IL-18, IL-18Rα, IL-18Rβ, COX-II and β-actin. A representative of six samples is shown.

IL-18Rα-positive, infiltrating cells were stained with anti-CD68 antibody (Figure 4D), but not with toluidine blue (Figue 4E), indicating that they were not mast cells but probably macrophages. Similar results were observed with endometrioma (data not shown).

RT–PCR analysis of expression of IL-18, IL-18R and COX-II

RT-PCR analysis showed that endometriotic tissues of endometriosis patients expressed mRNAs of IL-18, IL-18 receptors (IL-18Rα, IL-18Rβ) and COX-II (Figure 5).

Induction of COX-II in endometriotic cells:

To analyse the effect of IL-18 on the expression of COX-II in ectopic endometrium, endometriotic tissues were treated with DNase and collagenase, and released cells were incubated with IL-18. Western blot analysis showed that IL-18 stimulated COX-II expression (Figure 6). IL-1β was also found to enhance COX-II expression (Figure 6).

Discussion

In the present study, we estimated levels of IL-18 in the serum and the peritoneal fluid of endometriosis patients since a

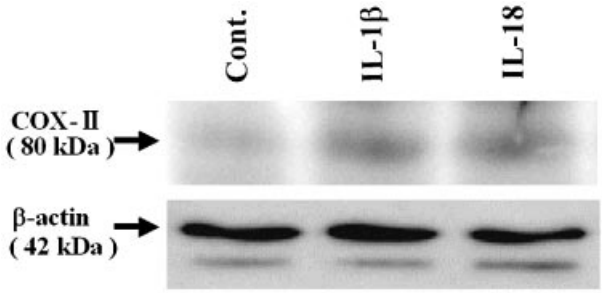

Figure 6. Induction of COX-II expression in endometriotic cells by IL-18 and IL-1β. Endometriotic cells were incubated with IL-18 or IL-1β for 16 h, and COX-II was analysed by western blotting. β-Actin was used as an internal control. Arrows indicate bands specific to COX-II and β-actin. A representative of six experiments is shown.

number of studies have reported elevation of various cytokines, particularly IL-6, in the peritoneal fluid of endometriosis patients. We did not observe elevation of IL-6 levels in either the serum or the peritoneal fluid of endometriosis patients, but found marked increases in IL-18 levels in their peritoneal fluid, but not in their serum. No significant correlation was found between IL-18 levels in the peritoneal fluid and the r-ASRM score, raising the possibility that r-ASRM score may not closely reflect the current pathological status of endometriosis.

Endometriosis patients generally accumulate peritoneal fluids rich in macrophages in the pelvic cavity (Chacho *et al*., 1986; Harada *et al*., 1997). Our fluorescence-activated cell sorting (FACS) analysis using FITC-labelled anti-human-CD14 antibody confirmed that the majority of cells in the peritoneal fluid of endometriosis patients were macrophages. We found that these macrophages express IL-18Rα and produce COX-II in response to treatment with IL-18. We also showed the expression of IL-18 and IL-18Rα in endometriotic tissue and found that COX-II was induced by IL-18 in endometriotic cells. In addition, COX-II was found to be expressed in CD68-positive cells, probably macrophages, infiltrating into endometriotic tissues. Finally, we detected caspase-1, which converts inactive IL-18 precursors to an active form (Gu *et al*., 1997), in the stroma of endometriotic tissues (data not shown). These results suggest that functional IL-18 is generated *in situ* and induces COX-II, which would lead to the production of PGs, eventually causing pain.

Elevation of IL-18 levels in endometriosis is of interest from the standpoint of the production of autoantibodies in endometriosis, since IL-18 has been shown to enhance both Th1 and Th2 immune responses (Hoshino *et al*., 1999; Nakanishi *et al*., 2001; Ogura *et al*., 2001). With IL-12, IL-18 induces IFN-γ as a Th1 cytokine, but without IL-12, it induces IL-10 as a Th2 cytokine (Nakanishi et al., 2001; Ogura *et al*., 2001). It has been shown that simultaneous treatment of systemic lupus erythematosis (SLE) model mice MRL/1pr with IL-18 and IL-12 exacerbates bufferfly facial rash but reduces autoantibody levels (Esfandiari *et al*., 2001). On the other hand, treatment of these mice with IL-18 alone results in remission of facial rash and increases in autoantibody levels. It is possible that, in endometriosis, IL-18 may induce PGs in ectopic endometriotic cells, converting their environment into a Th2 dominant status to favour local autoimmunity leading to autoantibody production. In this way, IL-18 may play a crucial role in the pathogenic network in endometriosis.

In conclusion, our study shows for the first time that IL-18 might be a key cytokine in developing the pathogenesis of endometriosis. This raises the possibility that certain chemical agents which block IL-18 functions may be of use for the treatment of endometriosis patients.

Acknowledgements

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