Secretion of interleukin-6 by human endometriotic cells and regulation by proinflammatory cytokines and sex steroids

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Endometriosis is generally associated with an immunoinflammatory process that takes place in the peritoneal cavity of patients. Interleukin (IL)-6, a multifunctional cytokine involved in numerous immunological and proliferative processes, has been found at high concentrations in the peritoneal fluid of endometriosis patients. The purpose of this study was to investigate the ability of endometriotic cells to produce IL-6 and to assess the regulation of its secretion by proinflammatory cytokines and sex steroids. Cultures of human endometriotic cells were exposed to different concentrations of cytokines and sex steroids for varying periods of time. IL-6 secretion was measured using an enzyme-linked immunosorbent assay. Endometriotic cells spontaneously released IL-6 in culture. IL-6 and tumour necrosis factor (TNF)-α (0.1–100.0 ng/ml) potentiated IL-6 secretion in a time- and dose-dependent manner. Interferon-γ (0.4–400 ng/ml) induced a dose-related increase in IL-6 secretion and showed a synergistic effect on that secretion in combination with TNF-α (10 ng/ml). Either spontaneous or cytokine-induced IL-6 secretion was inhibited by progesterone (10^-8–10^-5 M) and danazol (10^-6 M), whereas oestradiol (10^-4–10^-5 M) had a limited inhibitory effect. The anti-progesterin RU486 (10^-8–10^-4 M) antagonized the inhibitory effects of progesterone and danazol, but showed agonist action when used alone. These findings indicate that endometriotic tissue may actively contribute to the biological changes observed in the peritoneal fluid of endometriosis patients. They also provide new insights into the mechanisms of action of progesterone and those of danazol and RU486 used in the treatment of endometriosis.

Key words: cytokines/endometriosis/interleukin-6

Introduction

Endometriosis is generally associated with an immunoinflammatory process that takes place in the peritoneal cavity of patients. Numerous studies indicate that the peritoneal fluid of endometriosis patients contains increased concentrations of activated macrophages (Halme et al., 1983), elevated levels of lysozymes (Halme et al., 1983), prostaglandins (Vernon et al., 1986), proinflammatory cytokines (Fakih et al., 1987; Taketani et al., 1992) and auto-antibodies organ-specific and specific to endometrial antigens (Gleicher et al., 1987; Badawy et al., 1990).

There are several physiological sources for an inflammatory response in the peritoneal environment. They include endometrial debris regurgitated in the peritoneal cavity at each menstrual cycle, and rupture of the pre-ovulatory follicle that leads to the release of follicle content and bleeding. Non-physiological sources of inflammation would include an altered biological response by ectopic endometrial implants in the pelvis. According to available data, it is quite obvious that ectopic endometrial tissue is biologically active, particularly in the milder stages of the disease (Vernon et al., 1986), and may have a significant role in alteration of the peritoneal environment. Reciprocally, components of the peritoneal milieu may exert a direct action on the development of endometriotic implants and their biological function. Endometriotic implants have the capacity to produce prostaglandins (Vernon et al., 1986). They display the ability to synthesize and to secrete complement component 3, which possesses a chemotactic activity for macrophages (Isaacson et al., 1989). Using cell culture, we were able to demonstrate in a previous study that, in response to local proinflammatory stimuli such as interleukin (IL)-1β and tumour necrosis factor (TNF)-α, endometriotic cells synthesize and secrete monocyte chemotactic peptide-1 (MCP-1), which is known to exert a potent and specific action on monocyte recruitment and activation (Akoum et al., 1995).

IL-6 is a multifunctional cytokine involved in numerous immunological, proliferative and neoplastic processes (Miki et al., 1989; Motro et al., 1990). It appears to play a critical role in differentiation and the production of immunoglobulin by B cells (Hirano et al., 1990). This cytokine may have a significant role in the pathophysiology of endometriosis because it has been shown to be a normal constituent of the peritoneal fluid (Peters et al., 1985), and its concentration has been shown to increase in endometriosis (Rier et al., 1994).

Here we have evaluated the ability of endometriotic cells to produce IL-6 following in-vitro stimulation with IL-1β and TNF-α, cytokines that are mainly produced by activated macrophages and found in elevated concentrations in the peritoneal fluid of endometriosis patients (Fakih et al., 1987; Taketani et al., 1992). In addition, we have assessed the effects of ovarian steroids and progestational agents currently used in the treatment of endometriosis on the regulation of IL-6 secretion.
Endometriotic tissue was minced into small pieces and dissociated with collagenase as described previously (Akoum et al., 1995). Collagenase IA, deoxyribonuclease and danazol (17β-hydroxy-2,4,17α-pregnenadien-20-yno[2,3-d]isoxazole) were purchased from Sigma (St Louis, MO, USA). Monoclonal anti-epithelial keratin-AE1, AE3 mix (lot no. 10H1), fluorescein isothiocyanate-conjugated, affinity-purified antibodies to mouse Fc (lot no. 0011) and fetal bovine serum (FBS) were obtained from ICN Biomedicals (St Laurent, Quebec, Canada). Anti-CD45 mouse monoclonal antibody was purchased from Becton-Dickinson (Mississauga, Ontario, Canada). Trypsin/ethylenediamine tetraacetic acid, Hanks' balanced salt solution (HBSS) without calcium and magnesium, RPMI 1640 medium, Dulbecco's modified Eagle's medium-F12 (DMEM-F12) and antibiotics-antimycotic (×100) liquid were purchased from Gibco (Burlington, Ontario, Canada). IL-6 concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) having a sensitivity of 0.35 pg/ml (Quantikine IL-6, R&D Systems Inc., Minneapolis, MN, USA). Oestradiol [1,3,5,(10)-estratrien-3,17β-diol-3-benzoate] and progesterone (4-pregnene-3,20-dione) were purchased from Steraloids Inc. (Wilton, NH, USA). RU486 (11β-(4-dimethylaminophenyl)-17α-hydroxy-17α-(1-propynyl)oestradiol-4,9-dien-3-one) was a gift from Roussel-Uclaf (Romainville, France). Monoclonal mouse anti-vimentin antibody was a generous gift from Dr Michel Vincent (Le Centre Hospitalier de l'Université Laval, Quebec, Canada).

Materials and methods

Chemicals

Recombinant human IL-1β (10⁶ IU/mg), TNF-α (2.0×10⁷ IU/mg) and interferon (IFN)-γ (2.5×10⁷ IU/mg) were supplied by Genzyme, Intermedico (Markham, Ontario, Canada) Insulin, transferrin, collagenase IA, deoxyribonuclease and danazol (17β-hydroxy-2,4,17α-pregnenadien-20-yno[2,3-d]isoxazole) were purchased from Sigma (St Louis, MO, USA). Monoclonal anti-epithelial keratin-AE1, AE3 mix (lot no. 10H1), fluorescein isothiocyanate-conjugated, affinity-purified antibodies to mouse Fc (lot no. 0011) and fetal bovine serum (FBS) were obtained from ICN Biomedicals (St Laurent, Quebec, Canada). Anti-CD45 mouse monoclonal antibody was purchased from Becton-Dickinson (Mississauga, Ontario, Canada). Trypsin/ethylenediamine tetraacetic acid, Hanks' balanced salt solution (HBSS) without calcium and magnesium, RPMI 1640 medium, Dulbecco's modified Eagle's medium-F12 (DMEM-F12) and antibiotics-antimycotic (×100) liquid were purchased from Gibco (Burlington, Ontario, Canada). IL-6 concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) having a sensitivity of 0.35 pg/ml (Quantikine IL-6, R&D Systems Inc., Minneapolis, MN, USA). Oestradiol [1,3,5,(10)-estratrien-3,17β-diol-3-benzoate] and progesterone (4-pregnene-3,20-dione) were purchased from Steraloids Inc. (Wilton, NH, USA). RU486 (11β-(4-dimethylaminophenyl)-17α-hydroxy-17α-(1-propynyl)oestradiol-4,9-dien-3-one) was a gift from Roussel-Uclaf (Romainville, France). Monoclonal mouse anti-vimentin antibody was a generous gift from Dr Michel Vincent (Le Centre Hospitalier de l'Université Laval, Quebec, Canada).

Source and handling of tissue

Tissue specimens used in this study were obtained from six women with endometriosis who had given informed consent before laparoscopy. Three women had revised American Fertility Society (AFS, 1985) stage II, two had stage III and one had stage IV endometriosis. The original location of endometriotic tissue was the ovary (two endometromas) and the peritoneum (three red vesicles and one undescribed lesion). Menstrual cycle dating was determined according to the regularity of the cycle and the date of the previous menses. Age, cycle phase, infertility, pain, stage of endometriosis and location of endometriotic tissue were the main clinical characteristics (listed in Table I). Ovarian endometrioma cyst linings and endometriotic foci were placed at 4°C in sterile HBSS containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin, and were transported to the laboratory.

Tissue dissociation and cell culture

Endometriotic tissue was minced into small pieces and dissociated with collagenase as described previously (Akoum et al., 1995). Cells were pelleted by centrifugation (200 g for 10 min), resuspended in DMEM-F12 containing 10 μg/ml insulin, 5 μg/ml transferrin and 10% FBS, and plated in 100 mm diameter culture dishes at 37°C, 5% carbon dioxide. In this study, no attempt was made to separate epithelial cells from fibroblast-like cells. These were identified morphologically in culture by light microscopy and immunocytochemically with specific monoclonal antibodies to cytokeratins and vimentin, as described previously (Akoum et al., 1995).

Culture stimulation

Cultures grown to confluence were trypsinized and harvested by centrifugation (200 g for 10 min). After resuspension and homogenous dispersion in the culture medium, they were replated at 100 000 cells per well in 24-well tissue culture plates (Costar, Cambridge, MA, USA). Confluent cultures were then washed twice with 500 μl per well RPMI 1640 medium free of phenol red and incubated for 16 h at 37°C, 5% carbon dioxide with the same medium. After washing twice with RPMI 1640 medium, the cultures were incubated with 1 ml RPMI 1640 medium containing different concentrations of cytokines or hormones for various periods of time. In some experiments, endometriotic cells were first pre-incubated with various concentrations of hormones. At 24 h later, 25 μl IL-1β were added at a final concentration of 1 ng/ml/well, and the cells were incubated for a further 24 h at 37°C, 5% carbon dioxide. The culture supernatants were then collected, centrifuged (400 g for 10 min) to remove cell debris and kept in small aliquots at −80°C until used for the assay of IL-6.

Statistical analysis

The results were expressed as means ± SEM. A one-way analysis of variance (ANOVA) was used to determine whether there were any differences found in IL-6 concentration in the culture medium of cells following treatment with different concentrations of a cytokine or a hormone, or after exposure to a cytokine for different periods of time. The Tukey test of significant differences was used post hoc for multiple comparisons. A probability value <0.05 was considered to be statistically significant.

Results

Characterization of endometriotic cell cultures

Endometriotic cell cultures were first examined morphologically by phase-contrast microscopy. Epithelial cells exhibited a large and polygonal morphology, whereas fibroblast-like cells were more elongated and spindle shaped (Figure 1). The cells were also characterized by immunocytochemistry with specific monoclonal antibodies to cytokeratins and vimentin.

### Table I. Patient characteristics at the time of laparoscopy

<table>
<thead>
<tr>
<th>Patient no</th>
<th>Age (years)</th>
<th>Cycle phase</th>
<th>Duration of infertility (years)</th>
<th>Pain</th>
<th>Stage of endometriosis</th>
<th>Location of endometriotic tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>Luteal</td>
<td>1.5</td>
<td>Dysmenorrhoea</td>
<td>II</td>
<td>Peritoneum (red vesicle)</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>Luteal</td>
<td>No</td>
<td>Dyspareuma</td>
<td>II</td>
<td>Peritoneum (red vesicle)</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>Luteal</td>
<td>No</td>
<td>Dyspareuma</td>
<td>II</td>
<td>Right ovary (endometroma)</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>Luteal</td>
<td>No</td>
<td>Dysmenorrhoea</td>
<td>III</td>
<td>Right ovary (endometroma)</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>Luteal</td>
<td>1.5</td>
<td>Dysmenorrhoea</td>
<td>II</td>
<td>Right ovary (endometroma)</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>Unknown</td>
<td>No</td>
<td>Dysmenorrhoea</td>
<td>IV</td>
<td>Peritoneum</td>
</tr>
</tbody>
</table>

*According to the revised scale of the American Fertility Society (1985)
Regulation of IL-6 secretion by endometriotic cells

Epithelial cells were strongly positive for cytokeratins, whereas fibroblast-like cells were only stained positively for vimentin. Using phycoerythrin-labelled anti-CD45 mouse monoclonal antibody, no leukocytes were detected among endometriotic cells detached from culture dishes and assessed by flow cytometry (data not shown).

Cytokine-induced IL-6 production by endometriotic cells

The effect of IL-1β and TNF-α on IL-6 secretion by endometriotic cells was examined as a function of dose and duration of exposure to the cytokines. The results (Figure 2) represent the means ± SEM of duplicate determinations in three different patients. Incubation of endometriotic cells with increasing concentrations of IL-1β or TNF-α (0–100 ng/ml) resulted in a dose-dependent increase of IL-6 concentrations measured in the culture medium after 24 h of treatment (Figure 2A). During the incubation of endometriotic cells over increasing periods of time, there was a spontaneous release of IL-6 in the culture medium, particularly after 24 h of culture. Such a secretion was potentiated by the presence of IL-1β (1 ng/ml) and TNF-α (10 ng/ml), and gradually increased throughout the 24 h of incubation with the cytokines (Figure 2B).

IL-6 secretion by endometriotic cells was also investigated
Hormonal regulation of IL-6 production by endometriotic cells

Figure 3. Hormonal regulation of interleukin (IL)-6 secretion by endometriotic cells. (A) Dose effect of progesterone, oestradiol and danazol (10^-8-10^-5 M) on the spontaneous release of IL-6 by endometriotic cells in the culture medium. Cells were grown to confluence in 24-well culture plates and incubated with different concentrations of hormones for 24 h at 37°C, 5% carbon dioxide. IL-6 released in the culture supernatants was measured by an enzyme-linked immunosorbent assay. (B) Dose effect of progesterone, oestradiol and danazol (0-10^-5 M) on cytokine-induced IL-6 secretion by endometriotic cells. Confluent cultures were pre-incubated for 24 h with varying concentrations of hormones prior to stimulation with IL-1β (1 ng/ml) for an additional 24 h. The culture supernatants were then collected and IL-6 was measured as described previously. IL-6 secretion was expressed as the percentage above or below control (medium without hormones), minus IL-6 secretion by cells incubated with hormones, divided by the former, ×100. Results were expressed as means ± SEM of duplicate determinations performed with the Tukey test of significant differences.

Discussion

The study shows that endometriotic cells spontaneously release IL-6 into the culture medium, and such a secretion is potentiated upon stimulation with IFN-γ. The results showed that IFN-γ induced a detectable increase of IL-6 secretion at concentrations ≥4 ng/ml. However, co-incubation of the cells with IFN-γ (0.4-400.0 ng/ml) and TNF-α (10 ng/ml) resulted in a synergistic and dose-dependent increase in IL-6 secretion (Figure 2C).

Hormonal regulation of IL-6 production by endometriotic cells

Figure 3A illustrates the effects of oestradiol, progesterone and danazol, used at various concentrations, on the spontaneous release of IL-6 by endometriotic cells during 24 h of incubation. IL-6 secretion was expressed as the percentage above or below control: IL-6 secretion by cells incubated with the control medium (unsupplemented with hormones), minus IL-6 secretion by cells incubated with hormones, divided by the former, ×100. Results were expressed as means ± SEM of duplicate determinations in five different patients. A statistical analysis of the results using ANOVA indicated that IL-6 secretion was significantly inhibited when endometriotic cells were incubated with progesterone (10^-6-10^-5 M; P < 0.001) and danazol (10^-6-10^-3 M; P < 0.0001), whereas no significant change following incubation of cells with oestradiol (10^-8-10^-5 M) was noted. Post-hoc multiple comparisons using the Tukey test of significant differences showed that IL-6 secretion was lower than control for all progesterone concentrations (P < 0.05) and for danazol at 10^-6 and 10^-5 M (P < 0.01). Furthermore, the inhibition of IL-6 secretion increased as progesterone and danazol concentrations increased, reaching highest levels of statistical significance with 10^-5 M progesterone (P < 0.001) and 10^-5 M danazol (P < 0.001).

Figure 3B depicts IL-6 secretion by endometriotic cells pre-incubated with various concentrations of oestradiol, progesterone and danazol (10^-6-10^-5 M) for 24 h prior to stimulation with 1 ng/ml IL-1β for a further 24 h. Statistical analysis of the results with ANOVA showed that IL-1β-induced IL-6 secretion was significantly inhibited by progesterone (P < 0.01) and danazol (P < 0.001), and that oestradiol had no significant inhibitory action. Moreover, a dose-dependent inhibition of IL-6 secretion by progesterone and danazol was observed. Such an inhibitory effect achieved statistical significance at 10^-5 M progesterone (P < 0.05) and 10^-5 M danazol (P < 0.01), as analysed post hoc with the Tukey test of significant differences.

Table II shows the effects of oestradiol (10^-8 M), progesterone (10^-7 M) and danazol (10^-6 M) on IL-1β-induced IL-6 secretion by endometriotic cells of each of the six endometriosis patients included in the study. Treatment of endometriotic cells with progesterone and danazol led to a significant inhibition of IL-6 secretion in all patients (~9 to ~11% and ~18 to ~24% respectively), whereas treatment with oestradiol had a small but significant inhibitory effect (~11 to ~24%) in three of the six patients.

In some experiments, endometriotic cell cultures were pre-incubated for 24 h with increasing concentrations of the antiprogestrone RU486 alone (0-10^-4 M) or in combination with oestradiol (10^-8 M), progesterone (10^-7 M) or danazol (10^-6 M) prior to stimulation with IL-1β (1 ng/ml) for an additional 24 h. The results illustrated in Figure 4 represent means ± SEM of duplicate determinations performed with cells from three different endometriosis patients. They show that RU486 alone inhibited IL-1β-induced IL-6 production by endometriotic cells in a dose-dependent manner, reaching statistical significance at 10^-6 (P < 0.05) and at 10^-4 M (P < 0.001). On the other hand, RU486 reversed the inhibitory effect of progesterone and danazol on IL-6 secretion when used at 10^-8 and 10^-6 M, while at higher concentrations (10^-4 M) it displayed an inhibitory effect.
by the presence of IL-1β and TNF-α. These proinflammatory cytokines are mainly produced by activated macrophages, and because their amounts are increased in the peritoneal fluid of endometriosis patients (Fakih et al., 1987; Taketani et al., 1989), they may up-regulate IL-6 production by endometriotic tissue. Furthermore, this study demonstrates that IFN-γ acts in a synergistic manner with TNF-α to enhance IL-6 production by endometriotic cells. IFN-γ, a 20 kDa cytokine mainly produced by activated T lymphocytes, is also known to potentiate the activation and accessory cell function of macrophages (Peters et al., 1985). Higher numbers of activated T lymphocytes have been found in the peritoneal fluid of endometriosis patients (Khorram et al., 1993), and therefore these cell interactions may play an important role in the pathophysiology of endometriosis.

Like the endometrium, endometriotic tissue is composed of endometrial-like glands and stroma, although with various and frequently disrupted structural organization (Metzger et al., 1993). It is hormonally dependent and contains sex steroid receptors, but in lower concentrations than the corresponding intrauterine endometrium (Prentice et al., 1992). Our results show that IL-6 secretion by endometriotic cells is hormonally regulated. Progesterone inhibits either spontaneous or cytokine-induced IL-6 secretion by endometriotic cells in a dose/response manner. These results have an interesting physiological significance because a progesterone derivative, medroxyprogesterone acetate, has been shown to reduce the peritoneal inflammatory reaction in patients having endometriosis (Haney and Weinberg, 1988). They also suggest that progesterone may exert a direct inhibitory action on endometriotic cells. The inhibitory effect of progesterone on IL-6 secretion by endometriotic cells is reversed by the concomitant addition of an equimolar or a 100-fold higher concentration of RU486. This synthetic steroidal antiprogestosterone has a 4-fold higher affinity for progesterone receptors than progesterone itself (Baulieu, 1989), and could thereby antagonize the action of progesterone by competitive binding to its receptor. On the basis of this data, it could therefore be suggested that the inhibitory effect of progesterone on IL-6 secretion by endometriotic cells is receptor mediated. On the other hand, RU486 alone exerted a direct dose-related inhibitory effect on IL-6 secretion, which may be due to its progesteromimetic properties as reported previously (van Uem et al., 1989).

Danazol exerts a dose-dependent inhibitory action on either spontaneous or cytokine-induced IL-6 secretion by endometriotic cells. Concentrations of danazol comparable with or higher than therapeutic amounts found in the serum of endometriosis patients treated with 600 mg danazol per day (10⁻⁶ M; Stillman et al., 1980; Barbieri and Ryan, 1981) significantly inhibit IL-6 secretion. Danazol, a testosterone analogue widely used in the medical management of endometriosis, mainly acts by creating a hypo-oestrogenic milieu inadequate for the development of endometriotic tissue (Barbieri and Ryan, 1981). Besides its hypo-oestrogenic action, danazol has been shown to interact with progesterone, glucocorticoid and androgen receptors on endometrial cells (Stillman et al., 1980; Barbieri and Ryan, 1981) and to exert a direct inhibitory action on their growth (Stillman et al., 1980).

Table II. Effects of oestradiol, progesterone and danazol on interleukin (IL)-1β-induced IL-6 secretion* by endometriotic cells obtained from different endometriosis patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>IL-1β (1 ng/ml)</th>
<th>IL-1β (1 ng/ml) + oestradiol (10⁻⁶ M)</th>
<th>IL-1β (1 ng/ml) + progesterone (10⁻⁶ M)</th>
<th>IL-1β (1 ng/ml) + danazol (10⁻⁶ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>971 ± 34</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>1125 ± 10</td>
<td>1107 ± 13</td>
<td>987 ± 15b</td>
<td>817 ± 21c</td>
</tr>
<tr>
<td>3</td>
<td>5339 ± 84</td>
<td>4758 ± 76b</td>
<td>4587 ± 115c</td>
<td>4081 ± 153c</td>
</tr>
<tr>
<td>4</td>
<td>5240 ± 92</td>
<td>5165 ± 71</td>
<td>4446 ± 63c</td>
<td>4267 ± 45c</td>
</tr>
<tr>
<td>5</td>
<td>4406 ± 127</td>
<td>3803 ± 111b</td>
<td>4014 ± 72b</td>
<td>3606 ± 29c</td>
</tr>
<tr>
<td>6</td>
<td>746 ± 58</td>
<td>564 ± 31b</td>
<td>443 ± 54c</td>
<td>425 ± 34c</td>
</tr>
</tbody>
</table>

ND = not determined.

*p/μl, mean ± SEM.

bValues significantly different from that for IL-1β alone in the same patient (P < 0.05 and P < 0.01 respectively).

Figure 4. Dose effect of RU486 on cytokine-induced interleukin (IL)-6 secretion by endometriotic cells with and without progesterone (10⁻⁶ M), oestradiol (10⁻⁸ M) or danazol (10⁻⁶ M) in the culture medium. Confluent cultures were pre-incubated for 24 h at 37°C, 5% carbon dioxide with different concentrations of RU486 alone or in combination with progesterone, oestradiol and danazol at the indicated concentrations. IL-1β was then added to the culture medium to give a final concentration of 1 ng/ml. The incubation was pursued for an additional 24 h. IL-6 concentrations found in the culture supernatants were then quantified by an enzyme-linked immunosorbent assay. IL-6 secretion was expressed as the percentage above or below control (without treatment with hormones). Values are means ± SEM of duplicate determinations in three different patients. (*, **) Significantly different from control (medium without hormones) (P < 0.05 and P < 0.01 respectively).
Danazol also displays immunoregulatory properties and has been shown to inhibit the proliferation of T lymphocytes (Surrey and Halme, 1992), the production of IL-1 and TNF-\(\alpha\) by peripheral blood monocytes (Mori et al., 1990), and their endometrial cell growth-promoting activity (Braun et al., 1994). Patients treated with danazol show a reduced immunoinflammatory reaction in the peritoneal cavity and decreased numbers of autoantibodies in the serum and the peritoneal fluid as well (El-Roey et al., 1988). These results are consistent with our data showing an inhibitory effect of danazol on IL-6 secretion by endometriotic cells. RU486, used in equimolar or 100-fold lower concentrations, antagonizes this inhibitory effect of danazol, which might be explained by a competitive binding of the two drugs to progesterone and glucocorticoid receptors on endometriotic cells.

In this study we have observed that oestradiol used at a near-physiological concentration (10\(^{-8}\) M) exhibited some inhibitory action in three of the six patients included in the study. The meaning of this observation is difficult to explain as oestradiol is known to play a key role in the proliferation of endometrial cells and in the maintenance and progression of endometriosis. Nevertheless, according to previous investigations with eutopic endometrial cells, cytokine-induced expression of IL-6 by endometrial stromal cells was found to be inhibited by oestradiol (Tabibzadeh et al., 1989), and immunoreactive IL-6 in the endometrium was weak in the follicular phase of the menstrual cycle, suggesting a down-regulation by oestradiol (Tabibzadeh et al., 1995).

IL-6 is a pleiotropic cytokine whose main biological property is to induce B cell differentiation and antibody production (Hirano et al., 1990). It has also been shown to induce cell proliferation and angiogenesis (Miki et al., 1989; Motro et al., 1990). According to recent evidence, this cytokine may play an active role in reproductive physiology, including the regulation of ovarian steroid production and folliculogenesis (Motro et al., 1990; Gorospe and Hugher, 1992). Increased concentrations of IL-6 were found in the peritoneal fluid of patients having endometriosis (Rier et al., 1994). In addition, IL-6 production by peritoneal macrophages is higher in patients with endometriosis than in normal women with no evidence of the disease (Rier et al., 1994). Interestingly, it has been reported that IL-6 inhibits eutopic endometrial stromal cell proliferation (Zarmakoupis et al., 1995), while ectopic endometrial stromal cells exhibit a resistance to IL-6-mediated growth inhibition (Rier et al., 1995). Taken together, these results emphasize the role of IL-6 in the pathophysiology of endometriosis, while our results provide additional evidence that endometriotic tissue itself is involved in IL-6 secretion, which is regulated by proinflammatory cytokines and sex steroid hormones.

IL-6 is produced by a wide variety of cells, including macrophages, fibroblasts and epithelial cells (Hirano et al., 1990). In the present study epithelial and fibroblast-like cells isolated from endometriotic lesions were cultured without any further separation. According to previous data, indicating that both epithelial and stromal cells of human endometrium have the ability to produce IL-6 (Laird et al., 1993), it could be postulated that both epithelial and fibroblast-like endometriotic cells in our cultures may contribute to IL-6 secretion. It would have been of interest to investigate the regulation of IL-6 secretion by each type of cell cultured separately. However, such an investigation could on the one hand be limited by the number of epithelial cells that can be isolated from endometriotic implants, while on the other, there is evidence indicating that mutual interactions between stromal and epithelial cells are necessary for maintaining cell differentiation and hormonal responsiveness in vitro (Cunha et al., 1985; Metzger et al., 1993).

Endometriotic tissue can produce and release numerous products that may have a direct impact on the local inflammatory reaction. Vernon et al. (1986) have described the synthesis and secretion of prostaglandins by human endometriotic implants in vitro. Isaacson et al. (1989) have reported that glandular epithelial cells found in endometriotic implants produce and secrete complement component 3, which possesses a chemotactic activity for macrophages. Sharpe-Timms et al. (1994) have localized granulocyte-macrophage colony-stimulating factor, which may contribute to peritoneal macrophage migration, proliferation and activation, in endometriotic epithelial cells. According to our recent data, the stimulation of either fibroblast-like or epithelial cells isolated from endometriotic lesions with IL-1\(\beta\) and TNF-\(\alpha\) induces the secretion of high concentrations of MCP-1, a potent chemotactic and activating factor specific for monocytes (Akoum et al., 1995).

In conclusion, our study provides additional evidence that endometriotic tissue contributes actively to the biological changes observed in the peritoneal cavity of endometriosis patients. It also provides new insights into the mechanisms of action of progesterone and those of danazol and RU486 used in the treatment of endometriosis. These hormones might also reduce the inflammatory reaction by down-regulating the production of proinflammatory cytokines by endometriotic lesions. It is still unclear, however, how these hormones can affect the responsiveness of ectopic endometrial tissue to peritoneal inflammatory stimuli. Possible mechanisms may include interactions with progesterone, oestradiol and glucocorticoid receptors on endometriotic cells. The resolution of these investigations will be necessary to clarify how these hormones may influence IL-6 production by endometriotic cells.

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References

