Peritoneal fluid cytokines and the relationship with endometriosis and pain*

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It is generally accepted that the current scoring system for endometriosis has little correlation with clinical symptoms such as pain, and therefore we may deduce that either endometriosis does not cause pain, or that the current scoring system does not indicate the biological activity of the disease. Pain may occur because the presence of endometriosis produces an intraperitoneal inflammatory response, and several studies have shown that the cytokine content of peritoneal fluid differs between women with and without endometriosis. We studied the relationship between tumour necrosis factor α (TNFα), platelet-derived growth factor (PDGF), interleukin (IL)-6, IL-4 and TNF (α and β) activity in peritoneal fluid and the clinical history of pain and infertility. TNFα concentrations were increased in peritoneal fluid of women with endometriosis and of infertile women; PDGF concentrations were increased in peritoneal fluid of parous women; IL-6 was increased in peritoneal fluid of women with adhesions; IL-4 was absent from peritoneal fluid. PDGF and IL-6 concentrations were cycle related, with the highest amounts in the menstrual and proliferative phases respectively. We failed to demonstrate any association between concentrations of cytokines in vivo and pain symptoms or severity of endometriosis.

Key words: cytokines/endometriosis/infertility/pain/peritoneal fluid

Introduction

Several studies have shown that the peritoneal fluid from women with endometriosis differs from that of women without the disease. Cytokines have been measured in peritoneal fluid and higher concentrations of tumour necrosis factor β (TNFβ) (Eisermann et al., 1988) and interleukin (IL)-1 (Fakhri et al., 1987; Mori et al., 1991) have been found in peritoneal fluid of women with endometriosis compared to normal controls. Peritoneal fluid concentrations of epidermal growth factor (EGF) (DeLeon et al., 1986), IL-6 (Boutten et al., 1992; Buyalos et al., 1992) and macrophage colony-stimulating factor (M-CSF) (Weinberg et al., 1991) have also been measured and similar amounts found in women with and without endometriosis.

Some researchers have postulated that these phenomena in peritoneal fluid occur because the presence of endometriosis produces an intraperitoneal inflammatory response, and studies have demonstrated an increase in macrophage number (Haney et al., 1981) and activation (Halme et al., 1987) in peritoneal fluid of women with endometriosis. Activated macrophages may produce elevated concentrations of cytokines, including IL-1, IL-4, IL-6 and colony-stimulating factors.

Cytokines may be directly involved in the aetiology of pelvic pain by the stimulation of the nociceptor, or they may be indirectly involved, e.g. by stimulating the production of prostaglandins. IL-1 is a primary mediator of the inflammatory response and has been shown to induce prostaglandin synthesis (Rossi et al., 1985). Several cytokines and their receptors have been shown to be present in the endometrium, including platelet-derived growth factor (PDGF), EGF, transforming growth factor β (TGFβ), IL-1, TNFα and IL-6, or in decidua (IL-2, IL-4, and M-CSF) and some have been related to the proliferation of cells in those tissues. Cytokines have also been found in peritoneal fluid, where they may have the potential to affect the growth of endometrium inside the peritoneal cavity and may be implicated in the aetiology of pain.

Our aim was to study different cytokines in peritoneal fluid in relation to activity of disease and the clinical symptoms of pain and infertility. For this purpose, concentrations of TNFα, IL-4, IL-6 and PDGF and TNFα and β activity in peritoneal fluid were measured.

Materials and methods

Peritoneal fluid was obtained at laparoscopy from 51 women. The study group consisted of 43 women under investigation for infertility (defined as a delay in conception of ≥12 months) and pain; 32 women had primary and 11 secondary infertility. The control group consisted of eight parous, pain-free women undergoing sterilization. The women in the control group had no pelvic pathology at laparoscopy. The laparoscopic findings of the 43 women in the study group were as follows: three had a normal pelvis, 34 had minimal-mild endometriosis without adhesions and six had moderate-severe endometriosis with adhesions.

All the women had regular menstrual cycles of 23–31 days (median 28 days) and had not taken corticosteroids, hormones, danazol or gonadotrophin-releasing hormone agonists in the month prior to laparoscopy. Women in the study group were aged 24–44 years (median 31 years), had menarche at 9–16 years (median 12 years)
and their duration of infertility was 15–96 months (median 34 months). There were no significant differences between the study and control group women.

Peritoneal fluid was aspirated from the Pouch of Douglas with the patient in a 15° Trendelenburg position at the time of laparoscopy and was collected into plain glass tubes. The samples were kept on ice (according to the manufacturer’s instructions) and centrifuged (670 g for 10 min) as soon as possible after collection. The supernatant was divided into 500 µl aliquots, labelled and stored at −20°C until assay. Freeze–thaw cycles were avoided. Samples were divided into proliferative (n = 24), secretory (n = 15) or menstrual (n = 4) phase on the basis of the last menstrual period and collected in accordance with the requirements of the Central Oxfordshire Research Ethics Committee.

A detailed pain history was taken from the 51 women participating in this study. Women were specifically questioned about pre-menstrual pain, dysmenorrhoea, dyspareunia and pelvic pain unrelated to menses or sexual intercourse. The severity of pain was evaluated using a scoring system modified from that of Andersch and Milsom (1982). The system defines pain according to limitation of working ability. Pelvic pain and dysmenorrhoea were classified as mild (no analgesia required and no interference with lifestyle), moderate (analgesia required, but the woman was still able to perform her daily tasks) or severe (despite analgesia, the woman was unable to carry out her daily tasks, or was confined to the house). Dyspareunia was graded as mild (momentary pain, not interfering with sexual intercourse), moderate (pain interfering with sexual intercourse, requiring an alteration in some aspect of intercourse) or severe (sexual intercourse avoided because of pain). An arbitrary scoring system was devised, allocating 0 for no pain, 1 for mild, 2 for moderate and 3 for severe, similar to that proposed by Brosens et al. (1993).

Peritoneal fluid concentrations of PDGF, TNFα, IL-4 and IL-6 were measured using commercially available enzyme-linked immuno- sorbent assay (ELISA) kits. The assays were performed according to the manufacturer’s instructions, with all standards and peritoneal fluid samples run in duplicate. The TNFα and β bioassay was performed in triplicate using a method available in our department.

**Tumour necrosis factor α and β bioassay**

A total of 24 peritoneal fluid samples were bioassayed. The biological activity of TNF (α and β) was assayed by its cytotoxic activity against WeHi cells (Espevik and Nissen-Meyer, 1987), with cell number being measured by a colorimetric assay (Mosmann, 1983). The specificity of the assay was confirmed by neutralization of cytotoxicity with anti-TNFα antibodies (101–4; gift of Dr A Meager, NIBSC, Herts, UK). Cells were maintained in RPMI 1640 containing 20 mM glutamine (Gibco Europe, Uxbridge, UK) supplemented with 50 µg/ml of gentamicin (Roche Products Limited, Herts, UK), 100 µg/ml of streptomycin (Evans Medical Limited, Surrey, UK) and 100 uI/ml benzylpenicillin (Britannia Pharmaceuticals Limited, Surrey, UK) and 10% heat-inactivated fetal calf serum (hiFCS; Imperial Laboratories, Andover, UK). Peritoneal fluid samples were serially diluted in 96-well flat-bottomed microtitre plates (Nunc, Gibco Europe) and 4×10^4 WeHi cells were added to each well. After incubation for 20 h at 37°C, 10 µl of 10 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Poole, Dorset, UK) was added to each well. After a further 4 h at 37°C, the supernatant was removed and the blue formazan crystals (reaction product) dissolved in acid isopropanol. The absorbance at 570 nm was measured (with reference set at 630 nm) using a multwell scanning spectrophotometer (Titertek Multiskan MC; Flow Labs, Herts, UK). Recombinant TNFα was used as a standard (specific activity 2×10^7 U/mg) (British Biotechnology Ltd, Oxford, UK). The limit of detection of the assay was 50 pg/ml.

**Tumour necrosis factor α ELISA**

TNFα was measured by an ELISA. Microtitre plates were coated with murine monoclonal antibody to human TNFα (Rockefeller University: SDW 18.1.1) in carbonate buffer. After washing plates with phosphate-buffered saline (PBS) supplemented with Tween 20 (PBS-T), peritoneal fluid samples were incubated in the wells. The following reagents were then applied successively, with PBS-T washes before each step: (i) ammonium sulphate-precipitated rabbit antiserum to recombinant human TNFα (rhTNF) with 2% goat serum in PBS-T; (ii) affinity-purified, alkaline phosphatase-conjugated goat antibodies to IgG (adsorbed to mouse and human immunoglobulin, supplied by American Qualex, La Mirada, CA 90638, USA) with 2% human peritoneal fluid in PBS-T; and (iii) p-nitrophenylphosphate in diethanolamine buffer. Optical densities at 405 nm were measured by an automated dual-beam ELISA reader (Titertek Multiskan MC; Flow Labs), and TNF concentrations in the peritoneal fluid samples were determined by reference to a serially diluted recombinant human TNFα standard (Amersham International, UK) included on each plate. The detection limit of this assay was 10 pg/ml.

**Interleukin-6 ELISA**

Medgenix IL-6-EASIA (Medgenix Diagnostics, Milton Keynes, UK) is an enzyme-amplified sensitivity immunoassay. Standards contained 0, 20, 50, 150, 500, 1000 and 2000 pg/ml of recombinant human IL-6 in a buffered protein base (borate buffer with bovine serum albumin and preservative). The optical density of each well was measured immediately, using a spectrophotometer set to 450 nm with wavelength correction set to 620 nm. The concentration of IL-6 in the unknown peritoneal fluid sample was determined by comparing the optical density of the samples to the standard curve.

**Interleukin-4 ELISA**

The Quantikine IL-4 immunoassay (Research and Diagnostics Systems, Minneapolis, MN, USA) is a solid-phase ELISA. Standards contained 0, 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/ml IL-4 in animal serum. The optical density of each well was measured immediately, using a spectrophotometer set to 450 nm and wavelength correction 540 nm. The concentration of the IL-4 in the unknown peritoneal fluid sample was determined by comparing the optical density of the samples to the standard curve.

**Platelet-derived growth factor ELISA**

Quantikine Human PDGF-AB immunoassay (Research and Diagnostic Systems) is a solid-phase ELISA which measures PDGF-AB. Peritoneal fluid samples were diluted 1:10 with a buffered, lyophilized protein base. Standards contained 0, 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/ml of recombinant human PDGF-AB in a buffered protein base. The optical density of each well was determined immediately, using a spectrophotometer set to 450 nm and wavelength correction 540 nm. The concentration of PDGF in the unknown peritoneal fluid sample was determined by comparing the optical density of the samples to the standard curve and multiplying by the dilution factor. 10. PDGF was found only in low concentrations in peritoneal fluid and, therefore, the ELISA was repeated with undiluted samples.

The inter- and intra-assay variation of the IL-6, IL-4 and PDGF ELISA was <10%, and the minimum detectable dose in peritoneal fluid was 3.0 pg/ml for IL-6, 4.1 pg/ml for IL-4 and 84 pg/ml for PDGF assayed at a 10-fold dilution and 8.4 pg/ml for undiluted samples. The ELISA for IL-4 demonstrated no measurable cross-
reactivity with other cytokines, but IL-6 demonstrated some cross-reactivity with G-CSF and PDGF some cross-reactivity with PDGF-AA (9.8%) and PDGF-BB (1.6%). In order to determine that the collection and storage of peritoneal samples did not alter cytokine concentrations, known amounts of the TNFα, IL-6 or PDGF were added to peritoneal fluid samples and re-assayed after centrifugation and storage for 1 week and 1 month. The collection and storage of peritoneal samples did not alter cytokine concentrations, and recovery of the different cytokines added to control peritoneal fluid was 95% for TNFα, 85% for IL-6, 94% for IL-4 and 89% for PDGF.

Statistical analysis
Continuous variables were analysed by means of analysis of variance which, when only two treatment groups are being examined, corresponds to Student’s r-test. The distribution of many of the concentration variables studied, which were of course bounded below by zero and yet contained some moderate values, displayed severe positive skewness. In such cases a natural logarithmic conversion was applied, in order to render the variables more amenable to statistical analysis. Contingency tables and proportions were analysed by means of Fisher’s exact test for a contingency table, this being a distribution-free analogue of the familiar χ² test.

Results
The mean values and the SE (on the logarithmic scale) of the total pain score and peritoneal fluid concentrations of TNFα, PDGF and IL-6 for the different grades of endometriosis are shown in Table I. Analysis of variance provided statistical evidence (P < 0.001) of an association between endometriosis and pain score, with increased pain among women with endometriosis compared to women without.

No clear relationship between TNFα concentrations and pain or AFS score was found. There was no significant difference between women with moderate and severe endometriosis compared to women with minimal and mild disease. It was noted, however, that highest peritoneal fluid TNFα concentrations were found in women with mild endometriosis and this group also had the highest pain score.

TNF concentrations were generally low (<60 pg/ml) except for those high values in the study group (>800 pg/ml), which were unexplained. This erratic pattern of values for TNFα prompted an alternative representation, simply the presence or absence of TNFα (Table II). A clear positive association was detected between the presence of TNFα and endometriosis. A higher incidence of endometriosis was found among women with detectable concentrations of TNFα in the peritoneal fluid

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Endometriosis score</th>
<th>Total pain score</th>
<th>TNFα (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>PDGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0</td>
<td>0.43 ± 0.16</td>
<td>0.72 ± 0.72</td>
<td>4.20 ± 0.42</td>
<td>3.63 ± 0.13</td>
</tr>
<tr>
<td>21</td>
<td>1-5</td>
<td>1.29 ± 0.10</td>
<td>1.66 ± 0.31</td>
<td>3.93 ± 0.45</td>
<td>3.57 ± 0.09</td>
</tr>
<tr>
<td>13</td>
<td>6-15</td>
<td>1.73 ± 0.20</td>
<td>1.81 ± 0.52</td>
<td>4.37 ± 0.60</td>
<td>3.34 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>16-39</td>
<td>1.27 ± 0.17</td>
<td>0.97 ± 0.17</td>
<td>4.37 ± 1.83</td>
<td>3.86 ± 0.35</td>
</tr>
<tr>
<td>2</td>
<td>&gt;39</td>
<td>1.59 ± 0.20</td>
<td>0.55 ± 0.55</td>
<td>5.60 ± 0.04</td>
<td>3.78 (n = 2)</td>
</tr>
</tbody>
</table>

*Endometriosis score 0 versus endometriosis score >0; P < 0.001. There was no significant difference between women with endometriosis and those without for TNFα, IL-6 and PDGF concentrations.

Table I. Mean values and SE of the variables of total pain, interleukin (IL)-6, tumour necrosis factor α (TNFα) and platelet-derived growth factor (PDGF) (on the logarithmic scale) in 51 patients grouped according to their endometriosis scores

Table II. The presence or absence of TNFα in peritoneal fluid from women with and without endometriosis

<table>
<thead>
<tr>
<th>Women with a normal pelvis</th>
<th>Women with endometriosis</th>
<th>Proportion with endometriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 11)</td>
<td>(n = 40)</td>
<td></td>
</tr>
<tr>
<td>TNFα absent (n = 16)</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>TNFα present (n = 35)</td>
<td>1</td>
<td>34</td>
</tr>
</tbody>
</table>

*P < 0.001 by Fisher’s exact test comparing 34/35 (97%) women with endometriosis and undetectable amounts of TNFα in peritoneal fluid and 34/35 (97%) women with endometriosis and detectable concentrations of TNFα.

Table III. The negative association between the presence of TNFα in peritoneal fluid and a history of pregnancy

<table>
<thead>
<tr>
<th>Nulliparous (n = 32)</th>
<th>Parous (n = 19)</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα absent (n = 16)</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>TNFα present (n = 27)</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

P = 0.025 by Fisher’s exact test comparing 69% of parous women with undetectable amounts of TNFα and 23% of parous women with detectable concentrations of TNFα in peritoneal fluid.
TNFα absent

Table IV. Contingency tables of peritoneal fluid TNFα by cycle phase

<table>
<thead>
<tr>
<th>Phase</th>
<th>Proliferative (n = 24)</th>
<th>Secretory (n = 15)</th>
<th>Menstrual (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα absent</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TNFα present</td>
<td>19</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Proportion of women with undetectable TNFα</td>
<td>5/24 (21%)</td>
<td>2/15 (13%)</td>
<td>1/4 (25%)</td>
</tr>
</tbody>
</table>

Table V. The association between PDGF in peritoneal fluid and a history of parity

<table>
<thead>
<tr>
<th>Phase</th>
<th>Nulliparous (n = 25)</th>
<th>Multiparous (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal fluid PDGF (pg/ml)</td>
<td>34.0 ± 3.19</td>
<td>45.8 ± 5.12*</td>
</tr>
<tr>
<td>Proportion of women with endometriosis</td>
<td>24/25 (96%)</td>
<td>9/14 (64%)</td>
</tr>
</tbody>
</table>

*P < 0.05 by Student’s t-test.

Table VI. Mean values of peritoneal fluid PDGF by cycle phase

<table>
<thead>
<tr>
<th>Phase</th>
<th>No. of patients</th>
<th>PDGF (pg/ml)</th>
<th>Log scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative</td>
<td>18</td>
<td>33.17 ± 2.58</td>
<td>3.44 ± 0.09</td>
</tr>
<tr>
<td>Secretory</td>
<td>13</td>
<td>38.92 ± 5.05</td>
<td>3.57 ± 0.12</td>
</tr>
<tr>
<td>Menstrual</td>
<td>4</td>
<td>65.00 ± 14.50</td>
<td>4.08 ± 0.26</td>
</tr>
</tbody>
</table>

P = 0.0324 (menstrual versus proliferative/secretory phase).

Table VII. Contingency tables of peritoneal fluid concentrations of interleukin (IL)-6 by cycle phase

<table>
<thead>
<tr>
<th>Phase</th>
<th>Proliferative (n = 23)</th>
<th>Secretory (n = 15)</th>
<th>Menstrual (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–100</td>
<td>18</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>101–500</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>&gt;500</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Proportion &lt;100</td>
<td>0.783</td>
<td>0.533</td>
<td>0.250</td>
</tr>
</tbody>
</table>

P < 0.05 by Fisher’s exact test (menstrual versus secretory/proliferative phase).

Discussion

The aetiology of endometriosis continues to be debated. Cytokines are present in peritoneal fluid and have been shown to differ between women with and without endometriosis. This altered peritoneal environment may interfere with fertility and provide the mechanism for pain. This study attempts to define the relationship between peritoneal fluid cytokines and clinical symptoms.

Anx study of endometriosis and pain has difficulty with the classification of the disease. The current American Fertility Society (AFS, 1985) classification has only a poor correlation with pain; it weights for ovarian disease and attempts to predict fertility. It is deeply infiltrating endometriosis which correlates strongly with pain, not pelvic area (Koninckx et al., 1991) and, while the AFS system weights deep endometriosis more strongly than superficial disease, the assessment of depth by laparoscopy is unreliable. A recent study abandoned the AFS classification and found that dysmenorrhea was related to the number of implants (Perper et al., 1995). We have used the AFS classification rather than introduce another variable.

In the classification of endometriosis and pain, current opinion suggests that dysmenorrhea, pelvic pain and dyspareunia should be scored according to severity (as described above), giving a total score for pelvic symptoms and excluding other causes for pain (Brosens et al., 1993). It has been questioned whether mild endometriosis should be considered a disease or whether it is a condition occurring intermittently in all women. It is suggested that endometriosis exists in two forms, the subtle form, without clinical significance, and the aggressive disease associated with infertility and pain. The subtle form of endometriosis may progress to endometriotic disease, and cytokines may play some part in this process (Koninckx, 1994). It may be that mild endometriosis is an accidental finding without relevance to the presenting problem and that other causes of pain should be excluded (Moen, 1995).

We chose to measure TNFα, PDGF, IL-4 and IL-6, since these cytokines and their receptors have been detected in either endometrium or decidua and are known to be involved in inflammation or wound healing. TNFα is secreted primarily compared to none of the normal women. TNF (α and β) concentrations as detected by bioassay were not directly comparable with concentrations of TNFα detected by ELISA.

There was no significant difference between peritoneal fluid PDGF concentrations of women with (mean 44 pg/ml, range 25–37) and without endometriosis (mean 44 pg/ml, range 12–88) and no evidence of a relationship between PDGF concentrations and pain (Table I). However, PDGF values were on average higher in the women who achieved a pregnancy (P < 0.05 by Student’s t-test) (Table V). PDGF concentrations in nulliparous women were 12–72 pg/ml (mean 34 pg/ml) and in multiparous women 27–88 pg/ml (mean 46 pg/ml). There was evidence that the PDGF values were highest in the menstrual phase of the cycle (mean 65 pg/ml) compared to the secretory (mean 38.92 pg/ml) or proliferative (mean 33.17 pg/ml) phases (P < 0.05 by analysis of variance), as shown in Table VI.

IL-6 was found in the peritoneal fluid of women with (mean 320.5 pg/ml, range 3–1932) and without endometriosis (mean 53.5 pg/ml, range 32–81). There was no evidence of an association between IL-6 concentrations and pain. Elevated concentrations of IL-6 (>150 pg/ml) were found in four of six women (67%) with endometriosis and adhesions, compared to 11 of 36 women (31%) with endometriosis without adhesions (not statistically significant). All women with a normal pelvis had peritoneal fluid IL-6 <150 pg/ml. There was evidence of an association between IL-6 and the phase of the cycle, with lowest amounts of IL-6 detected in the menstrual phase (P < 0.05) (Table VII).
by macrophages and mediates macrophage cytotoxicity against susceptible cells (Urban et al., 1986). TNFα receptors have been detected in stromal cells by immunostaining (Fernandez-Shaw, 1993) and in full-term decidua, where they were found only on macrophages (using immunostaining and flow cytometry) (Vince et al., 1992). IL-6 is a multifunctional cytokine and is produced by a variety of cells, including T- and B-cells (Hirano and Kishimoto, 1989). It regulates growth and differentiation within a complex cytokine network with major actions on the immune system and inflammation (Kishimoto, 1989). PDGF is a secretory product of activated macrophages and plays a major role in the inflammatory response and wound healing. It has been demonstrated to accelerate the rate of wound healing of various types of wounds (Raines, 1990; Pierce et al., 1991). IL-4 is produced by T-lymphocytes and affects the proliferation and differentiation of T- and B-lymphocytes and other cell types, including macrophages and mast cells. Studies of the effect of IL-4 in vivo have been limited, but it has been suggested that IL-4 is involved in the regulation of IgE synthesis (deVries et al., 1991).

Measurement of cytokine concentrations in peritoneal fluid is complicated by the fact that cytokines are never produced in isolation, but as a mixture of cytokines which may have similar or opposing actions. We measured TNFα, PDGF, IL-4 and IL-6 by ELISA and repeated the measurement of TNFα by bioassay. Monoclonal antibodies and polyclonal antisera in ELISA identify not only important regions of the TNFα molecule but also biologically inactive moieties. We chose ELISA rather than bioassay, since cytokine is more stable as an antigen and the ELISA is easier to perform. The biologically active form of the cytokine has a half-life of seconds or minutes *in vivo* and may be affected by the collection technique; the assay can also be insensitive when used with complex biological fluids such as peritoneal fluid due to non-specific protein binding and the presence of antagonists. In order to reduce the inter- and intra-sample variation, samples were collected by one investigator. To determine if the collection or storage of samples altered the cytokine content, known amounts of TNFα, IL-6 and PDGF were added to control peritoneal fluid samples and re-assayed. The recovery rate indicated that the collection methods did not alter the cytokine concentrations. However, it may not be appropriate to equate results for human recombinant cytokines to cytokines *in vivo*.

Previous studies have been criticized both for the control group selection and for failing to allow for cycle effects. It is well recognized that peritoneal fluid volume alters with the day of the menstrual cycle, with an increase in the early luteal phase of the cycle (Maathuis et al., 1978; Koninckx et al., 1980a). Our control group was selected on the basis of normal pelvic anatomy, negative pain history, parity and no drug or hormone treatment in the month prior to laparoscopy. The strict entry criteria for women into this group meant that the sample numbers were small (*n* = 8), but previous studies have been criticized for including infertile women without endometriosis (DeLeon et al., 1986; Eierman et al., 1988; Surrey and Halme, 1991). Classification was based on visual inspection and biopsies were not taken; therefore microscopic lesions may have been missed in these women. The number of control samples was necessarily small, since the fluid obtained from these women was of such small volume that several aliquots had to be pooled in order to produce sufficient volume for the determination of several cytokines. We chose to have strict criteria for the control group at the expense of numbers.

We found that concentrations of TNFα in peritoneal fluid, as detected by ELISA, were very variable, with no obvious relationship between these concentrations and pain or severity of endometriosis. There was a significant correlation between TNFα concentrations and women with endometriosis as compared to those with a normal pelvis and between TNFα and nulliparity compared with parity. The association between TNF and endometriosis may be one of parity rather than endometriosis, since it is generally accepted that parity is protective against the development of endometriosis (Moen, 1995). The frequent absence of TNFα in peritoneal fluid of parous women may be as a result of this protective effect, rather than any effect of parity on TNFα concentrations.

It is worthy of comment that maximum peritoneal fluid TNFα concentrations and maximum pain scores were found in women with mild endometriosis. This may suggest that different conditions prevail in minimal–mild disease compared to more severe disease and could explain the enigma of severe pain in women with minimal disease. This would fit with the hypothesis that endometriosis exists in two forms, the aggressive being associated with clinical symptoms such as pain (Koninckx, 1994). There appeared to be no relationship between TNFα concentrations and phase of the menstrual cycle.

Biologically active TNF may not completely identify with TNF as an antigen and it was of interest to compare the two. We bioassayed TNFα for 24 of the peritoneal fluid samples by means of its cytotoxic effect on WeHi cells. The trend was similar, with TNF absent from peritoneal fluid of parous women and detectable TNF concentrations associated with the presence of endometriosis. Eierman et al. (1988) measured TNFα in peritoneal fluid by means of a 51Cr release assay which measured lysis of the TNF-sensitive cell line L929. They reported an absence of TNF in peritoneal fluid of normal women, but maximum TNFα concentrations in women with moderate–severe disease. We were unable to demonstrate any significant difference in peritoneal fluid concentration of TNF between minimal–mild and moderate–severe disease.

We found PDGF in peritoneal fluid of women with normal pelvic anatomy (range 25–37 pg/ml) and women with endometriosis (12–88 pg/ml) in lower amounts than found in serum (1023–17326 pg/ml) and plasma (226–4877 pg/ml) in normal, healthy blood donors (Quantikine data sheet). We found no association between PDGF concentrations and severity of endometriosis or pain symptoms, but did demonstrate significant evidence of increased PDGF concentrations in fertile women. We found that concentrations of PDGF were significantly higher in the menstrual phase of the cycle, with lowest concentrations being detected in the proliferative phase of the
cycle. This may reflect increased macrophage activity in peritoneal fluid around the time of menstruation.

IL-6 was a normal constituent of peritoneal fluid present in women with and without endometriosis. An elevated peritoneal fluid IL-6 concentration was associated with pelvic adhesions (not significant), but there was no evidence of a relationship between concentrations of IL-6 in peritoneal fluid and severity of endometriosis, pain symptoms or pregnancy. This confirms previous reports by Boutten et al. (1992) and Buyalos et al. (1992), who also measured IL-6 in peritoneal fluid. Boutten et al. (1992) showed no differences between women with minimal to mild endometriosis, women with infertility and fertile women with a normal pelvis. Buyalos et al. (1992) reported similar findings, but demonstrated a significant relationship between concentrations of IL-6 and pelvic adhesions. They validated their storage conditions by demonstrating that the freezing and thawing of cell-free peritoneal fluid did not alter IL-6 measurement. However, they reported no effect of cycle phase on IL-6 concentrations, while we found that concentrations of IL-6 were highest in the proliferative phase and lowest in the menstrual phase of the cycle (P < 0.05).

There are no previous studies on the measurement of IL-4 in peritoneal fluid, although normal, healthy donor serum contains no IL-4 (Quantikine data sheet). Previous studies have shown that IL-4 affects the growth of endometrial gland and stromal cells (Fernandez-Shaw, 1993) and IL-4 receptor is present in endometrium (Starkey, 1991). We did not detect IL-4 in peritoneal fluid from normal women or from women with endometriosis. Perhaps it is not surprising that a cytokine involved in allergic reactions and anaphylaxis is not present in peritoneal fluid.

In conclusion, we have studied the relationship between concentrations of TNFα, IL-6 and PDGF in peritoneal fluid and pain. We have shown significant associations between peritoneal fluid TNFα, endometriosis and nulliparity, and between PDGF and parity. We were unable to demonstrate a significant association between IL-6 and adhesions. PDGF and IL-6 were cycle related, with highest concentrations being detected in the menstrual and proliferative phases respectively. Cytokines have been implicated in the pathogenesis of pain and infertility associated with endometriosis but, disappointingly, we have failed to demonstrate any association between concentrations of cytokines in vitro and pain symptoms or severity of endometriosis. If cytokines are not related to pain or severity of endometriosis, then it may be that some other factor(s) is involved or that different conditions exist in women with pain than in women with no symptoms.

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