

# Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells

Aydin Arici<sup>1,3</sup>, Salli I.Tazuke<sup>1</sup>, Erkut Attar<sup>1</sup>, Harvey J.Kliman<sup>1,2</sup> and David L.Olive<sup>1</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, and <sup>2</sup>Department of Pathology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

<sup>3</sup>To whom correspondence should be addressed

**Interleukin-8 (IL-8) is a chemoattractant and activating factor for human neutrophils and a potent angiogenic agent. The peritoneal fluid of women with endometriosis has been shown to have increased neutrophil chemotactic activity. We postulate that IL-8 may be an important modulator in the pathogenesis of endometriosis and adhesion formation. We first investigated IL-8 concentrations in the peritoneal fluid of women with or without endometriosis, then assessed peritoneal mesothelial cells as a potential source of peritoneal fluid IL-8. Northern blot analysis and enzyme-linked immunosorbent assay (ELISA) were used to investigate IL-8 mRNA and protein modulation. The mean concentration of IL-8 in samples obtained from control patients ( $n = 28$ ) was  $4.8 \pm 0.5$  pg/ml; from patients with minimal-mild endometriosis ( $n = 24$ ) was  $27.5 \pm 2.6$  pg/ml; and from patients with moderate-severe endometriosis ( $n = 21$ ) was  $530.2 \pm 65.1$  pg/ml. Confluent mesothelial cells were incubated with human recombinant IL-1 $\alpha$  (0.01–100 IU/ml) or tumour necrosis factor (TNF)- $\alpha$  (0.01 to 100 ng/ml) for 2–24 h. IL-8 mRNA was detectable in non-treated cells, however both IL-1 $\alpha$  and TNF- $\alpha$  induced higher amounts of IL-8 mRNA in a dose- and time-dependent manner. Non-treated mesothelial cells in culture also produced and secreted IL-8 protein quantified by ELISA, but again higher concentrations were induced by IL-1 $\alpha$  and TNF- $\alpha$  treatment. In conclusion, we found that IL-8 concentrations were elevated in peritoneal fluids from women with endometriosis. Cultured mesothelial cells expressed cytokine-inducible IL-8 mRNA and secreted IL-8 protein. The regulated expression of this angiogenic factor may play a role in pathogenesis of endometriosis.**

**Key words:** endometriosis/interleukin-8/mesothelial cells/peritoneal fluid

## Introduction

Endometriosis is among the most common of gynaecological entities, with a prevalence among reproductive age women estimated to be 9–33% (Duignan *et al.*, 1972; Peterson and Behrman, 1970). Despite extensive investigation the pathogenesis of endometriosis is still poorly understood. Considerable circumstantial evidence supports the role of retrograde menstruation with subsequent endometrial implantation as an aetiological factor in most cases (Olive and Henderson, 1987). Retrograde menstruation is a nearly universal phenomenon among cycling women (Blumenkrantz *et al.*, 1981; Halme *et al.*, 1984; Kruitwagen *et al.*, 1991) but it is not clear why endometrial tissue will implant and grow in the peritoneal cavity of only a subgroup of women.

Immunological dysfunction at various levels has been observed in women with endometriosis; this dysfunction may be either a causal factor in the development of the disease or may simply be a result of the disorder. In the peritoneal fluid of women with endometriosis, an increased number, concentration, and activation of macrophages has been described (Haney *et al.*, 1981; Halme *et al.*, 1982; Dunselman *et al.*, 1988). Secretory products of macrophages such as interleukin-1 (Fakih *et al.*, 1987), tumour necrosis factor (TNF) (Eisermann *et al.*, 1988; Halme, 1989) and growth factors

(Halme *et al.*, 1988; Koutsilieris *et al.*, 1993) are also found at increased concentrations in the peritoneal fluid of these women. Macrophage-conditioned medium has been shown to enhance mouse endometrial stromal cell proliferation *in vitro* (Olive *et al.*, 1991).

Recently, the peritoneal fluid of women with endometriosis has also been shown to have increased neutrophil chemotactic activity (Leiva *et al.*, 1993). However, the nature and source of this chemotactic factor remains to be determined. One of the candidates is interleukin-8 (IL-8), a chemoattractant and activating cytokine for neutrophils (Baggiolini *et al.*, 1989) and a potent angiogenic agent (Koch *et al.*, 1992). IL-8 is produced by a number of cell types, including monocytes (Yoshimura *et al.*, 1987), endothelial cells (Strieter *et al.*, 1989), fibroblasts (Larsen *et al.*, 1989), mesothelial cells (Goodman *et al.*, 1992), and endometrial stromal cells (Arici *et al.*, 1993). We postulated that IL-8 may be an important modulator in the pathogenesis of endometriosis by not only attracting and activating neutrophils but also by stimulating new blood vessel formation. In the present study we have investigated IL-8 concentrations in the peritoneal fluid of women with or without endometriosis, then assessed peritoneal mesothelial cells as a potential source of peritoneal fluid IL-8.

## Materials and methods

### Peritoneal fluid collection

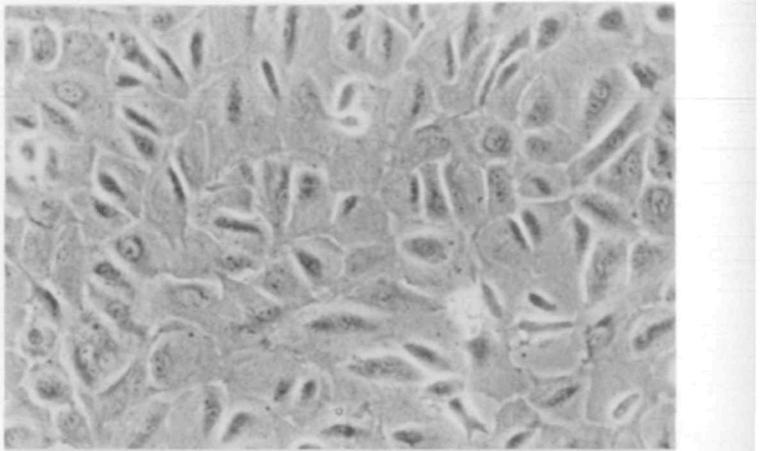
Peritoneal fluid samples were obtained from women undergoing diagnostic laparoscopy. Informed consent was obtained from each woman prior to surgery using consent forms and protocols approved by the Human Investigation Committee of Yale University, CT, USA. The day of the menstrual cycle was established from the woman's menstrual history and verified in a subset by histological examination of the endometrium. Patients were classified into three groups according to anatomical findings observed during the procedure: normal fertile patients (mostly undergoing laparoscopic tubal ligation), patients with minimal to mild endometriosis, and patients with moderate to severe endometriosis as defined by the revised American Fertility Society (1985) classification. Prior to any intervention, peritoneal fluid was aspirated from the anterior and posterior cul-de-sac into a sterile syringe and centrifuged at 600 g for 10 min at 4°C to remove cells, aliquoted, and frozen at -80°C until assayed. Cell pellets were resuspended in Hank's balanced salt solution (HBSS) for mesothelial cell culture as described below.

### Mesothelial cell cultures

Peritoneal cell pellets that were resuspended in HBSS were layered over Ficoll-Paque (Pharmacia LKB, Piscataway, NJ, USA) and centrifuged at 400 g for 30 min, according to techniques modified from Baumann *et al.* (1987). Cells at the interface were pelleted once in HBSS then plated in plastic flasks (25 cm<sup>2</sup>), and allowed to replicate to confluence in M-199 medium that contained fetal bovine serum (FBS, 10%, v/v), antibiotics-antimycotics (1%, v/v), and epidermal growth factor (EGF, 20 ng/ml) at 37°C in a humidified atmosphere (5% CO<sub>2</sub> in air). Thereafter, the cells were passed by standard methods of trypsinization, plated in culture dishes (10 mm diameter or 24-well plates) as appropriate for the experimental design, and allowed to replicate to confluence prior to commencement of each experiment (Figure 1). Cultured human peritoneal mesothelial cells were flat, polygonal when confluent, and grew with a doubling time of 7–12 days. The mesothelial cells grew and formed a homogeneous population after three passages, whereas contaminating macrophages, because they were terminal cells, stopped dividing. These observations were similar to those of Zhang *et al.* (1993). All experiments were done using cells at third passage. Experiments were commenced 1–3 days after confluence was attained. Because previously we have shown in endometrial stromal cells in culture that serum does have direct stimulatory effect on the production of IL-8 (Arici *et al.*, 1993), the confluent cells were treated with serum-free, EGF-free M-199 medium for 24 h before treatment with test agents was initiated and all experiments were conducted using the serum-free, EGF-free medium. At the end of each experiment, the culture media were collected and frozen at -80°C for quantification of IL-8 by enzyme-linked immunosorbent assay (ELISA). Cells were used for quantification of total protein or for isolation of RNA.

### Immunocytochemical analyses of the cultured cells

Peritoneal cells in culture were characterized by use of immunocytochemistry. Cells were plated in 6-well dishes containing flamed 22 mm<sup>2</sup> coverslips. At preconfluence, cells were washed with phosphate-buffered saline (PBS) pH 7.4, fixed with Bouin's fixative for 10 min, then washed again with PBS. Using the avidin-biotin method (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA), cells were stained with the following mouse monoclonal antibodies: low molecular weight cytokeratin [1.3 µg immunoglobulin (Ig)G/ml; Dako, Carpinteria, CA, USA] as a marker of epithelial cells, vimentin (0.5 µg protein/ml; BioGenex, San Ramon, CA, USA) as a marker



**Figure 1.** Light microscopy of unstained peritoneal mesothelial cell monolayer. The mesothelial cells show their characteristic cobble-stone appearance.

of fibroblasts, and for HAM45 (0.8 µg IgG/ml; Dako) as a marker of macrophages. Approximately 95% of the cells were positive for both vimentin and cytokeratin, a finding characteristic of mesothelial cells (LaRocca and Rheinwald, 1984). In all, <1% of the cells were positive for HAM56 after 2 or 3 passages. We also pelleted cells from peritoneal fluid without culturing them and fixed then stained immunohistochemically as described above. The staining pattern seen in ~70% of freshly collected cells was consistent with mesothelial nature.

### Preparation of total RNA and northern analysis

Total RNA was prepared by the guanidinium isothiocyanate-caesium chloride ultracentrifugation method of Chirgwin *et al.* Total RNA (5 or 10 µg per lane) was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N<sup>+</sup> membrane (Amersham; Arlington Heights, IL, USA), and cross-linked to the membrane by use of UV light. Prehybridization was conducted for 5 h at 65°C in buffer comprised of NaCl (0.9 M), Tris-Cl (90 mM, pH 8.3), EDTA (6 mM), 5× Denhardt solution, sodium dodecyl sulphate (SDS, 0.1%), sodium pyrophosphate (0.1%, w/v), and salmon sperm DNA (0.2 mg/ml). Hybridizations were conducted for 16 h at 65°C in buffer that contained an IL-8-specific oligonucleotide probe (5'-TGT TGG CGC AGT GTG GTC CAC TCT CAA TCA-3') end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP; the sequence of this probe corresponds to a portion of exon 2 in the coding region of the IL-8 gene (Matsushima *et al.*, 1988). Thereafter, the blots were washed with 6× standard saline citrate (SSC) and SDS (0.1%, w/v) for 15 min at room temperature, once with 2× SSC and SDS (0.1%, w/v) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membranes was performed at -70°C using Kodak X-Omat AR film. The presence of equal amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits and by analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, using a cDNA probe (Clontech Laboratories, Palo Alto, CA, USA) radiolabelled with [ $\alpha$ -<sup>32</sup>P]dCTP by random hexamer priming. The autoradiographic bands were quantified by using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Each IL-8 band was normalized by using the value for the corresponding G3PDH mRNA, thus correcting for any variation in amounts of RNA applied to each lane. Similar experiments were conducted on three different occasions with cells prepared from different peritoneal fluids.

### IL-8 immunoassay

Immunoreactive IL-8 in peritoneal fluid samples and culture supernatant was quantified using an ELISA from R&D Systems (Minneapolis, MN, USA). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. The sensitivity for IL-8 was 0.47 pg/100 µl sample. All of the peritoneal fluid samples were evaluated in a duplicate assay. Validation of its use for human peritoneal fluid was also performed: recombinant IL-8 was diluted in assay buffer and pooled peritoneal fluid and parallelism was observed between the standard curve of buffer and peritoneal fluid dilutions. Each experiment was done using three replicate wells for each condition and supernatant from each well was tested in a single ELISA assay. Each experimental setup was repeated at least on three occasions using mesothelial cells obtained from three different patients. The intra-assay and interassay coefficients of variation were 7.95 and 10.2% respectively.

### Statistical analyses

Because the concentrations of IL-8 in the peritoneal fluid were not normally distributed, they were analysed with non-parametric analysis of variance by ranks (Kruskal-Wallis). Individual groups were compared *post hoc* with the non-parametric Mann-Whitney test, using the Bonferroni correction for multiple comparisons. Data from the ELISA assays were evaluated by analysis of variance with Bonferroni *post hoc* analysis for multiple comparisons.

### Reagents

Culture media, antibiotics-antimycotics, and fetal bovine serum from Sigma Chemical Co. (St Louis, MO, USA). EGF, TNF- $\alpha$ , IL-8 (all recombinant), anti-IL-1, and anti-TNF- $\alpha$  were from R&D Systems.

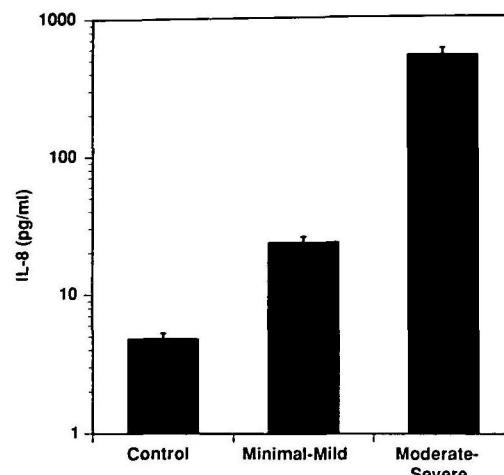
## Results

### Immunoreactive IL-8 in peritoneal fluid

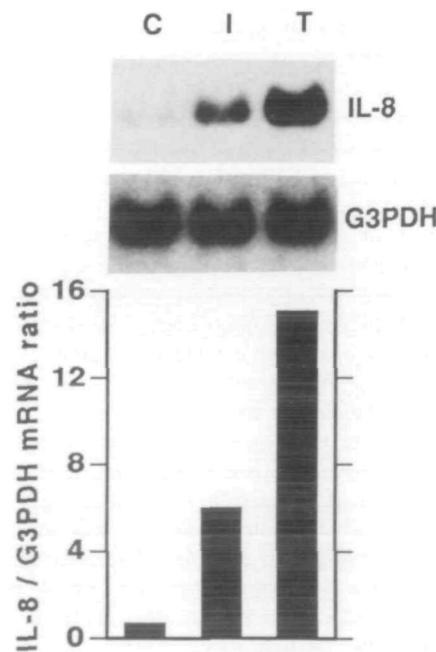
The mean concentration of IL-8 in peritoneal fluid obtained from control patients ( $n = 28$ ) was  $4.8 \pm 0.4$  pg/ml ( $\pm$  SEM); from patients with minimal-mild endometriosis ( $n = 24$ ) was  $27.5 \pm 2.6$  pg/ml; from patients with moderate-severe endometriosis ( $n = 21$ ) was  $530.2 \pm 65.1$  pg/ml (Table 2). Peritoneal fluid IL-8 concentrations differed among the three groups ( $P = 0.023$ ), with the moderate-severe group having significantly higher IL-8 concentration than the others ( $P = 0.021$ ).

### IL-8 production by mesothelial cells

Confluent mesothelial cells in serum-free medium were incubated with human recombinant IL-1 $\alpha$  (10 IU/ml) or TNF- $\alpha$  (10 ng/ml) for 6 h. IL-8 mRNA was detectable by northern analysis in non-treated cells. Both IL-1 $\alpha$  and TNF- $\alpha$  induced higher concentrations of IL-8 mRNA (Figure 3). The increase in the concentrations of IL-8 mRNA in mesothelial cells was dependent upon the concentration of IL-1 $\alpha$  (0.01–100 IU/ml) and was seen starting at concentration of 1 IU/ml (detectable at longer exposed autoradiographs) (Figure 4). Similarly a concentration-dependent increase in the concentrations of IL-8 mRNA was observed with TNF- $\alpha$  (0.1–10 ng/ml) starting at concentration of 1 ng/ml (data not presented). We also found that non-treated mesothelial cells in culture produce and secrete IL-8 protein quantified by ELISA. Treatment with IL-1 $\alpha$

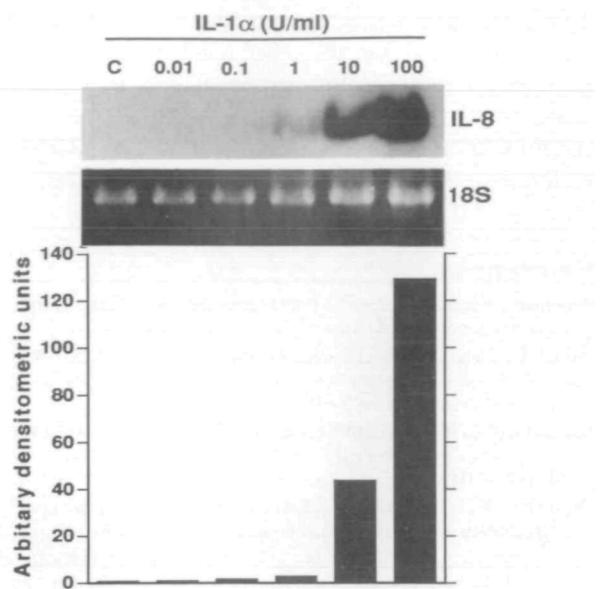


**Figure 2.** Immunoreactive interleukin-8 concentrations according to endometriosis: control, minimal-mild, and moderate-severe. Values are mean  $\pm$  SEM.  $P = 0.023$  overall;  $P = 0.021$  for control versus moderate-severe groups;  $P$  not significant for control versus minimal-mild groups and for minimal-mild versus moderate-severe groups.

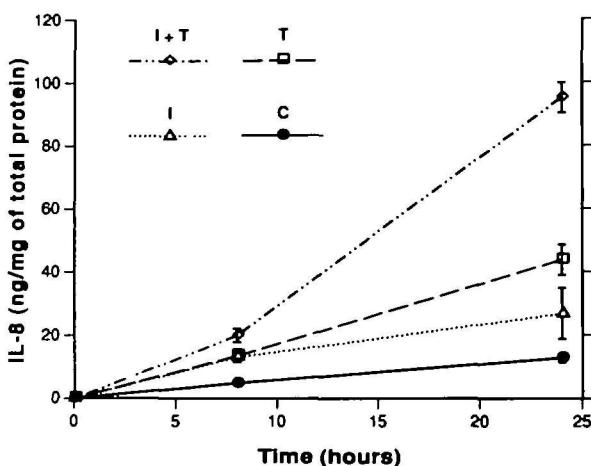


**Figure 3.** Northern analysis of interleukin (IL)-8 mRNA in mesothelial cells treated with IL-1 $\alpha$  and tumour necrosis factor (TNF)- $\alpha$ . Confluent mesothelial cells in culture were incubated for 6 h with IL-1 $\alpha$  (10 IU/ml) and TNF- $\alpha$  (10 ng/ml). Total RNA (10 µg per lane) was evaluated. C = control; I = IL-1 $\alpha$ ; T = TNF- $\alpha$ .

(10 IU/ml) or TNF- $\alpha$  (10 ng/ml) caused increases in the accumulation of immunoreactive IL-8 in the media; this difference was significant by 8 h treatment ( $P = 0.00001$ ). After 24 h IL-1 $\alpha$  and TNF- $\alpha$  treatment, the longest duration of the experiments, maximal accumulation of IL-8 in the media was observed (Figure 5). Similar, though lower increase in the production of IL-8 was also observed when cells were treated at lower concentration of IL-1 $\alpha$  (1 IU/ml) or TNF- $\alpha$  (1 ng/ml) (data not presented).

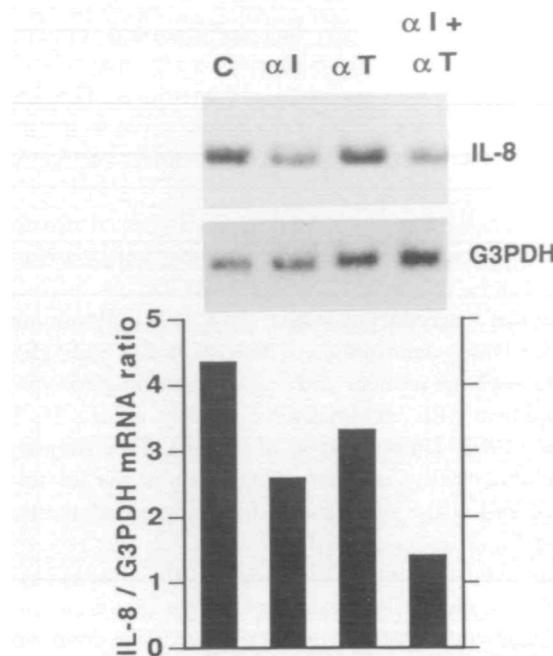


**Figure 4.** Induction of interleukin (IL)-8 mRNA in mesothelial cells by IL-1 $\alpha$ : dose response. Confluent mesothelial cells in culture were incubated for 6 h in serum-free culture medium with IL-1 $\alpha$  (0.01–100 IU/ml). Total RNA (10  $\mu$ g per lane) was evaluated. C = control.



**Figure 5.** Stimulation of immunoreactive interleukin (IL)-8 production by mesothelial cells in culture by IL-1 $\alpha$  and tumour necrosis factor (TNF)- $\alpha$ . Confluent mesothelial cells were treated with serum-free medium alone (control) or with medium containing IL-1 $\alpha$  (10 IU/ml) or TNF- $\alpha$  (10 ng/ml) or both for 8 and 24 h. The culture media were collected, and IL-8 was quantified by enzyme-linked immunosorbent assay (ELISA). Data are mean  $\pm$  SEM for four replicates.

To investigate the aetiology of constitutive IL-8 gene expression in mesothelial cells in culture, cells were treated for 6 h with rabbit polyclonal anti-human IL-1 and TNF- $\alpha$  neutralizing IgG antibodies (10  $\mu$ g/ml) alone or simultaneously. Anti-IL-1 antibody treatment caused a decrease in the constitutive IL-8 mRNA. Anti-TNF- $\alpha$  antibody treatment also induced a decrease in IL-8 mRNA. Both antibodies together caused a 70% decrease in constitutive IL-8 mRNA (Figure 6). This finding suggests a role for an autocrine action of IL-1 and TNF- $\alpha$  in mesothelial cells.



**Figure 6.** Effect of neutralizing antibodies on the interleukin (IL)-8 gene expression in mesothelial cells. Confluent mesothelial cells in culture were placed in serum-free medium for 24 h prior to incubation for 6 h in culture medium containing rabbit polyclonal anti-human IL-1 $\beta$  and tumour necrosis factor (TNF)- $\alpha$  neutralizing immunoglobulin (Ig)G antibodies (10  $\mu$ g/ml) alone or simultaneously. Total RNA (10  $\mu$ g per lane) was evaluated. C = control;  $\alpha$ I = anti-IL-1 $\beta$  antibody;  $\alpha$ T = anti-TNF- $\alpha$  antibody.

## Discussion

Although endometriosis is a relatively common gynaecological disorder, a lack of understanding of its pathogenesis has hindered research into prevention and treatment. Many theories for pathogenesis have surfaced over the years, but the mechanism appears to require endometrium and retrograde menstruation in most cases of the disease. This is consistent with epidemiologic data correlating a higher risk of the disorder with decreased parity, increased number of menses, early menarche, and heavier menstrual flow (Mahmood and Templeton, 1991). The i.p. distribution is also consistent with this process, as endometriosis is found primarily in gravity-dependent areas of the pelvis and abdomen (Jenkins *et al.*, 1986). In women with Müllerian anomalies, functioning endometrium and antegrade outflow obstruction yield a high rate of disease, while patients without both conditions do not (Olive and Henderson, 1987). Finally, in the baboon model, i.p. placement of menstrual endometrium causes a high resulting implantation rate; this is not seen with endometrial fragments from the remainder of the menstrual cycle (D'Hooghe *et al.*, 1994).

It thus appears that retrograde menstruation is a necessary condition for most endometriosis. However, it is also clear that retrograde menstrual flow is a nearly universal phenomenon, while endometriosis is found in a distinct minority of reproductive age women. Thus another factor is involved: the initiation of endometriosis may be a function of the quantity of refluxed endometrium that is viable, or it may depend upon

particular characteristics of the shed endometrium. Furthermore, the environment of the peritoneal cavity may play a decisive role in the development of endometriosis. This last concept, that the peritoneal milieu itself may serve to initiate and/or maintain the disease, has been the subject of intense investigation.

Several authors have demonstrated that endometrial stromal cell growth can be optimized in an environment of oestrogen and growth factors, particularly those growth factors secreted by the peritoneal macrophages (Olive *et al.*, 1991; Hammond *et al.*, 1993). These data are consistent with the finding of increased macrophage number and activation in the peritoneal cavity of women with endometriosis (Haney *et al.*, 1981; Halme *et al.*, 1982; Dunselman *et al.*, 1988). This suggests that such an abnormality in macrophages may at the least be supportive of and at the most causal for ectopic endometrial growth.

Given this scenario, we asked whether the macrophage abnormality in endometriosis subjects might represent the primary pathogenetic factor or whether a pre-existing condition promotes leukocyte migration and activation. One of the candidates for this latter possibility is IL-8, a chemoattractant and activating cytokine for neutrophils (Baggiolini *et al.*, 1989) and a potent angiogenic agent (Koch *et al.*, 1992). If IL-8 is truly involved in the pathogenesis of endometriosis, the concentration of IL-8 should be increased in peritoneal fluids of patients with endometriosis versus controls; in addition, a greater increase should be seen with more severe disease. This is precisely the finding of this study: IL-8 is found in greater quantity in women with endometriosis, and the worse the disease the higher the IL-8 concentration.

If IL-8 is a participant in the pathogenetic pathway, the next question to be asked is where this cytokine originates. Endometrial cells themselves have been shown to produce IL-8 (Arici *et al.*, 1993), and more recently Critchley *et al.* (1994) have shown the perivascular location of IL-8 in human endometrium. Thus, the refluxed tissue or associated fluid may well represent a source of the IL-8 in endometriosis patients. Other potential sources are macrophages and peritoneal mesothelial cells. We found that cultured mesothelial cells constitutively express IL-8 mRNA and secrete IL-8 protein. Thus, sources of IL-8 are abundant in these women.

The final question we asked was how IL-8 secretion might be regulated. This study presents evidence that the expression of this leukocyte chemoattractant from mesothelium is modulated by other cytokines such as IL-1 and TNF- $\alpha$ . These latter cytokines appear to play some role in the constitutive secretion of IL-8 as well as being capable of greatly stimulating further production and secretion. Sources of IL-1 and TNF- $\alpha$  may therefore play an important role in the initiation of the pathogenic cascade: peritoneal mesothelium, endometrium, follicular fluid (Wang and Norman, 1992; Jasper and Norman, 1995), and peritoneal macrophages are all candidate sources. To our knowledge, although there is no report on the production of TNF- $\alpha$  by peritoneal mesothelial cells, these cells synthesize IL-1 $\alpha$  and IL-1 $\beta$  (Douvdevani *et al.*, 1994). There is also a potential for an interplay between leukocytes and mesothelial cells: macrophages by producing cytokines such as IL-1 and

TNF- $\alpha$  may lead to increased production of IL-8 by mesothelial cells in response. On the other hand, whether the increased IL-8 concentration in the peritoneal fluid is a cause or consequence of the disease is difficult to answer, but it will certainly have an adverse effect on the local environment and facilitate further growth of endometriotic implants.

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