

# Peritoneal fluid-mediated enhancement of eutopic and ectopic endometrial cell proliferation is dependent on tumor necrosis factor- $\alpha$ in women with endometriosis

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**Objective:** To determine the effect of autologous peritoneal fluid and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on proliferation of endometrial cells from women with endometriosis.

**Design:** Endometrial cells from eutopic and ectopic endometrium were cultured in vitro with peritoneal fluids or recombinant TNF- $\alpha$  for 72 hours before DNA synthesis determination by  $^3\text{H}$ -thymidine labeling and liquid scintillation counting.

**Setting:** An institute for the study and treatment of endometriosis and university-based research laboratories.

**Patient(s):** Thirty-five women with endometriosis and 17 controls without endometriosis.

**Main Outcome Measure(s):** In vitro incorporation of  $^3\text{H}$ -thymidine in endometrial cells was examined.

**Result(s):** Peritoneal fluid from women with endometriosis enhanced proliferation of autologous and heterologous endometrial cell cultures from women with endometriosis. The soluble TNF-receptor etanercept blocked the ability of peritoneal fluid from women with endometriosis to enhance proliferation of eutopic or ectopic endometrial cells. Recombinant TNF- $\alpha$  also enhanced proliferation of eutopic and ectopic endometrial cells from women with endometriosis. In contrast, autologous peritoneal fluid, heterologous peritoneal fluid from women with endometriosis, and recombinant TNF- $\alpha$  failed to enhance, and often inhibited, the proliferation of eutopic endometrial cells from controls without endometriosis.

**Conclusion(s):** Endometrial cells from women with endometriosis can utilize factors in peritoneal fluids, such as TNF- $\alpha$ , to facilitate proliferation in ectopic environments. Endometrial cells from women without endometriosis do not share this ability, suggesting that this abnormality is etiologically related to development of the disease. Therapy with agents that block the effects of TNF- $\alpha$  may be warranted. (*Fertil Steril*® 2002; 78:727–32. ©2002 by American Society for Reproductive Medicine.)

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The activational state of mononuclear phagocytes in women with endometriosis is increased compared to healthy controls (1). This effect has been shown for circulating monocytes in the peripheral blood and regional macrophages in the pelvic and peritoneal cavities by using assays that measure cytokine biosynthesis, oxidative metabolism, and cytotoxic function (2–5). Although it is theoretically possible that such macrophage functions contribute to the etiology or pathophysiology of endometriosis, evidence of a causative relationship is lacking.

To investigate the possible pathophysio-

logic implications of macrophage activation in endometriosis, we developed an in vitro coculture system to measure the effects of mononuclear phagocytes on the proliferation of autologous endometrial cells (6). The results of those studies demonstrated that monocytes from women with endometriosis stimulate the in vitro proliferation of autologous endometrial cells, whereas monocytes from controls without endometriosis inhibit autologous endometrial cell proliferation. Moreover, heterologous cocultures of monocytes and endometrial cells from unrelated donors revealed that monocytes from women with endometriosis enhance the proliferation of endometrial cells from women

with endometriosis but fail to enhance proliferation of endometrial cells from controls without endometriosis. This differential response between controls and patients with endometriosis suggests intrinsic differences in the response of these cells to normal growth regulation. This possibility is further substantiated by our demonstration of the relative resistance of endometrial cells from women with endometriosis to spontaneous apoptosis (7) and macrophage-mediated cytolysis (8).

The hypothesis that emerges from these collective studies is that endometriosis is a manifestation of intrinsic endometrial cell abnormalities that permit these cells to resist and exploit normal growth controls in the ectopic environment of the peritoneal cavity. In the current study, we investigated this hypothesis further by testing the influence of peritoneal fluids from women with endometriosis on the proliferation of eutopic and ectopic endometrial cells. We found that peritoneal fluid from women with endometriosis enhances autologous or heterologous endometrial cell proliferation in women with the disease, whereas peritoneal fluid from fertile controls without endometriosis does not. Furthermore, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) appears to play a prominent role in the proliferation-enhancing activity of peritoneal fluids from women with endometriosis.

## MATERIALS AND METHODS

The study was approved by the institutional review board and subjects gave written informed consent.

### Sample

Participants were women of reproductive age who were undergoing laparoscopic tubal ligation or diagnostic laparoscopy for evaluation of suspected endometriosis. Pelvic organs were carefully examined for endometriosis, which was staged according to the revised American Fertility Society classification (9). Thirty-five patients were found to have endometriosis (3 with stage I disease, 17 with stage II disease, 6 with stage III disease, 5 with stage IV disease, and 4 whose disease was confirmed histologically but not classified). These patients and 17 controls without endometriosis were tested at the time of initial diagnosis and staging. The procedures were performed during the proliferative (14) and secretory (11) phases of the cycle with 10 patients accrued who had not had a menstrual period for more than 3 months.

### Tissue Sampling and Histologic Examination

During laparoscopy, peritoneal fluid from all participants was aspirated into a sterile container with 0.2 mL of heparin at a concentration of 1,000 U/mL, and an endometrial biopsy sample was obtained by using a Novak curette. Peritoneal fluid was centrifuged at 1,000 rpm for 10 minutes to remove cells and cellular debris before addition to cell cultures. Endometrial tissues were handled as described below.

In a subgroup of eight patients, exophytic endometriotic

implants were identified during laparoscopy. These were typically surface exophytic lesions that were fleshy in appearance and microscopically consisted of endometrial glands and stroma devoid of fibrous tissue reaction. These implants were removed with biopsy forceps, placed in normal saline, and processed as described below.

Each eutopic and ectopic endometrial specimen was examined histologically to confirm the presence of endometrial glands and stroma and to classify the menstrual phase of the tissue. In each participant, endometriotic lesions had a characteristic but variable appearance.

### Endometrial Cell Preparation

Uterine and ectopic endometrium obtained at the time of laparoscopy was subjected to one 20-minute cycle of digestion with a mixture of collagenase (0.14% wt/vol) and DNAase (0.1% wt/vol) in Hank's balanced salt solution (Whittaker) at 37°C to prepare single cells. After digestion, the endometrial cells were filtered through sterile mesh (3-163T Nitex mesh; Martin Supply, Baltimore, MD), collected by centrifugation, and resuspended in RPMI 1640 medium (Whittaker) containing 10% fetal bovine serum (Whittaker), 100 U/mL of penicillin, and 100  $\mu$ g/mL of streptomycin.

The cell suspensions contained both stromal cells and glandular epithelial cells, as determined on the basis of morphology. No attempt was made to separate stromal cells from epithelial cells and, thus, the results represent the net proliferation obtained with a mixture of both endometrial cell types.

### Endometrial Cell Proliferation Assay

Endometrial cells (100  $\mu$ L of a  $5 \times 10^5$ /mL suspension) suspended in RPMI medium (Whittaker) and 10% fetal bovine serum, 100 IU/mL of penicillin and 100  $\mu$ g/mL of streptomycin were dispensed into 96-well microtiter plates. Subsequently, 100  $\mu$ L of complete medium (control) or complete medium supplemented with peritoneal fluid (25 and 50  $\mu$ L) or recombinant TNF- $\alpha$  (100 U/mL [ $9.1 \times 10^2$  pg/mL]; Genzyme, Cambridge, MA) was added (total volumes per well adjusted to 250  $\mu$ L), and endometrial cells were cultured for 72 hours in an humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. At the end of the incubation period, the cultures were pulsed with <sup>3</sup>H-thymidine, 1  $\mu$ Ci/well (Amersham, Arlington Heights, IL) for 24 hours before harvesting by using a multiple automated sample harvester and liquid scintillation counting.

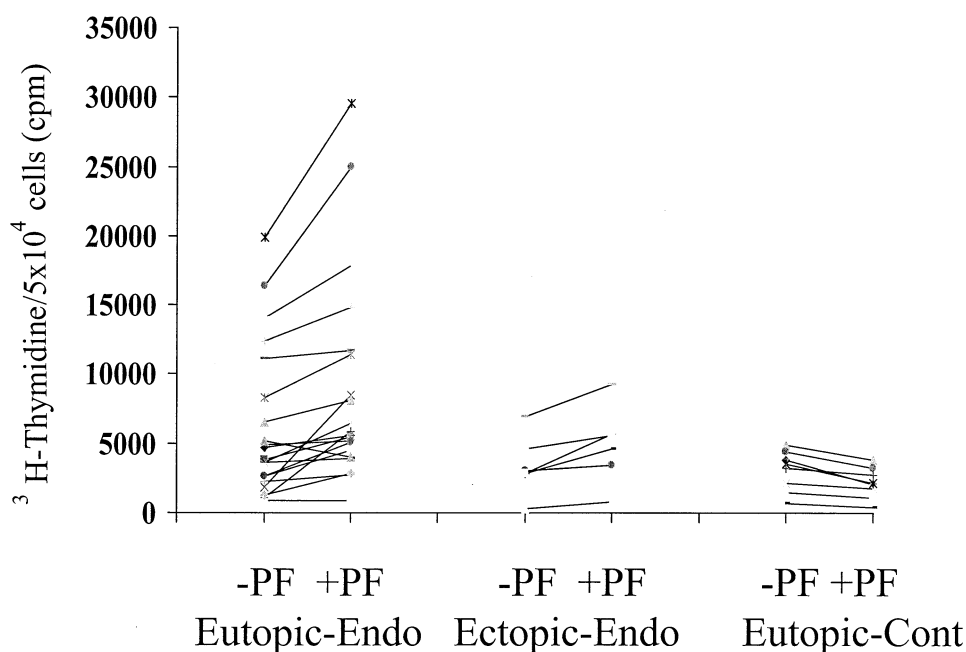
Endometrial cell proliferation for each culture was expressed as the mean count per minute (cpm). Whenever replicate cultures were tested with both 25 and 50  $\mu$ L peritoneal fluid, the results presented represent the largest effect seen.

### Statistical Analysis

The effect of peritoneal fluid or recombinant TNF- $\alpha$  on endometrial cell proliferation was evaluated by using paired two-tailed *t*-tests.

**FIGURE 1**

Effect of autologous peritoneal fluid (PF) on endometrial cell (*Endo*) proliferation in fertile controls (*Cont*) and patients with endometriosis. Eutopic and ectopic endometrial cells from endometriosis patients, and eutopic endometrial cells from controls were incubated in the absence (-PF) and presence (+PF) of autologous peritoneal fluid for 72 hours before addition of <sup>3</sup>H-thymidine for 24 hours and assessment of incorporation of label in cellular DNA by liquid scintillation counting.



Braun. TNF and endometrium in endometriosis. *Fertil Steril* 2002.

## RESULTS

### Proliferation of Eutopic and Ectopic Endometrial Cells in the Presence of Autologous and Heterologous Peritoneal Fluids From Women With Endometriosis

Peritoneal fluid from women with endometriosis significantly enhanced the proliferation of autologous eutopic endometrial cells (mean [ $\pm$ SD] incorporation,  $6,337 \pm 5,537$  cpm vs.  $8,963 \pm 7,558$  cpm in the absence and presence of autologous peritoneal fluid, respectively;  $P=.001$ ) and ectopic endometrial cells (mean incorporation,  $3,458 \pm 2,196$  cpm vs.  $4,710 \pm 2,857$  cpm in the absence and presence of autologous peritoneal fluid, respectively;  $P=.03$ ) (Fig. 1). In contrast, peritoneal fluids from controls significantly suppressed the proliferation of autologous eutopic endometrial cells (mean incorporation,  $3,011 \pm 1,462$  cpm vs.  $2,160 \pm 1,115$  cpm in the absence and presence of autologous peritoneal fluid respectively;  $P=.003$ ).

The magnitude of effects on endometrial cell proliferation by autologous peritoneal fluids varied substantially among individual participants. The effects were qualitatively consistent when values for participants in different phases of the menstrual cycle were considered separately (data not shown).

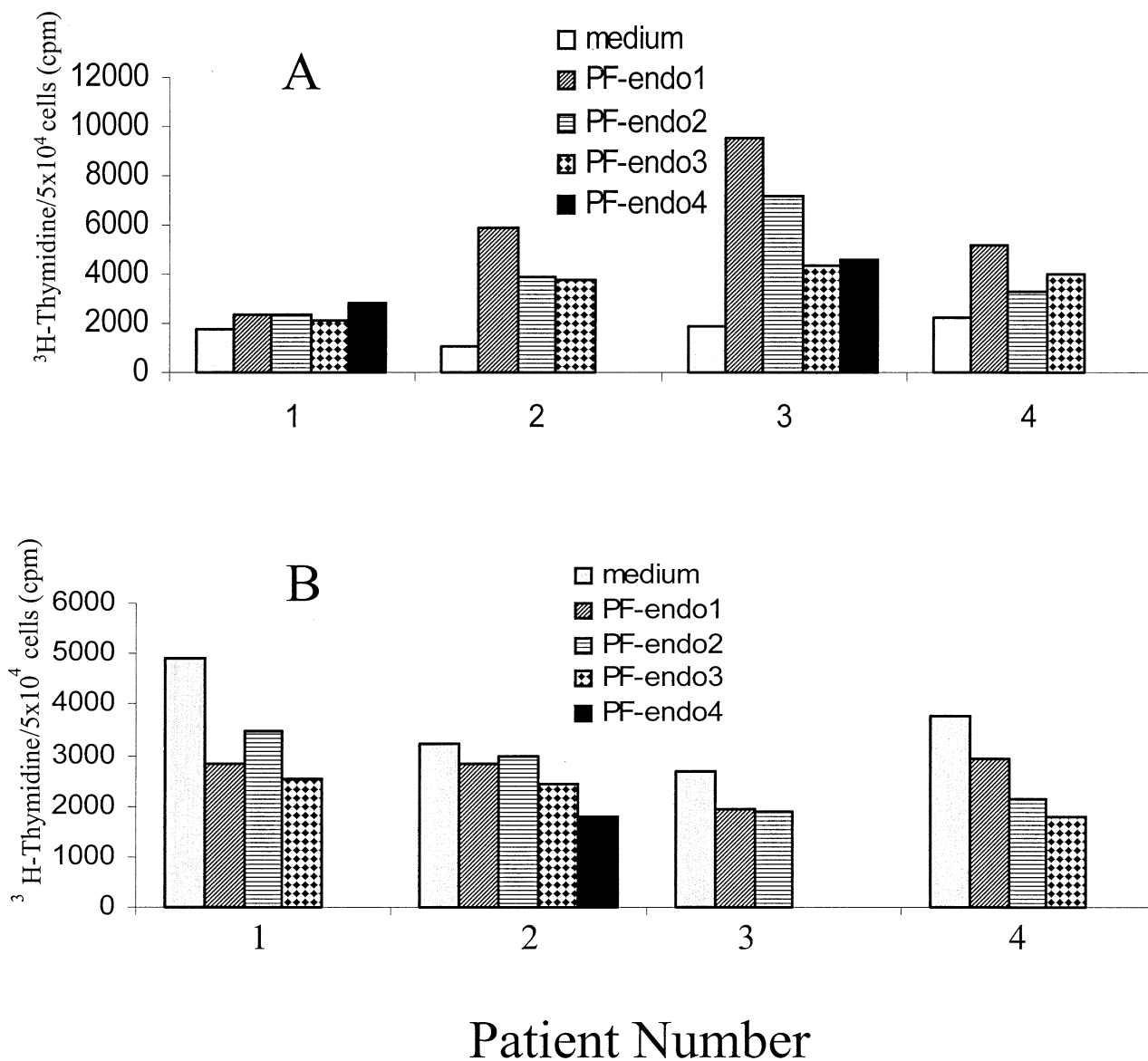
The modulatory effect of peritoneal fluids from women with endometriosis depended on the source of endometrial cells (Fig. 2). Thus, peritoneal fluid from four donors with endometriosis had little effect on endometrial cells from one patient but enhanced to a variable extent the proliferation of eutopic endometrial cells from the other three heterologous patients with endometriosis. In contrast, these fluids had no effect or suppressed proliferation of endometrial cells in four controls.

### Effect of Soluble TNF- $\alpha$ Receptor on Proliferation of Endometrial Cells in the Presence of Autologous Peritoneal Fluids

Because studies have demonstrated increased levels of TNF- $\alpha$  in the peritoneal fluid of women with endometriosis (10), we examined the effect of the soluble TNF receptor etanercept (Immunex, Seattle, WA) on endometrial cell proliferation in the presence of autologous peritoneal fluid (Fig. 3). Etanercept reduced or eliminated the proliferation-enhancing activity of peritoneal fluid from women with endometriosis in four of five eutopic endometrial cell cultures and three of three ectopic endometrial cell cultures. Limited amounts of peritoneal fluid from controls without endometriosis did not permit similar studies in this group.

**FIGURE 2**

Effect of heterologous peritoneal fluid (PF) from patients with endometriosis (A) and four controls (B) were incubated for 72 hours in the presence of peritoneal fluids from four unrelated patients with endometriosis (PF-endo1, PF-endo2, PF-endo3, and PF-endo4) before addition of  $^3\text{H}$ -thymidine for 24 hours and assessment of incorporation of label in cellular DNA by liquid scintillation counting.



Braun. TNF and endometrium in endometriosis. *Fertil Steril* 2002.

**Effect of Recombinant TNF- $\alpha$  on Eutopic and Ectopic Endometrial Cell Proliferation in Women With Endometriosis**

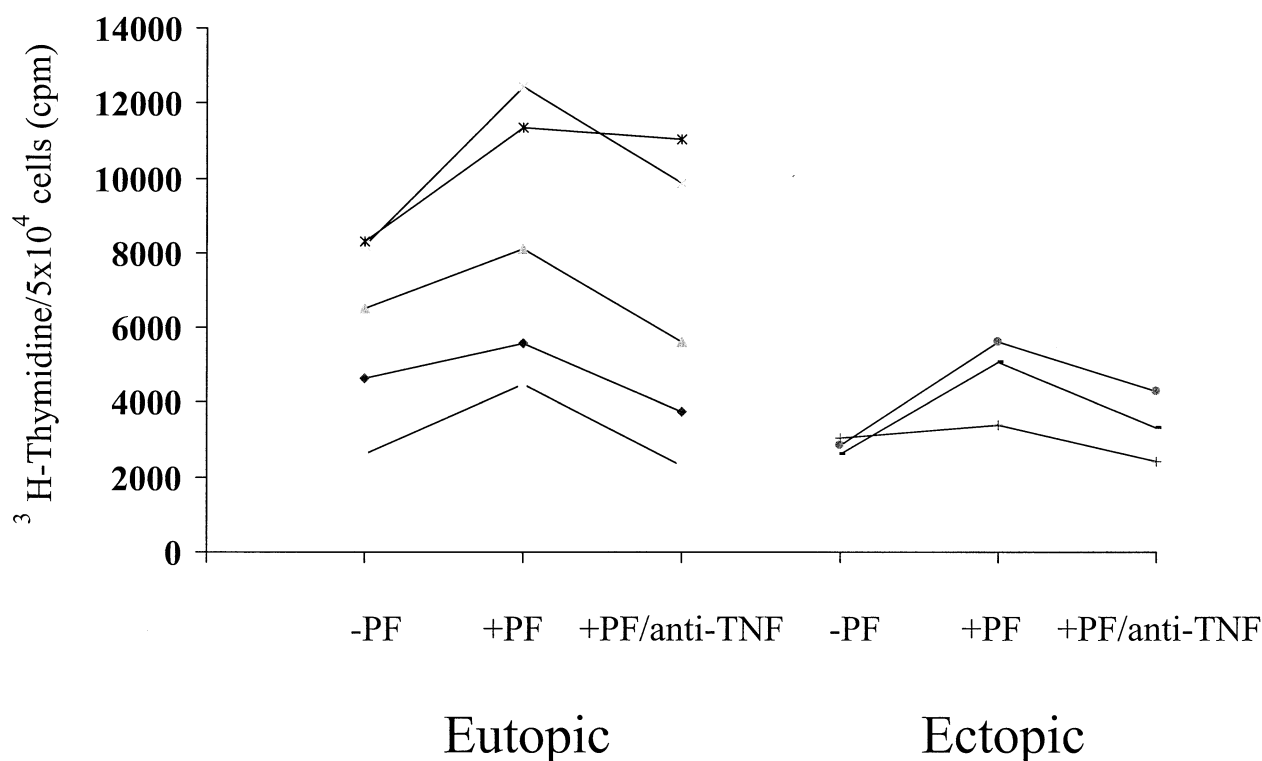
We also tested the effect of recombinant TNF- $\alpha$  on endometrial cell proliferation. In specimens from women with endometriosis, recombinant TNF- $\alpha$  significantly enhanced the proliferation of eutopic (mean incorporation,  $4,982 \pm 4,558$  cpm vs.  $5,921 \pm 5,800$  cpm;  $P=.005$ ) and ectopic ( $3,145 \pm$

$2,006$  cpm vs.  $5,344 \pm 3,101$  cpm;  $P=.03$ ) endometrial cells in the absence and presence of recombinant TNF- $\alpha$ , respectively. In contrast, recombinant TNF- $\alpha$  suppressed the proliferation of eutopic endometrial cells from controls ( $3,312 \pm 1,841$  cpm vs.  $2,999 \pm 1,642$  cpm in the absence and presence of recombinant TNF- $\alpha$  respectively;  $P=.016$ ).

As seen with peritoneal fluids, the magnitude of effects of

**FIGURE 3**

Effect of the soluble tumor necrosis factor (*TNF*) receptor etanercept on enhancement of eutopic and ectopic endometrial cell proliferation by peritoneal fluid (*PF*) in women with endometriosis. Endometrial cells were incubated in the absence (*-PF*) or presence (*+PF*) of autologous peritoneal fluid, with and without etanercept, for 72 hours before addition of  $^3\text{H}$ -thymidine for 24 hours and assessment of incorporation of label in cellular DNA by liquid scintillation counting. The effect of etanercept in the absence of peritoneal fluid was negligible, and data are therefore not shown.



Braun. *TNF* and endometrium in endometriosis. *Fertil Steril* 2002.

recombinant  $\text{TNF-}\alpha$  varied substantially among individual participants.

## DISCUSSION

Our findings show that factors in peritoneal fluids enhance the proliferation of endometrial cells in women with endometriosis but fail to do so in endometrial cells of controls without endometriosis. The enhancing effect of peritoneal fluids was seen in autologous or heterologous eutopic endometrial cells from women with endometriosis and in ectopic endometrial cells from peritoneal implants.

Similar proliferation-enhancing effects were seen with recombinant  $\text{TNF-}\alpha$ , a cytokine that is increased in the peritoneal fluid of women with endometriosis, suggesting that  $\text{TNF-}\alpha$  may contribute to the etiology and pathogenicity of this disease. A role for  $\text{TNF-}\alpha$  in enhancement of endometrial cell proliferation by peritoneal fluids was also sug-

gested by the fact that the soluble  $\text{TNF}$ -receptor etanercept blocked this effect.

The differential response of endometrial cells from women with and without endometriosis to identical peritoneal fluids or to recombinant  $\text{TNF-}\alpha$  provides further evidence that the endometrium of women with this disease is abnormal. Moreover, the abnormality described herein is compatible with the proclivity of endometrial cells from women with endometriosis to grow in inflamed, ectopic anatomic locations. Thus, endometrial cells from women with endometriosis fail to undergo normal apoptosis and are stimulated to proliferate in the presence of factors in inflamed peritoneal and pelvic environments. Similarly, the reduced proliferation of endometrial cells from controls without endometriosis in response to the same factors reflects the behavior of these cells in the pelvic and peritoneal environments of such women.

Our results do not clarify whether the effects of peritoneal

fluids on endometrial cell proliferation are due exclusively to the TNF content of these fluids or reflect a more complex interaction between multiple stimulatory and inhibitory factors. Numerous factors with growth-regulating capacities are present in the extracellular fluid of patients with inflammatory conditions. Tumor necrosis factor- $\alpha$  is one such factor that has been found in increased amounts in peritoneal fluids from women with endometriosis (11). This cytokine regulates cellular proliferation and cellular apoptosis through ligation of distinct TNF receptors (TNFR1 for apoptosis and TNFR2 for proliferation) (12). Thus, the differential response of endometrial cells to TNF- $\alpha$  in women with and without endometriosis may reflect differential regulation of TNF-receptor expression or signaling by this cytokine.

Our data suggest that in women without endometriosis, endometrial cells do not implant in ectopic locations because normal apoptotic mechanisms are activated by TNF- $\alpha$  through the TNFR1 receptor and because the proliferation-enhancing effects of TNF- $\alpha$  are inhibited by down-regulation of the TNFR2 receptor. Disruption or dysregulation of the normal, cyclical expression of these two TNF- $\alpha$  receptors on endometrium from women with endometriosis may create cells that can grow in the presence of high concentrations of TNF- $\alpha$ , a possibility for which we have recently obtained evidence (13). Recent studies documenting increased levels of soluble TNFR1 and TNFR2 in the peritoneal fluids of patients with endometriosis support this concept (14). Thus, it appears that a fundamental attribute of endometrial cells from women with endometriosis is the ability to resist apoptosis and exploit factors such as TNF- $\alpha$  to thrive in environments that normally would select against their survival.

Our results also suggest that ectopic growth of endometrial cells and the physiological consequences of that growth in women with endometriosis may be retarded by agents that block the effects of TNF- $\alpha$ . Presumably, this could be achieved by blocking TNF- $\alpha$  production (e.g., administration of pentoxifylline or ciprofloxacin) or by blocking the effects of TNF- $\alpha$  on target tissues (e.g., administration of etanercept). The attenuation of the proliferation-enhancing activity in peritoneal fluid from women with endometriosis by etanercept that we observed, and recent results in an

animal model of endometriosis using a recombinant human TNF-binding protein (15), support this idea.

Blocking the effects of TNF- $\alpha$  on target tissues might be especially appropriate in patients with extensive or intractable disease and might be useful in the postsurgical adjuvant setting to reduce the likelihood of recurrence. Given the potential of TNF- $\alpha$  to play a prominent role in both the etiology and the pathogenicity of endometriosis, studies of such treatment are warranted.

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