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Cytokine Secretion and Estrogen Regulation in an *in vitro* Model of Endometriosis

by

Jennifer Fan-Yu Tseng

A Thesis

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Cytokine Secretion and Estrogen Regulation

in an *in vitro* Model of Endometriosis

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Abstract

Endometriosis is a common gynecologic disorder characterized by the growth of endometrial tissue external to the uterine cavity. Both normal and ectopic endometrium grow in response to estrogenic stimulation and regress when estrogen is withdrawn. Recent studies have suggested that peritoneal inflammation may play a greater role in the progression of endometriosis than the growth of the endometrial implants *per se*. The pathophysiology of endometriosis thus is conditioned by both ovarian function and immune activation. To explore the intersection of these two systems, this study investigated cytokine secretion and estrogenic responsiveness by endometrial stromal cells.

Stromal cells were isolated from normal human endometrium (NE), eutopic endometrium from women with endometriosis (EE), and endometriosis implants (EI). The cells were evaluated for estrogen responsiveness and production of the specific cytokines RANTES and interleukin-6 (IL-6) *in vitro*. RANTES is a potent chemotactic factor for macrophages, lymphocytes, NK cells, and eosinophils, and IL-6 has a variety of effects on cell growth and immune function. To assess estrogen receptor function, 17β-estradiol (E_2) was added to stromal cell cultures from NE, EE and EI tissues. In stromal cells from all sources, E_2 stimulated an increase in the concentration of progesterone receptors in a dose-dependent manner (EC_{50}~1 nM). However, differential secretion of tumor necrosis factor-α/interferon-γ-stimulated RANTES and basal and interleukin-1-stimulated IL-6 was observed. Stromal cells from NE expressed the lowest concentrations of RANTES and IL-6. Cells

from EE showed a trend toward increased secretion of cytokines compared to NE. Stromal cells cultured from EI secreted the highest levels of RANTES and IL-6; these levels were significantly greater than those from both NE and EE. Our results indicate that isolated stromal cells cultured from EI, EE, and NE respond similarly to estrogenic stimulation, but that cells from EI have the intrinsic capacity to secrete greater amounts of RANTES and IL-6 than cells derived from EE and NE. This enhanced responsiveness may link the estrogenic exacerbation of symptoms associated with endometriosis with known proinflammatory cytokines.

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Introduction

Endometriosis is a common gynecologic disorder believed to affect from 5-15% of women of reproductive age. The disease is often accompanied by symptoms of dysmenorrhea, pelvic pain, and reduced fertility. Endometriosis is characterized by the growth of endometrial tissue, made up of mesenchymal stromal and glandular epithelial cells, outside the uterine cavity. The disease is dependent upon ovarian function; symptoms do not occur until menarche, and regress spontaneously following menopause. Endometriosis can have profound effects, including sterility, pain, psychological as well as physical distress, and work absenteeism. It is a common indication for surgery and its attendant costs and complications. Thus, endometriosis is a disease with widespread prevalence and significant negative impact on women's health.

Multiple theories exist regarding the etiology of endometriosis, including the theory of "retrograde menstruation" first advanced by Sampson (1), whereby menstrual contents proceed backward through the fallopian tubes and seed the peritoneal cavity; the theory of coelomic metaplasia, whereby embryonic rests of endometrial tissue in ectopic locations develop at menarche into functional endometriotic implants; and the theory of induction, which combines elements from the first two theories. Based a number of animal and human studies, there is now cautious support for "retrograde menstruation" playing a role in the pathophysiology of endometriosis. The pathophysiology of endometriosis-associated pain and infertility remain enigmatic, but is believed to result from local inflammation at the implant site. Pelvic implants are associated with

evidence of local inflammatory changes: the accumulation of activated peritoneal macrophages, neovascularization, and fibrous scarring.

Hormonal modulation of the signs and symptoms of endometriosis have been recognized for many years. The traditional culprit in endometriosis is the ovary. More specifically, endometriosis waxes in states that are relatively high in estrogen, and wanes in low estrogen states. Medical treatments for endometriosis have utilized anti-gonadotropic agents such as danazol and down-regulating doses of GnRH analogs. Medical inhibition of ovarian function has been shown to decrease symptomatology and endometriotic lesion volume in prospective, randomized clinical trials (2, 3). However, the mechanism of action of estrogen on the disease remains unclear.

Recent studies indicate that peritoneal inflammation may play a greater role in the pathophysiology of endometriosis than the endometrial implants *per se*. Activated macrophages and lymphocytes are found in greater numbers in the peritoneal cavity of women with endometriosis (4, 5, 6). Elevated levels of several cytokines have been reported in the peritoneal cavity of women with endometriosis, including interleukin-1 (7), tumor necrosis factor-α (8), transforming growth factor-β (9), RANTES (10), and interleukin-8 (11). The roles of these soluble factors are currently unknown, but it has been postulated that they act as mediators of the inflammatory response elicited by endometriotic lesions.

Endometriosis thus depends on both immune activation and ovarian function. Our laboratory seeks to explore possible links between these two systems. To address the immune and endocrine pathophysiology of endometriosis, we have

isolated, purified, and cultured human endometrial stromal cells from three tissue sources: endometriosis implants (EI), eutopic endometrium from women with endometriosis (EE), and endometrium from normal controls (NE) (12). Figure 1 shows phase-contrast photomicrographs of epithelial and mesenchymal cells cultured from normal endometrium and from endometriosis implants.

Using this novel cell-culture system, we have examined cytokine secretion and estrogen regulation *in vitro* in stromal cells from endometriosis implants, eutopic endometrium from women with endometriosis, and endometrium from normal controls. We chose to study stromal cells rather than epithelial cells because of increasing evidence that mesenchymal cells have functional hormone receptors and can secrete factors which regulate and induce differentiation in epithelial-type cells. Specifically, we have focused on two inflammatory cytokines, RANTES and interleukin (IL)-6, which we postulate may be involved in the immune response in endometriosis.

RANTES is a recently cloned member of the C-C "chemokine" superfamily. It is a specific chemoattractant for monocytes and T-cells. RANTES was first described by Schall and colleagues as an activated T-cell product; however, RANTES expression has since been documented from multiple cell types (13, 14). Similar cytokines with significant sequence homology, MCAF (15), MCP-1 (16), and IL-8 have also have been identified. Our laboratory has reported elevated RANTES levels in the peritoneal fluid of women with endometriosis (10). In addition, we have recently shown that peritoneal fluid concentrations of IL-8 also correlate with the severity of endometriosis (11). A recent report demonstrates that MCP-1 levels are greater in the eutopic endometrium of women with endometriosis than in normal endometrium (17). Proteins of the

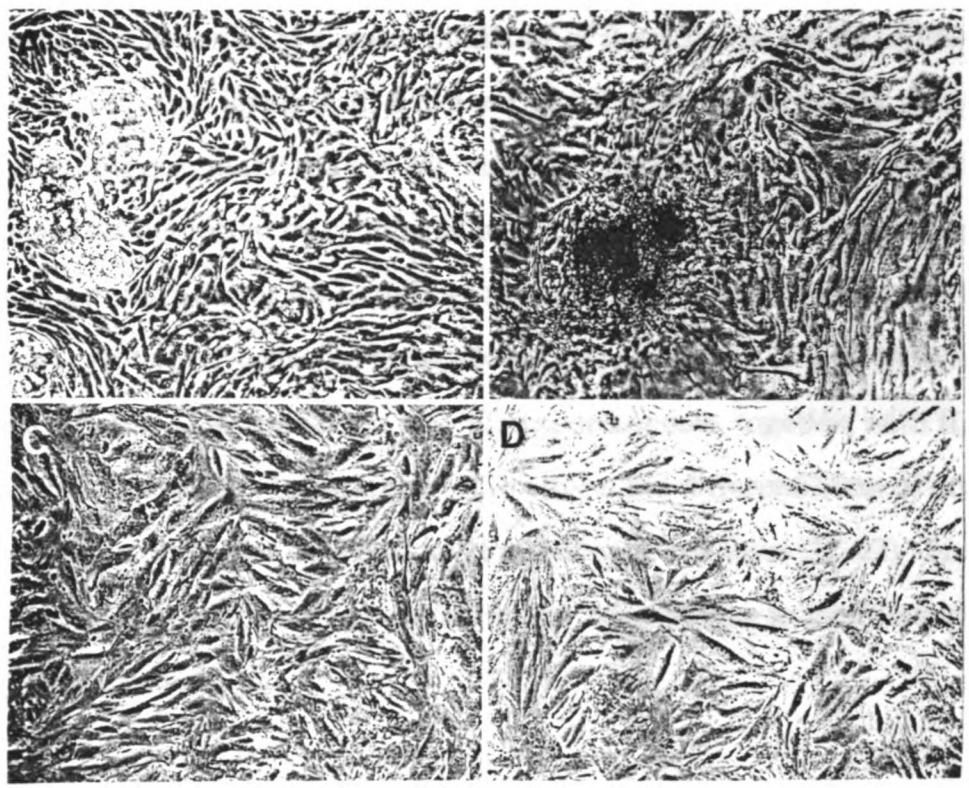


Figure 1: Phase contrast photomicrographs of cultured human endometrial and endometriosis cells. A. Normal endometrial epithelial cell outgrowth from gland. B, Endometriosis epithelial cell outgrowth from gland. C, Normal endometrial stromal cells. D, Endometriosis stromal cells. Magnification, x210.

"chemokine" family may play a fundamental role in the recruitment and activation of peritoneal macrophages in the pathogenesis of endometriosis.

Interleukin (IL)-6 is a multifunctional cytokine, produced by numerous cell types including mesenchymal cells, whose precise *in vivo* functions remain unclear. However, it is considered a major mediator of the acute phase inflammatory response. Elevated levels of IL-6 in a variety of diseases, including autoimmune disorders, suggest that it may be an important protein in the cascade of immune responses set in motion by cellular injury or infection (18). IL-6 has been shown to act as a growth factor for lymphocytes and induces differentiation of both B-cells and cytotoxic T-cells and is necessary for production of Ig-G in *in vitro* studies (19, 20). IL-6 appears to play an important role, together with IL-1, in early T-cell activation. Intriguingly, IL-6 affects the differentiation of normal hematopoetic precursor cells and myelomonocytic cell lines (21, 22). Studies in endometriosis have focused on the ability of this cytokine to stimulate peritoneal macrophage activation.

Our laboratory's work on endometriosis proposes that peritoneal macrophage recruitment and activation play a central role in the pathophysiology of endometriosis. We hypothesize that monocyte chemotaxis initiates a cascade of intraperitoneal inflammatory responses that result in pain and infertility in endometriosis. RANTES and IL-6 may be involved in this complex peritoneal cytokine network; a simplified proposed schema appears in Figure 2. Using our *in vitro* model, we seek to explore inflammatory cytokine secretion and estrogen regulation in endometrial stromal cells, and to ascertain whether any link between these two systems exists.

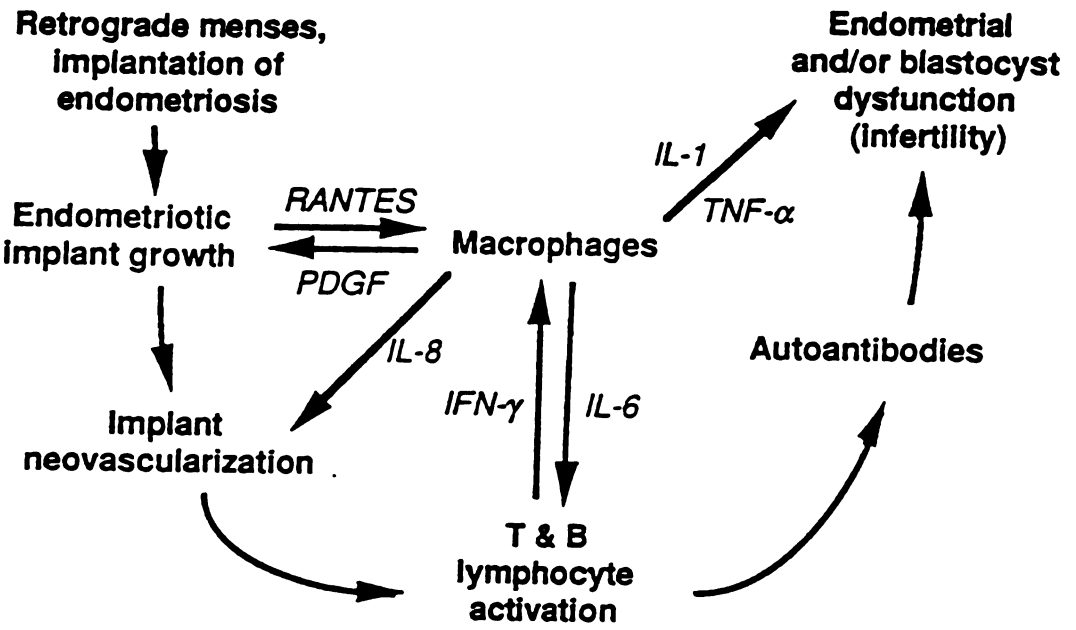


Figure 2. Postulated cascade of cytokine production in endometriosis pathophysiology.

Materials and Methods

Subjects

Normal ovulatory women undergoing laparoscopy for various indications, including evaluation of infertility, pelvic pain, pelvic mass, or elective tubal sterilization, were recruited. Women taking oral contraceptives or gonadotropin releasing hormone (GnRH) analogs were excluded. Informed consent was obtained under a protocol approved by the Committee on Human Research at the University of California, San Francisco. The presence or absence of endometriosis was identified at the time of operation by systematic observation of the pelvis. Women with active endometriosis lesions were assigned to the endometriosis group. Control subjects were women undergoing laparoscopy for tubal sterilization or for assessment of pelvic pain in whom no visible evidence of pelvic pathology was found. Women underwent laparoscopy and endometrial biopsy during the proliferative phase of their menstrual cycles. Subjects with an echogenic adnexal mass documented on ultrasonography were recruited for the possible retrieval of endometriomata biopsies at surgery.

Cell preparation and culture: The techniques for isolation and culture of human endometrial and endometriosis stromal cells *in vitro* have been discussed in detail elsewhere (12). Briefly, endometrial biopsies from control women and women with endometriosis were used to prepare cultures of eutopic endometrial stroma. Endometriosis stromal cell cultures were prepared from biopsies of ovarian endometrioma cyst linings. The tissues were rinsed, and cyst linings were dissected free from cyst walls. The specimens were minced, digested with collagenase, and then serially filtered through narrow gauge sieves with

apertures of 38-105 μm to trap the glandular epithelium. Stromal cells were plated and allowed to adhere to plastic cell-culture dishes for 30 minutes, at which time contaminating epithelial and blood cells and tissue debris were rinsed free. Cultures were allowed to proliferate in Minimum Essential Medium (MEM)-α supplemented with 10% fetal calf serum (FCS), nucleosides, and non-essential amino acids. All experiments were performed with cells at passage two, within 12 days of initial isolation.

Fig. 1 shows epithelial and stromal cells cultured from normal human endometrium and from human endometriosis. To identify cell type-specific cytoskeletal markers in situ, we performed immunohistochemistry experiments using monoclonal antibodies against cytokeratin (specific for epithelial cells) and vimentin (specific for stromal cells) intermediate filaments. The Vectastain Elite® kit (Vector laboratories, Burlingame, CA) was used to perform immunohistochemistry of normal proliferative endometrium. Glands stained for cytokeratin, while stromal cells stained for vimentin, as documented in Fig. 3. To ascertain that endometrial cells in culture maintained their specific cytoskeletal markers, we performed the same immunohistochemistry on cultured cells from normal and ectopic endometrial epithelial and stromal cells. As seen in Fig. 4, epithelial cells from normal endometrium and from endometriosis implants stained positively for cytokeratin, but not for vimentin; on the other hand, stromal cells from normal endometrium and endometriosis stained positively for vimentin, but not for cytokeratin. Staining with a pan-leukocyte monoclonal antibody (CD45), as well as monoclonal antibodies specific for macrophages (CD11b) and T-cells (CD3), documented the absence of contamination of the cell cultures with immune cells.

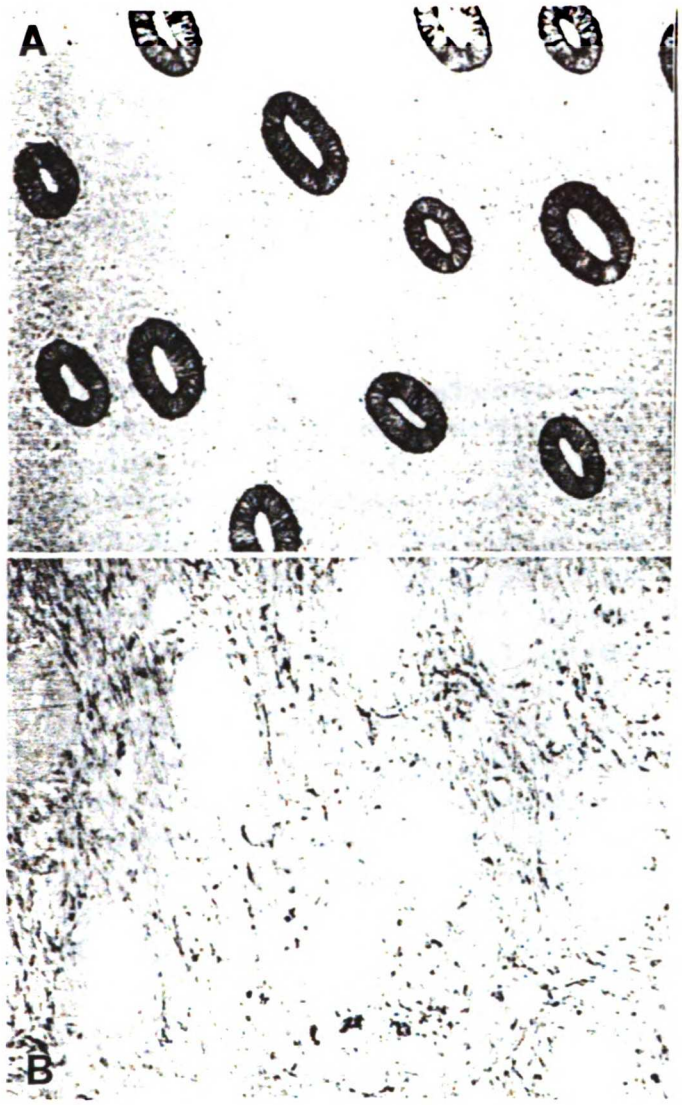
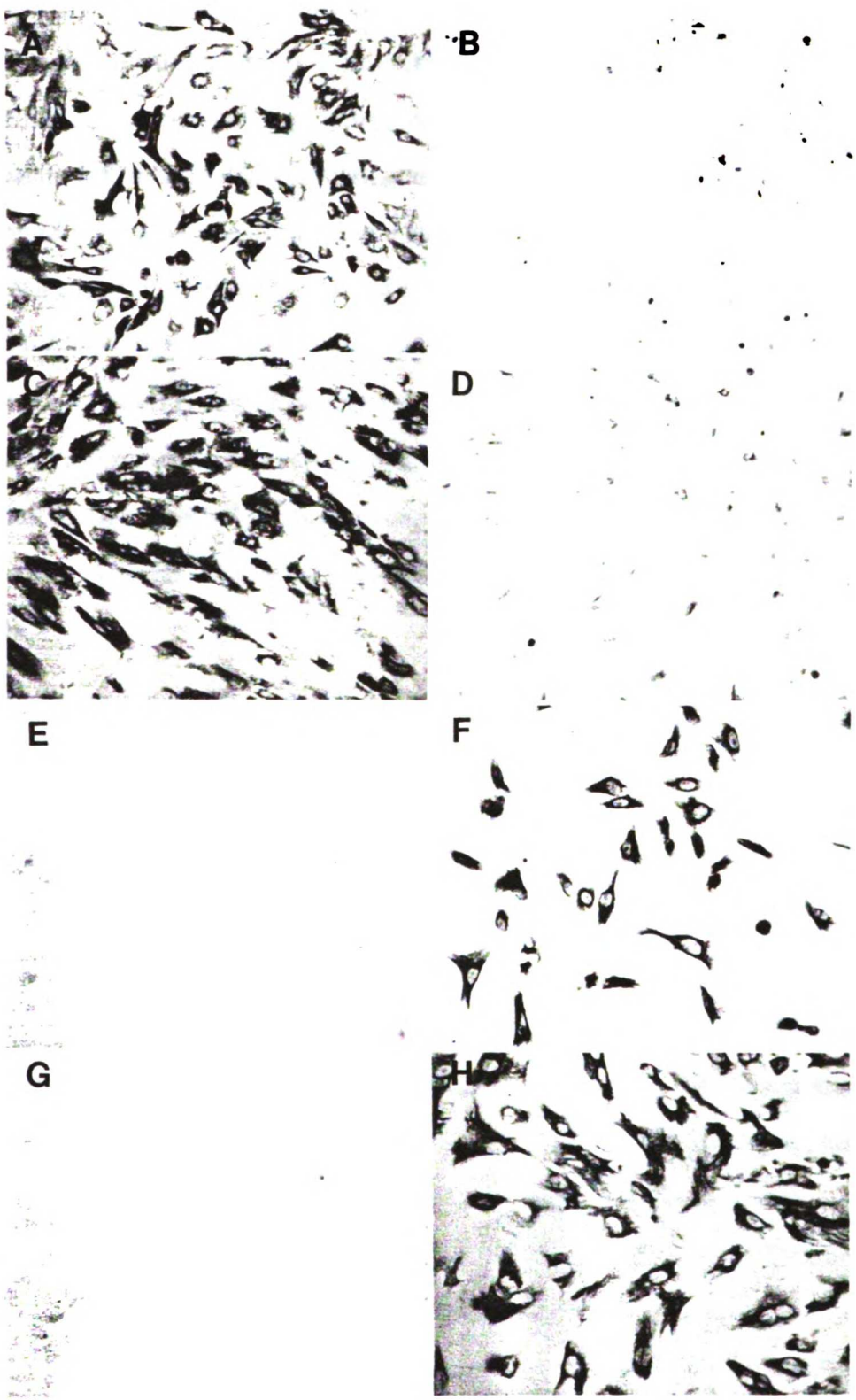


Figure 3: Normal proliferative human endometrium: immunohistochemistry. A, Anticytokeratin antibodies selectively stain epithelial gland intermediate filaments. B, Antivimentin antibodies selectively stain stromal intermediate filaments. Magnification, x130.

Figure 4: Immunocytochemistry of cultured human endometrial and endometriosis cells. Normal endometrial epithelial cells were immunopositive for cytokeratin (A), but negative for vimentin (B) intermediate filaments. Endometriosis epithelial cells were immunopositive for cytokeratin (C), but negative for vimentin (D). Normal endometrial stromal cells were immunonegative for cytokeratin (E), but positive for vimetin (F) intermediate filaments. Endometriosis stromal cells were immunonegative for cytokeratin (G), but positive for vimentin (H). Magnification, x210.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RANTES assay: Confluent cultures of stromal cells from normal endometrium of control women (NE), from eutopic endometrium of women with endometriosis (EE), and from endometriosis implants (EI) were plated in 24-well dishes (5×10^5 cells/well). Cells were then incubated overnight in low-serum medium (MEM-α supplemented with 2.5% FCS, nucleosides, and non-essential amino acids). Conditioned media were aspirated in a time-course fashion. Wells from all three stromal cell types were treated with tumor necrosis factor-α (1000 U/ml) and interferon-γ, (100 ng/ml) which have been shown to induce RANTES secretion in T-cells and other cell types. Assays for RANTES were performed on the conditioned media using a sensitive enzyme immunoassay developed by Dr. Tom Schall at Genentech, Inc., South San Francisco, CA. All aliquots were tested in duplicate at several dilutions and compared to reference standards of RANTES.

IL-6 assay: Confluent cultures of stromal cells from normal endometrium of control women (NE), from eutopic endometrium of women with endometriosis (EE), and from endometriosis implants (EI) were plated in 24-well dishes (5×10^5 cells/well). Cells were then incubated overnight in low-serum medium (MEM-α supplemented with 2.5% FCS, nucleosides, and non-essential amino acids). Conditioned media were aspirated in a time-course fashion. In addition, wells with all three stromal cell types were treated with IL-1β (10 U/mL), a potent inducer of IL-6 production. As a control for specificity of IL-6 secretion, the cells also were treated with *E. coli* lipopolysaccharide (LPS, 1 μg/mL), which stimulates IL-6 secretion by fibroblasts and monocytes but not by endometrial stromal cells. Enzyme immunoassays for IL-6 were performed on the conditioned media using a sensitive commercial kit (Quantikine®, R&D Systems, Minneapolis, MN) with demonstrated linearity in our samples. All aliquots were

tested in duplicate at several dilutions and compared to reference standards of recombinant human interleukin-6.

Data analysis and statistics: To most accurately compare cell cytokine expression between the different tissue sources of stromal cells, RANTES and IL-6 concentrations were normalized to total cell number per well, as determined by a Coulter apparatus. The data are expressed on a per cell basis and are presented as the mean ± SE of *n* independent determinations. As the RANTES and IL-6 data were not normally distributed, comparisons among the three types of stromal cells (NE, EE, and EI) were analyzed conservatively using nonparametric analysis of variance (ANOVA) by ranks (Kruskal-Wallis test). Correlation analysis by ranks (Spearman test) and paired comparisons using the Wilcoxon signed-rank test also were performed. Statistical significance was accepted at $P<0.05$ for two-tailed analyses.

17β-estradiol treatment and progestin receptor quantification: Previous studies from our laboratory indicated that endometrial stromal cells from normal and endometriosis tissues expressed estrogen receptor mRNA and protein. No differences in estrogen receptor affinity or concentration were detected between these cell types (12). However, presence may not necessarily indicate function. Estrogen classically induces progestin receptor up-regulation in responsive cells. To assess the presence of functional estrogen receptors in all three cell types, confluent cultures of stromal cells from NE, EE, and EI were plated in 6-well dishes (2×10^6 cells/well). Cells were quiesced overnight in serum-free medium (phenol-red-free MEM-α containing albumin, HEPES buffer, and an insulin-transferrin-selenium supplement). The medium was changed to serum-free medium with or without 17β-estradiol. After 48 hours, the cells were incubated

with increasing concentrations (50 pM to 5 nM) of the synthetic progestin [^{3}H]R5020 (New England Nuclear) for 60 minutes at 37° C. Free label was removed by two washes with cold PBS, and bound intracellular [^{3}H]R5020 was extracted with sodium hydroxide and quantified in a β-scintillation counter. Equilibrium saturation analysis was performed using an iterative, nonlinear curve-fitting program that analyzes bound ligand as a function of free ligand (23). Under the conditions of our assays, the ligand binding curves were resolved by three parameters, suggesting a single, saturable, high-affinity progestin binding site. The equilibium dissociation constant (K_d) is expressed as nM, and the receptor concentration (B_{max}) is expressed as receptors per cell. To clarify dosage effects of 17β-estradiol on progestin receptor concentrations, dose-response studies were carried out using increasing concentrations of estradiol in NE stromal cells.

RANTES luciferase transfection assay: Stromal cells from normal endometrium, eutopic endometrium from women with endometriosis, and from endometriosis implants were cultured in 6 cm plates. Cells were transfected with RANTES promoter-luciferase constructs when they had reached 50% confluence. Plasmid DNA was vortexed thoroughly with buffer containing 125 mM $CaCl_2$, 0.75 mM Na_2HPO_4 and 25 mM HEPES (pH 6.93) and allowed to precipitate on the cells for 18 hours at 37° C. The cells were washed with PBS, and fresh medium containing 2.5% FCS was added for 48 hours. Luciferase assays were initiated after washing in PBS and lysing the cells in buffer containing 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM KPO_4, 1 mM DTT, 2mM ATP, 0.8% Triton. Samples were added to a Monolight 1500 automatic luminometer that added D-luciferin to each sample and recorded luciferase

Results

RANTES production:

Unstimulated endometrial stromal cell cultures did not secrete detectable basal levels of RANTES *in vitro* (data not shown). However, when stimulated with TNF-α and interferon-γ, differential secretion of RANTES by NE, EE, and EI stromal cells was found (Fig. 5). As these responses were not normally distributed, conservative, nonparametric statistical analyses were performed. When stimulated with TNF-α (1000 U/ml) and interferon-γ (100 ng/ml), factors known to be produced by peritoneal leukocytes (8) (see Fig. 2), stromal cells isolated from endometriosis implants (EI) secreted more RANTES than normal endometrial stromal cells ($p<0.05$, Kruskal-Wallis ANOVA). Cells isolated from the eutopic endometrium of endometriosis patients had intermediate levels of stimulated RANTES secretion. RANTES production, even after stimulation with TNF-α and interferon-γ, was undetectable in epithelial cells from any of the three tissues (NE, EE, EI).

IL-6 production:

All unstimulated endometrial stromal cell cultures secreted detectable basal levels of IL-6 *in vitro*. Normal endometrial stromal cell IL-6 production, measured by EIA, was stimulated 193-fold above basal levels by IL-1β but <2-fold by LPS. By contrast, human skin fibroblast IL-6 secretion was stimulated 130- and 132-fold by IL-1β and LPS, respectively.

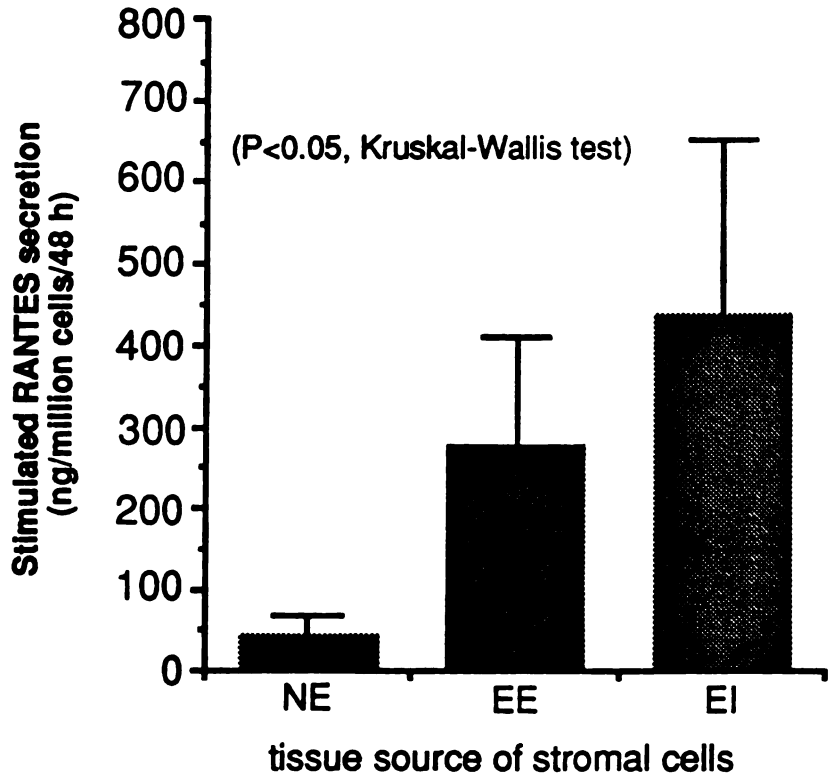

Figure 5: Stimulated RANTES secretion in endometrial and endometriosis cells.

In NE, EE, and EI stromal cells, differential responsiveness of IL-6 production was observed under basal and IL-1β stimulated conditions. As these responses were not normally distributed, conservative, nonparametric statistical analyses were performed. Under basal conditions (Fig. 6), the three experimental groups differed significantly by Kruskal-Wallis ANOVA ($H=17.56$, $P<0.01$, $n=21$) and a significant correlation between IL-6 concentrations and source of endometrial stromal cells was observed ($r=0.60$, Spearman correlation coefficient, $P<0.01$, $n=21$). In response to IL-1β stimulation, IL-6 secretion also was observed to differ significantly among the three sources of stromal cells ($H=7.12$, $P<0.03$, $n=21$ [Fig. 7]). IL-1β stimulated IL-6 secretion over basal levels by 193-, 46-, and 6-fold, respectively, in NE, EE, and EI cells.

Under basal and stimulated conditions, endometrial stromal cells secrete significantly greater amounts of IL-6 *in vitro* in a manner that correlates with the presence of endometriosis.

Progesterone receptor radioligand binding and Scatchard analysis: As previously discussed, endometriosis is an estrogen-dependent disease. Previous studies from our laboratory indicated that endometrial stromal cells from normal and endometriosis tissues expressed estrogen receptor mRNA and protein. No differences in estrogen receptor affinity or concentration were detected between these cell types (12). We sought to determine whether estrogen receptor function differed between stromal cells from NE, EE, and EI. Classically, exposure of cells with functional estrogen receptors to estrogen up-regulates progesterone receptors (PR). Thus, we used progesterone receptor radioligand binding and Scatchard analysis to assess for estrogen receptor function in endometrial stromal cells. Estradiol stimulation for 48 hours increased the concentration of progestin

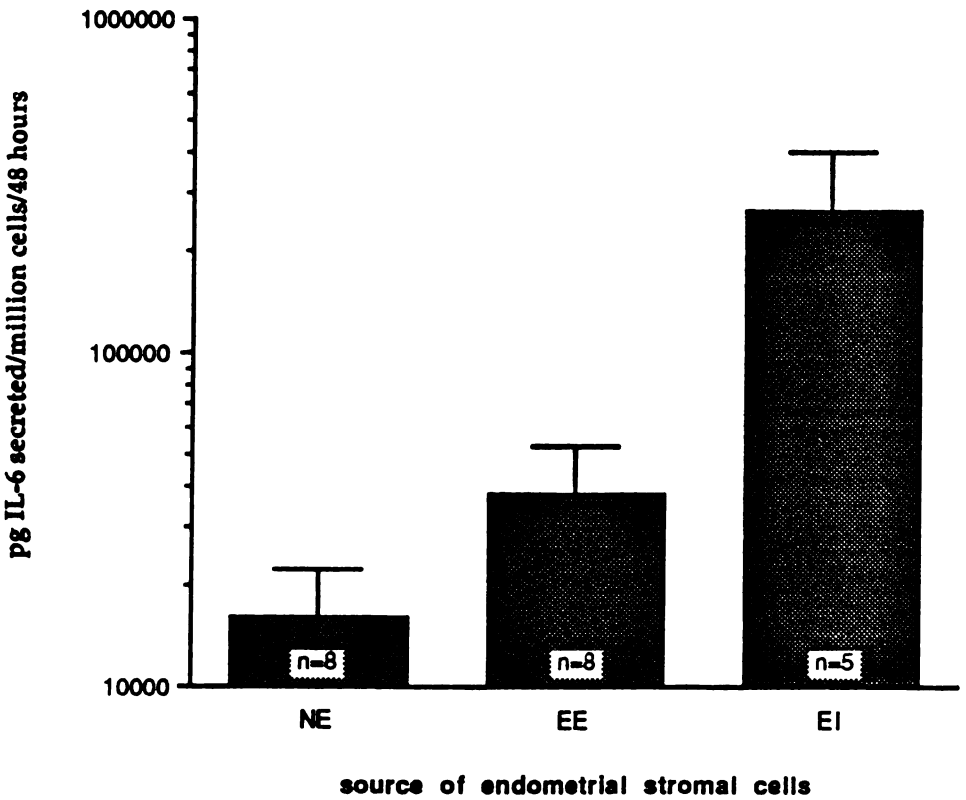

Figure 6: Basal IL-6 secretion in endometrial and endometriosis cells.

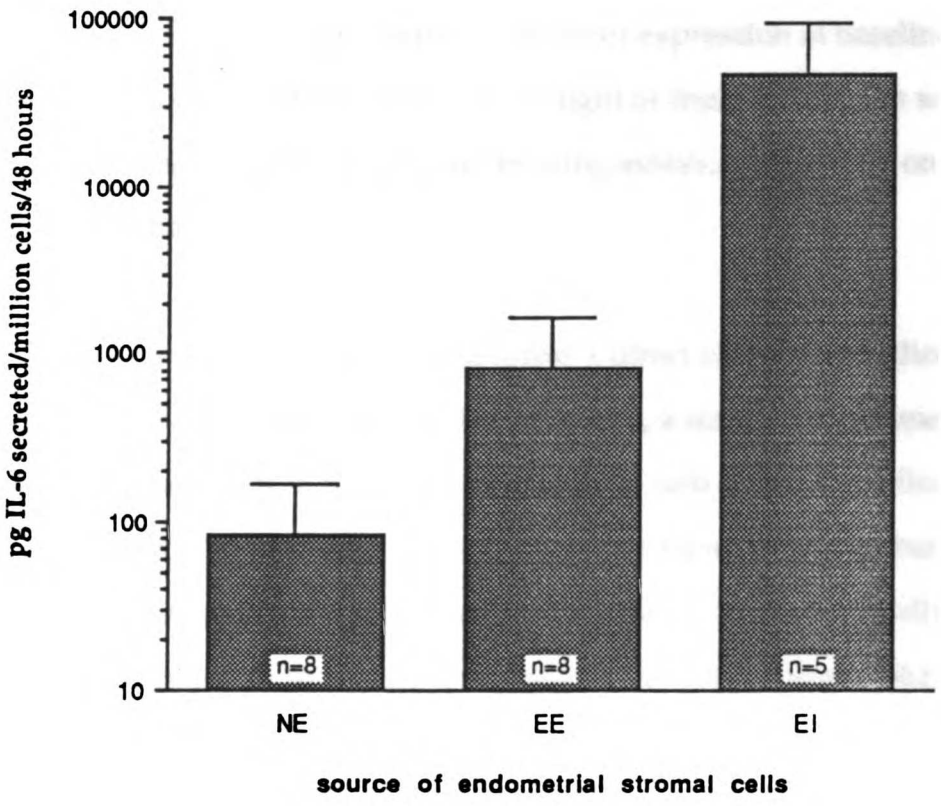

Figure 7: Stimulated IL-6 secretion in endometrial and endometriosis cells.

receptors approximately two-fold in a dose-dependent manner (EC_{50}~1 nM), consistent with the K_d of the ER detected in these cells (1.0 nM [12]). The results shown in Fig. 8 are the means of two independent series of Scatchard analyses performed on eutopic endometrial stromal cells. Table 1 summarizes the data from twelve sets of Scatchard analyses. Cells cultured from NE, EE, and EI demonstrated an increase in progesterone receptor concentration with estradiol stimulation in all three endometrial stromal cell types. In both NE (n=4) and EE (n=6) stromal cells, a 2.5-fold rise was observed after estradiol treatment; in EI cells (n=2), a rise of 1.5-fold was noted. The stromal cells cultured from EI were observed to have higher progesterone receptor expression at baseline, with less PR response to stimulation. However, in light of these results, the small number of progesterone receptor radioligand binding assays, particularly on EI cells, must be emphasized.

Estrogen effects on IL-6 secretion: To address a direct effect of estradiol on cytokine production by endometrial stromal cells *in vitro*, a series of experiments was performed to assess IL-6 production by stromal cells under the influence of 10 nM estradiol for 24-72 hours. In NE cells, which have the lowest basal IL-6 production, estradiol induced a small, but consistent and statistically significant rise in the secretion of this cytokine by paired NE cell cultures (246±76 *vs.* 282±88 pg/500,000 cells [15% increase], $P<0.05$, $n=11$, Wilcoxon test). However, cells derived from women with endometriosis (EI and EE), which have higher basal IL-6 production (Fig. 1) failed to show a significant direct estrogenic response (642±284 *vs.* 554±219 pg/500,000 cells [13% decrease], $P=0.93$, $n=11$, Wilcoxon test).

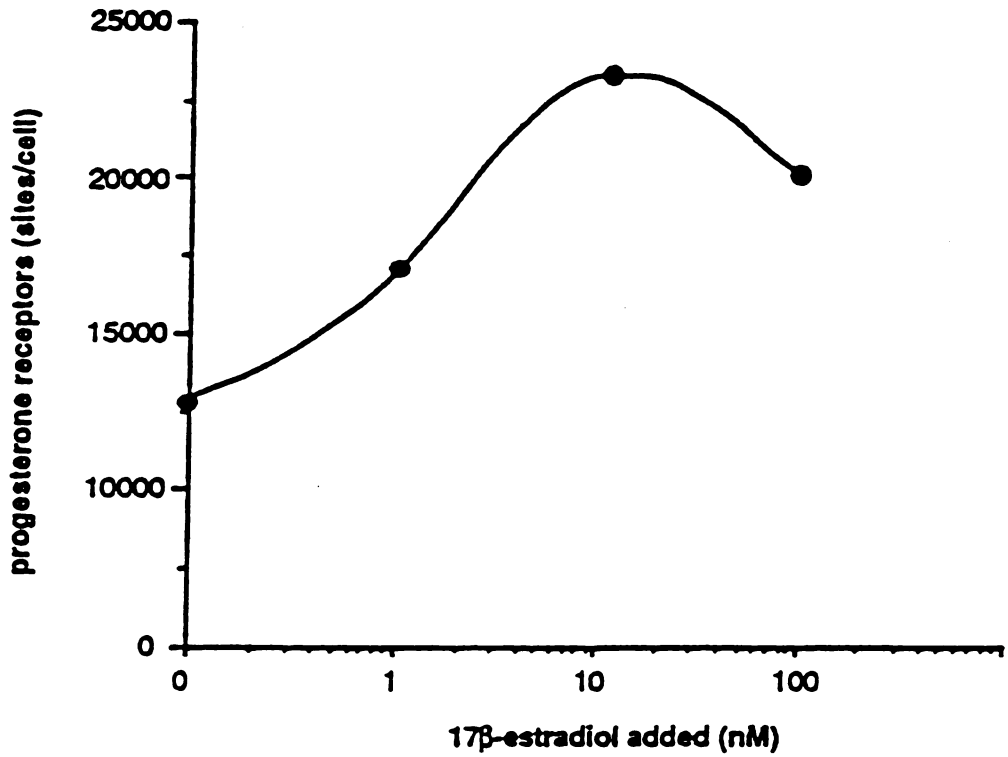

Figure 8: Effects of estradiol on endometrial stromal cell progesterone receptor expression.

Cell type	*Control* PR/ cell	*Control* K_d (nM)	*Estradiol treated* PR/ cell	*Estradiol treated* K_d (nM)	Increase after E_2 treatment
NE (n=4)	33161 (± 17243)	0.2	84093 (± 37841)	0.3	2.5x
EE (n=6)	27764 (± 8051)	0.2	68119 (± 25204)	0.2	2.5x
EI (n=2)	129538 (± 91972)	0.5	186532 (± 113786)	0.9	1.5x

Table 1: NE, EE, and EI progesterone receptor expression with and without 17β-estradiol (E_2) treatment.

RANTES gene promoter regulation: Preliminary data have been obtained concerning the RANTES gene promoter. A 961 bp sequence upstream from the RANTES gene transcription start site has been identified and cloned (24). Plasmids containing fusion constructs of the RANTES-promotor-luciferase reporter gene were obtained from Dr. Peter Nelson at the Stanford University School of Medicine. The promoter sequence contains several consensus elements for specific DNA transactivating proteins. Using the calcium phosphate co-precipitation method to transfect endometrial stromal cells, we found that these cells transiently express the fusion gene and after 48 hours activate the reporter in a sequence specific manner (Figure 10). Eutopic endometrial stromal cells activate the RANTES promoter-fusion gene 5-fold over the promoterless control. The positive control plasmids, containing the potent RSV promoter-luciferase construct, were stimulated 200-fold over the empty vector.

The luciferase activity results demonstrated in Figure 10 are averages of two independent transfection assays. The promoter activity of each construct was normalized to cell number and is expressed relative to that of untransfected stromal cells. Our observations indicate that endometrial stromal cells contain specific transactivating factors capable of initiating gene expression via a 961 bp promoter upstream from the RANTES trancriptional start site (24).

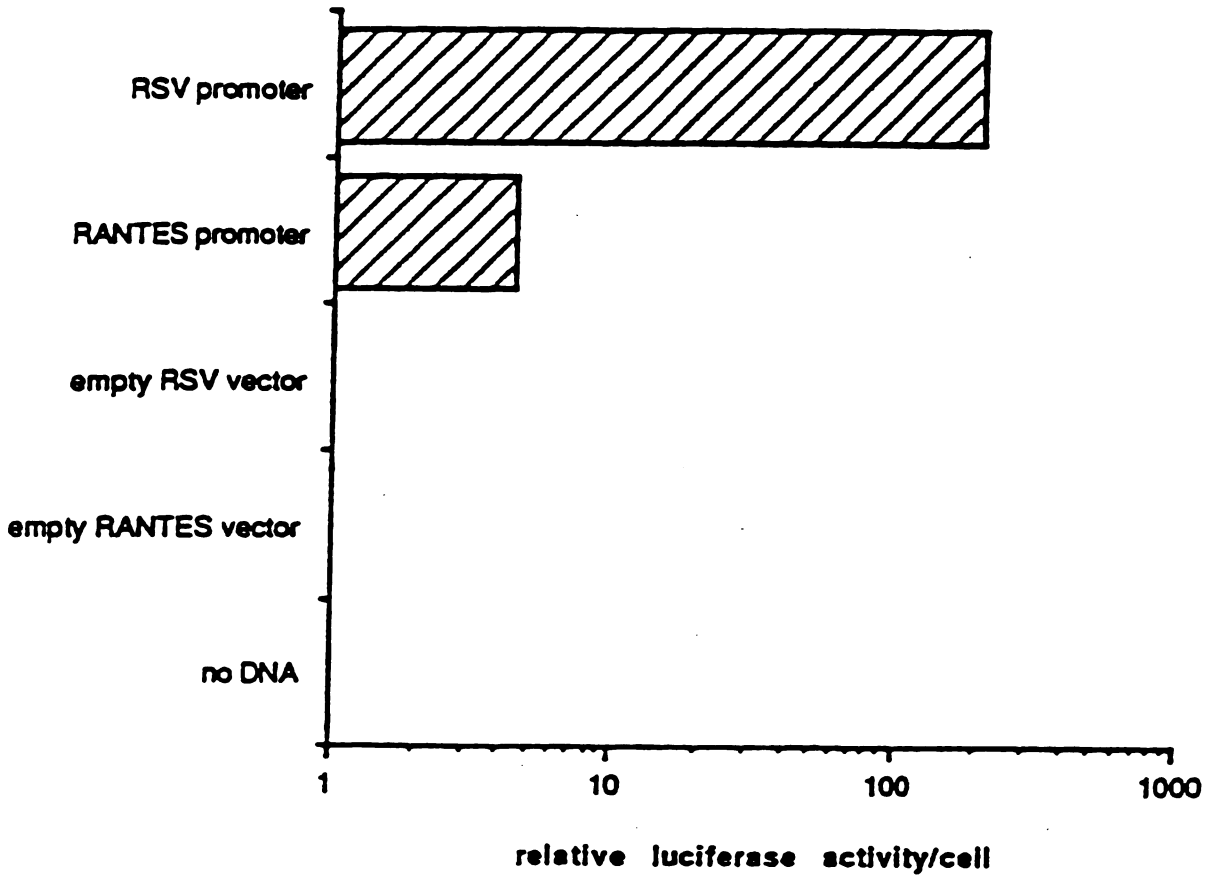

Figure 9: Functional analysis of RANTES promoter activation.

Discussion

In recent years, investigators have uncovered multiple connections between endometriosis and the immune system. Greater numbers of activated macrophages and lymphocytes have been described in the peritoneal fluid of women with endometriosis as compared with normal controls (4, 5, 6). T- and B-cells have been reported to be present in higher numbers, together with an elevated CD8:CD4 ratio, in the circulation of women with endometriosis (25). Some investigators have reported elevated titers of circulating anti-endometrial and other autoantibodies in patients with the disease, and have speculated that this finding may be related to increased polyclonal B-cell activation (26, 27, 28). Increased T-cell mediated cytotoxity to endometrial cell antigens in women with endometriosis has been described (29).

Regarding our own work on hormonal regulation and the inflammatory response in endometriosis, a number of experimental limitations deserve additional discussion. The *in vitro* system is based on a series of primary cell cultures from different subjects. A major issue with all primary cultures is that of culture purity. Contamination with immune cells could cause incorrect measurement of stromal cell cytokine secretion. Also, primary cell cultures of differentiated cell types can revert to less differentiated forms after repeated passages, *i.e.* mesenchymal cells such as endometrial stromal cells have been observed to lose their specialized characteristics, thus bearing a greater resemblance to fibroblasts than to their cell type of origin. To minimize both of these effects, all of the experiments previously described were performed at passage 2. Monoclonal antibody staining, as discussed in Materials and

Methods, demonstrated the absence of cells of immune lineages. Staining with antibodies to vimentin and cytokeratin showed that stromal cell cultures were free of epithelial cell contamination. Finally, *E.coli* lipopolysaccharide stimulated IL-6 production in fibroblasts but not endometrial stromal cells, supporting our assertion that these stromal cells at passage two remained true to their differentiated form, and had not reverted to a more primordial mesenchymal cell type.

Another issue with primary cell cultures is that of individual variation between human subjects. Differences in cytokine secretion might be attributable to the variable abilities of individual women's cells to secrete RANTES and IL-6, rather than to different characteristics of NE, EE, and EI stromal cell types. We plan to address this problem in two ways. First, we are collecting a series of paired specimens, with both eutopic and ectopic endometrial specimens from each woman. Thus, we will be able to compare each woman's eutopic endometrium cytokine secretion with cytokine secretion by her endometriosis implants. Second, as we increase our sample size, the individual variations will have less statistical effect on the overall trend. Although the restrictions of this laborious primary cell culture system are evident, we believe that our *in vitro* system will become increasingly useful and consistent as a tool to study the pathophysiology of endometriosis.

With this cell-culture model of endometriosis, we sought to answer two questions in the current studies: first, if RANTES and IL-6 production varies among stromal cells from NE, EE, and EI, and second, if estradiol alone *in vitro* can modulate cytokine production.

RANTES, as discussed earlier, is a potent chemotactic factor for macrophages. We have reported previously that levels of this cytokine are elevated in the peritoneal fluid of women with endometriosis (10). In the current work, we have shown that ectopic endometrial stromal cells are stimulated to secrete higher levels of this cytokine than normal and eutopic endometrial cells. We propose that intraperitoneal endometriosis implants secrete the soluble cytokine RANTES, which attracts and activates peritoneal macrophages. These macrophages, together with endometriosis stromal cells, may secrete interleukin-6 and other mediators of inflammation as part of the immune cascade that contributes to the pathophysiology of endometriosis. IL-6, with its myriad functions, has been suggested as an important modulator of human endometrial function (30). This cytokine may be particularly relevant to the study of endometriosis in terms of its ability to induce myelomonocytic differentiation in normal hematopoietic precursor cells, as well as its regulation of B- and T-cell function.

We further propose that ovarian hormones, either directly or via peptide growth factors, regulate the cellular production of RANTES and IL-6. Estrogen, the target hormone in endometriosis therapy, appears to have complex regulatory effects on IL-6. It has been recently reported that 17β-estradiol inhibits expression of the IL-6 promotor in HeLa and murine bone marrow stromal cells (31). Tabibzadeh and colleagues have described estradiol-mediated attenuation of IL-1β-induced increases in IL-6 production by endometrial stromal cells *in vitro*. Other investigators have presented a more complicated scenario. Laird and colleagues have reported variable effects of steroid hormones on IL-6 production depending on the phase of the menstrual cycle (32). It should be noted that although our experiments do not address menstrual cycle variability

per se, our specimens are obtained during the proliferative phase of the endometrium.

Using progesterone receptor radioligand binding and Scatchard analysis, we have demonstrated that endometrial stromal cells from normal endometrium from control women, from eutopic endometrium from women with endometriosis, and from endometriosis implants respond to estrogen treatment. Preliminary data suggest that endometriosis implant stromal cells may have constitutively increased concentrations of progesterone receptors, and be less sensitive to further up-regulation of PR by estradiol stimulation. These results can be compared to IL-1β effects on IL-6 secretion; EI stromal cells, which at baseline secrete the highest levels of IL-6, are stimulated in a lesser fashion by IL-1β than NE and EE stromal cells. In testing direct effects of estradiol on IL-6 secretion, NE cells showed a small but statistically significant increase in production of this cytokine. However, EE and EI stromal cells, with higher constitutive IL-6 production, did not show a direct response to estrogen treatment. Our results suggest that NE, EE, and EI stromal cells respond to estrogen stimulation, but do not support a direct effect of estradiol on stromal cell IL-6 production.

An ongoing debate in the study of endometriosis can be phrased in the following manner: are endometriotic cells simply normal endometrial cells in an abnormal location? Or, conversely, are endometriosis implants made up of cells that differ significantly from their eutopic counterparts in the endometrium? Answers to these queries may shed light on the still enigmatic pathogenesis of this important disease. In these experiments, we have demonstrated that cultured stromal cells from endometriosis implants secrete significantly more RANTES and IL-6 than

cells derived from eutopic endometrium from women with endometriosis and from control endometrium. Furthermore, eutopic endometrium from women with endometriosis appears to secrete an intermediate amount of these cytokines. Thus, intrinsic differences in cytokine secretion among the three endometrial stromal cell sources were noted. However, the mechanism for this variability remains elusive.

Further experiments will test the hypothesis that estrogen, the prime endocrine suspect in the pathogenesis of endometriosis, may have differential regulatory effects on both IL-6 and RANTES expression by different types of endometrial cells. The newly developed RANTES promoter-luciferase reporter transfection assay will allow us to examine the transcriptional regulation of the RANTES gene by hormones, anti-hormones, and peptide growth factors in stromal cells from normal endometrium, from eutopic endometrium from women with endometriosis, and from endometriosis implants. In addition, we hope to establish similar constructs to assess IL-6 transcriptional regulation.

In the almost seventy years since Sampson's seminal paper on endometriosis first appeared (1), the pathogenesis of the disease continues to baffle scientists and clinicians alike. The theory of retrograde menstruation has been supported more by observation and analogy than by direct scientific evidence. With the advent of laparoscopic surgery a diagnostic tool, investigators have documented that retrograde menstruation and peritoneal seeding of viable endometrial cells appear to occur frequently in menstruating women (33, 34). The inference can thus be drawn that retrograde menstruation may be necessary but not sufficient to establish clinically significant disease. It has been suggested that endometriosis may be an immunogenetic disease, albeit one not linked to a single

HLA haplotype (35), and with an apparently polygenic transmission pattern (36). Dmowski, Steele, and colleagues have proposed that women who are prediposed to developing endometriosis may have a different immune response which enhances ectopic endometriosis implant survival within the peritoneal mileu (37).

Our findings suggest that endometriosis stromal cells differ quantitatively in terms of their ability to produce specific mediators of inflammation. Furthermore, preliminary evidence indicates that eutopic endometrium from affected women may have greater cytokine-secreting potential than endometrium from control women. These data may further elaborate the emerging picture of endometriosis pathophysiology first begun by Sampson in 1927. Retrograde menstruation occurs; then, the menstrual contents interact with the complex peritoneal environment. Together with immunogenetic susceptibility factors including endometrial cell attachment, growth, invasion, and progression, the specific immunologic response, made up of both peritoneal and endometrial components, may determine which women are susceptible to developing endometriosis.

Through continuing laboratory investigation, we hope to clarify the relationship of normal ovarian hormonal cycling with the inflammatory response that leads to pain and infertility in women with endometriosis.

References

1. Sampson JA. 1927. Peritoneal endometriosis due to menstrual dissémination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol. 14:422-69.

2. Schriock E, Monroe SE, Henzl M *et al.* 1985. Treatment of endometriosis with a potent agonist of gonadotropin-releasing hormone (nafarelin). Fertil Steril. 44:583-8.

3. Henzl MR, Corson SL, Moghissi K *et al.* 1988. Administration of nasal nafarelin as compared with oral danazol for endometriosis. A multicenter double-blind comparative clinical trial. N Eng J Med. 318(8):485-9.

4. Haney AF, Muscato JJ and Weinberg JB. 1981. Peritoneal fluid cell populations in infertility patients. Fertil Steril. 35:696-8.

5. Halme J, Becker S, Hammond MG *et al.* 1983. Increased activation of pelvic macrophages in infertile women with mild endometriosis. Am J Obstet Gyncol. 145:333-7.

6. Hill JA and Anderson DJ. 1989. Lymphocyte activity in the presence of peritoneal fluid from fertile women and infertile women with and without endometriosis. Am J Obstet Gynecol. 161:861-4.

7. Fakih H, Baggett B, Holtz G *et al.* 1987. Interleukin-1: a possible role in the infertility associated with endometriosis. Fertil Steril. 47:213-7.

8. Halme J. 1989. Release of tumor necrosis factor-alpha by human peritoneal macrophages *in vivo* and *in vitro.* Am J Obstet Gynecol. 161:1718-25.

9. Oosterlynck DJ, Meuleman C, Waer M *et al.* 1994. Transforming growth factor-beta activity is increased in peritoneal fluid from women with endometriosis. Obstet Gynecol. 83(2):287-92.

10. Khorram O, Taylor RN, Ryan IP *et al.* 1993. Peritoneal fluid concentrations of the cytokine RANTES correlate with the severity of endometriosis. Am J Obstet Gynecol. 169:1545-9.

11. Ryan IP, Tseng JF, Schriock E *et al.* 1995. Interleukin-8 levels are elevated in the peritoneal fluid of women with endometriosis. Fertil Steril, in press.

12. Ryan IP, Schriock E and Taylor RN. 1994. Isolation, characterization, and comparison of human endometrial and endometriosis cells *in vitro*. J Clin Endocrinol Metab. 78:642-9.

13. Schall TJ. 1991. Biology of the RANTES/SIS cytokine family. Cytokine. 3:165-183.

14. Schall TJ, Bacon K, and Toy KJ. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature. 347:669-71.

15. Goodman SB, Rein MS and Hill JA. 1993. Hysterosalpingography contrast media and chromotubation dye inhibit peritoneal lymphocyte and macrophage function in vitro: a potential mechanism for fertility enhancement. Fertil Steril. 59:1022-7.

16. Arici A, Head JR, MacDonald PC *et al.* Cytokine modulation of monocyte chemotactic protein-1 (MCP-1) gene expression in endometrial stromal cells. American Fertility Society abstract #144.

17. Akoum A, Lemay A, Brunet C *et al.* 1994. Secretion of monocyte chemotactic protein-1 (MCP-1) by cytokine-stimulated endometrial cells of women having endometriosis. Endocrine Society abstract #1793.

18. Van Snick. 1990. Interleukin-6: an overview. Ann Rev Immunol. 8:253-78.

19. Le JM and Vilcek J. 1989. Interleukin 6: a multifunctional cytokine regulating immune reactions and the acute phase protein response. Laboratory Investigation. 61:588-602.

20. Houssiau FA, Coulie PG and Van Snick J. 1989. Distinct roles of IL-1 and IL-6 in human T cell activation. J Immunol. 143(8)2520-4.

21. Koike K, Nakahata T, Takagi M *et al.* 1988. Synergism of BSF-2/interleukin 6 and interluekin 3 on development of multipotential hemopoietic progenitors in serum-free culture. J Exp Med. 168(3):879-90.

22. Bot FJ, van Eijk L, Broeders L *et al.* 1989. Interleukin-6 synergizes with M-CSF in the formation of macrophage colonies from purified human marrow progenitor cells. Blood. 73(2)435-7.

23. Kilpatrick SJ, Roberts JM, Lykins DA *et al.* 1993. Characterization and ontogeny of endothelin receptors in human placenta. Am J Physiol. 264:E367-72.

24. Nelson PJ, Kim HT, Manning WC *et al.* 1993. Genomic organization and transcriptional regulation of the RANTES chemokine gene. J Immunol. 151:2601-12.

25. Badawy SZ, Cuenca V, Kaufman L *et al.* 1989. The regulation of immunoglobulin production by B cells in patients with endometriosis. Fertil Steril. 51:770-3.

26. Mathur S, Garza DE and Smith LF. 1990. Endometrial autoantigens eliciting immunoglobulin (Ig) G, IgA, and IgM responses in endometriosis. Fertil Steril. 54:56-63.

27. Wild RA, Hirisave V, Podczaski ES *et al.* 1989. Autoantibodies associated with endometriosis: can their detection predict presence of the disease? Obstet Gynecol. 77:927-31.

28. Gleicher N, el-Roeiy A, Confino E *et al.* 1987. Is endometriosis an autoimmune disease? Obstet Gynecol. 70:115-22.

29. Steele RW, Dmowski WP, and Marmer DJ. 1984. Immunologic aspects of human endometriosis. Am J Reprod Immunol. 6(1):33-6.

30. Tabibzadeh SS, Santhanam U, Sehgal PB *et al.* 1989. Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells: modulation by estradiol-17 beta. J Immunol. 142:3134-9.

31. Pottratz ST, Bellido T, Mocharla H *et al:* 1994. 17 beta-estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. J Clin Invest. 93(3)944-50.

32. Laird SM, Li TC and Bolton AE. 1993. The production of placental protein 14 and interleukin 6 by human endometrial stromal cells in culture. Human Reproduction. 8(6)793-8.

33. Halme J, Hammond MG, Hulka JF *et al.* 1984. Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol. 64:151-4.

34. Kruitwagen RFPM, Poels LG, Willemsen WNP *et al.* 1991. Endometrial epithelial cells in peritoneal fluid during the early follicular phase.

35. Moen M, Bralie A and Moen T. 1984. Distribution of HLA antigens among patients with endometriosis. Acta Obstet Gynecol Scand. 123:25-7.

36. Simpson JL, Elias S, Malinak LR *et al.* 1980. Heritable aspects of endometriosis: genetic studies. Am J Obstet Gynecol. 137:327-31.

37. Dmowski W, Steele RW and Baker GF. 1981. Deficient cellular immunity in endometriosis. Am J Obstet Gynecol. 141:377-83.

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