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Interleukin-8 Induces Proliferation of Endometrial Stromal Cells: a Potential Autocrine Growth Factor*

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ABSTRACT

Proliferation of endometrium is dependent on sex steroid hormones, but specific growth factors are likely to play an important role in regulating this process. A number of cytokines and growth factors are synthesized in the endometrium in response to sex steroid hormones and act to regulate endometrial function. Endometrial cells produce interleukin-8 (IL-8) both *in vivo* and *in vitro*. We hypothesized that IL-8, a neutrophil chemoattractant/activating factor and a potent angiogenic agent that has been shown to stimulate growth in other cell types, may directly stimulate proliferation of endometrial cells. We first investigated the effect of IL-8 and mouse antihuman-IL-8 neutralizing antibody on endometrial stromal cell proliferation using both a colorimetric assay and thymidine uptake. We then investigated the modulation of endometrial stromal cell IL-8 production and proliferation by antisense oligonucleotides specific for IL-8. There was a concentration-dependent increase of cell proliferation with IL-8 (2-fold at 1 ng/mL; $P < 0.01$ between control and concentrations above 0.01 ng/mL) and a concentration-dependent inhibition of cell proliferation with anti-IL-8 antibody (to 30% of the control at 1 μg/mL; $P < 0.01$ between control and concentrations above 0.1 μg/mL). IL-8 antisense oligonucleotide treatment decreased IL-8 production by endometrial stromal cells in culture as well as cell proliferation when it is compared with scrambled (nonsense) oligonucleotide treatment ($P < 0.01$). Addition of IL-8 (1 ng/mL) reversed the proliferation inhibitory effect of IL-8 antisense oligonucleotides. We propose that IL-8 may act as an autocrine growth factor in the endometrium, and suggest that it may also play a role in the pathogenesis of endometriosis. (*J Clin Endocrinol Metab* **83:** 1201–1205, 1998)

THE HUMAN endometrium undergoes remarkable cyclic growth and regeneration in response to sex steroid hormones. Tissue remodeling and neovascularization occurs during the proliferative phase, and predecidualization occurs during the late secretory phase of the endometrial cycle. Endometrial stromal mitoses are most abundant during the proliferative phase and absent during the mid-cycle, but reappear during late secretory phase (1). Although the proliferation and differentiation of endometrium are dependent on sex steroid hormones, in recent years it has become apparent that specific growth factors are likely to play an important role in regulating these processes. A number of cytokines and growth factors are synthesized in the endometrium in response to sex steroid hormones and act to regulate endometrial function.

Interleukin-8 (IL-8) is a polypeptide that induces chemotaxis of neutrophils (2) and is a potent angiogenic agent (3). It is produced by peripheral blood monocytes (4), endothelial cells (5), fibroblasts (6), mesothelial cells (7, 8), and endometrial cells (9). In addition to its chemotactic and activating properties for granulocytes, IL-8 was recently found to stimulate proliferation of various other cells such as epidermal cells (10), melanoma cells (11), and smooth muscle cells (12). Comparison of IL-8 messenger RNA levels throughout the menstrual cycle revealed that early-to-mid proliferative and late secretory phase IL-8 expression was significantly higher than mid-cycle expression (13). This cyclicity is similar to the one that is observed for the number of mitoses in the endometrial stroma throughout the menstrual phases.

We postulated that IL-8 may play a role in the growth and proliferation of endometrium not only by stimulating leukocytes to secrete growth factors and cytokines, but also by directly stimulating endometrial stromal cell proliferation. In the present study, we investigated the effect of IL-8 and anti-IL-8 neutralizing antibody on the proliferation of endometrial stromal cells in culture. We then evaluated the alteration of IL-8 production and cell proliferation in endometrial stromal cells in culture by IL-8 antisense oligodeoxynucleotides.

Materials and Methods

Tissue collection

Endometrial tissue was obtained from human uteri after hysterectomy conducted for reasons other than endometrial disease or from endometrial biopsies. Informed consent in writing was obtained from each woman before surgery using consent forms and protocols approved by the Human Investigation Committee of this university. The day of the menstrual cycle was established from the woman's menstrual history and was confirmed by histologic evaluation. Samples from 32 women (mean age: 38.7 yr; proliferative phase, n = 19; secretory phase, n = 13) were used. Endometrial tissues were placed in HBSS and transported to the laboratory for separation and culture of the endometrial stromal cells.

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Isolation and culture of human endometrial stromal cells

Endometrial epithelium and stromal cells were separated and maintained in monolayer culture as described previously (9). Briefly, endometrial tissue was digested by incubation of tissue minces in HBSS that contained HEPES (25 mM), penicillin (200 U/mL), streptomycin (200 mg/mL), collagenase (1 mg/mL, 15 U/mg), and DNase (0.1 mg/mL, 1500 U/mg) for 30 min at 37 C with agitation. The dispersed endometrial cells were separated by filtration through a wire sieve (73- μm diameter pore). The endometrial glands (largely undispersed) were retained by the sieve, whereas the dispersed stromal cells passed through the sieve into the filtrate. Because endometrial gland cells do not or only minimally proliferate in culture, we have not used gland cells in cell proliferation assays.

The stromal cells were plated in Ham's F12:DMEM (1:1, vol/vol) that contained antibiotics-antimycotics (1%, vol/vol) and FBS (10%, vol/vol). Cells were plated in plastic flasks (75 cm^2), maintained at 37 C in a humidified atmosphere (5% CO_2 in air), and allowed to replicate to confluence. Thereafter, stromal cells were passed by standard methods of trypsinization and were plated in 24- and 96-well plates at 5×10^4 and 10^4 cell per well concentrations, respectively. Cells became preconfluent (covering 80% of the culture well) in approximately 5–7 days. Endometrial stromal cells after the first passage were characterized as described previously (9) and were found to contain 0–7% epithelial cells, no endothelial cells, and 0.2% macrophages. In each experiment, cells were treated with serum-free medium for 24 h before treatment with test agents was initiated.

Cell proliferation assays

Endometrial stromal cell proliferation was measured by calculating [^{3}H]thymidine uptake. Briefly, endometrial stromal cells in 96-wells were allowed to replicate to preconfluence, were treated with serum-free media for 24 h, and then incubated with test agents in serum-free, phenol red-free medium for 20 h until methyl-[^{3}H]thymidine (1 μCi/well) was added. Four hours later, 0.1 M EDTA was added to each well. After that, cells were harvested using an automated cell harvester (PHD, Cambridge Technology, Watertown, MA), and cells were applied to glass-fiber filter disks (Cambridge Technology). The disks were washed and then dried with ethanol and air as the manufacturer recommended; radioactivity on each disk was quantified by liquid scintillation spectrophotometry. Data were expressed as counts per minute per well.

Cell proliferation was also determined by a colorimetric assay using MTT (3-[4,5-dimethythiazol-2-yl-)-2,5-diphenyl tetrazolium bromide] (14, 15). MTT is a water-soluble tetrazolium salt that yields a yellowish solution when prepared in media or salt solutions that lack phenol red. Dissolved MTT is converted to the colored product formazan in active mitochondria, and then can be solubilized using acid-isopropanol mixture (1 N HCl:isopropanol 4:96 vol/vol). Dual end optical density reading at 570–650 nm is directly proportional to the number of cells. The first column of each 96-well plate did not contain any cells and was used as a blank. Four hours before the end of each experiment, MTT solution was added into all wells (10 μL/100 μL medium per well), and plates were incubated at 37 C. At the end of the incubation period, acid-isopropanol mixture was added into each well (100 μL/well), and plates were read within 30 min with a multiwell plate reader (Thermomax, Molecular Devices Corp., Menlo Park, CA). Data were expressed in optical density units. Two assays of cell proliferation were compared and showed correlation in their results. Experiments were conducted with replicates of eight wells per treatment condition. Similar experiments were conducted on at least three different occasions with cells prepared from three different endometrial tissues.

Synthesis of IL-8 antisense oligodeoxynucleotides

Two different IL-8 antisense and two different scrambled (nonsense) oligonucleotides were used. The sequences of antisense oligonucleotides I (AS-I) and II (AS-II) and scrambled oligonucleotides I (S-I) and II (S-II) are shown in Table 1. AS-I and S-I were previously described (3). The second series were deducted from GeneBank Accession number M28130 and were designed using Primer Designer for Windows Version 3.0 (Scientific & Educational Software, Durham, NC) that allowed the choice of IL-8 specific sequences, by alignment, that are not capable of hybridizing to other known gene products. The single-strand oligonucleotide molecules were prepared at the Critical Technologies Laboratory at Yale University Department of Pathology, using Applied Biosystems 394 DNA/RNA synthesizer (Perkin Elmer, Foster City, CA) unmodified phosphodiester or phosphorothioate oligodeoxynucleotides.

For evaluation of IL-8 production, endometrial stromal cells were passed to 24-well plates as appropriate for the experimental design, and were allowed to replicate to confluence before starting the experiments. Cells were treated with various concentrations of IL-8 antisense and scrambled oligonucleotides in the absence or presence of FBS. 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).

IL-8 immunoassay

Immunoreactive IL-8 in culture supernatants of endometrial stromal cells was quantified using an ELISA from R & D systems (Minneapolis, MN). According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. Each experimental set-up was repeated on at least three occasions using endometrial stromal cells obtained from three different patients. All of the samples for individual experiments were evaluated in a triple assay. The sensitivity for IL-8 was 4.7 pg/mL. The intra- and interassay coefficients of variation were 7.9% and 10.2%, respectively.

Statistical analyses

Data from the ELISA and cell proliferation assays were normally distributed and evaluated with ANOVA. Individual groups were compared with Bonferroni *post hoc* analysis for multiple comparisons. Statistical calculations were performed using Statistical Package for Social Sciences (SPSS) Version 6.0 for Windows (SPSS, Chicago, IL).

Reagents

Culture media, antibiotics-antimycotics, inorganic chemicals, and FBS were from Sigma Chemical Co. (St. Louis, MO). Recombinant IL-8, monoclonal mouse antihuman IL-8 antibody, and nonspecific mouse IgG were from R & D Systems.

Results

Effect of IL-8 on endometrial stromal cell proliferation

Endometrial stromal cells were incubated in serum-free medium for 24 h, then were treated with various concentrations of IL-8 (0.001–10 ng/mL) or with PBS (control) for 24 h, and [^{3}H]thymidine uptake was measured. There was a concentration-dependent increase of cell proliferation with IL-8 (2-fold at 1 ng/mL; $P < 0.01$ between control and concentrations above 0.01 ng/mL) (Fig. 1). We observed similar

TABLE 1. Oligonucleotides used on endometrial stromal cells in culture to determine effect on IL-8 production and cell proliferation

Name	Sequence	Molecular modifications
AS-I	5′-GTT GGC GCA GTG TGG TCC ACT CTC AAT CAC-3′	Unmodified phosphodiester
S-I	5′-TTG TAT TAG TGT GAG CCT GAG AGT CCG ATG-3′	Unmodified phosphodiester
AS-II	5′-GCC AGC TTG GAA TGC A-3′	Cholesterol conjugated at 3′ end + phosphorothioate
S-II	5′-GCC AGT CTG GAG ACT A-3′	Cholesterol conjugated at 3′ end + phosphorothioate

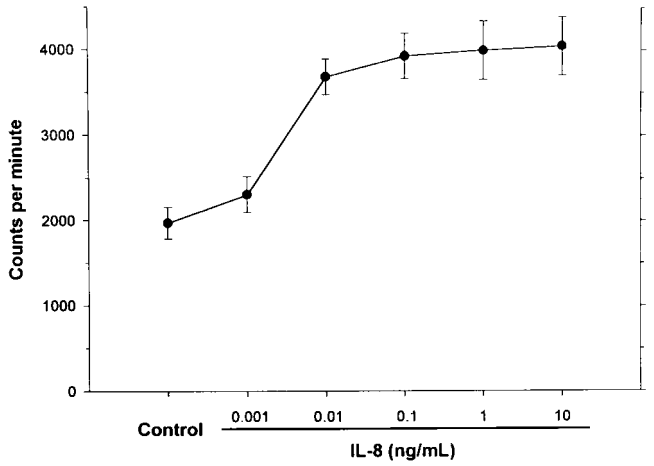

FIG. 1. Dose-dependent stimulation of proliferation of human endometrial stromal cells in culture by recombinant human IL-8 assayed by thymidine uptake method. Preconfluent cells were placed in serum-free medium for 24 h and then were incubated for 24 h in culture medium containing various concentrations of IL-8 (0.001–10 ng/mL). Data are mean ± SEM; $P < 0.01$ between control and concentrations above 0.01 ng/mL.

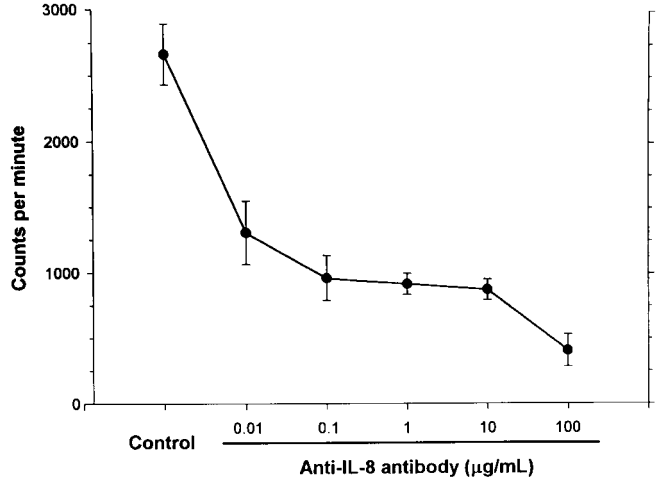

FIG. 2. Dose-dependent inhibition of proliferation of human endometrial stromal cells in culture by mouse antihuman IL-8 neutralizing antibody assessed by thymidine uptake method. Cells were placed in serum-free medium for 24 h before they were incubated for 24 h in culture medium containing various concentrations of anti-IL-8 antibody (0.01–100 μg/mL). Data are mean ± SEM; $P < 0.01$ between control and concentrations above 0.1 μg/mL.

findings with longer (48 and 72 h) treatments. Similar experiments performed with the MTT colorimetric assay for cellular proliferation also revealed the same results.

Because endometrial stromal cells in culture produce IL-8 constitutively (in the range of 0.1–1 ng/mL) (9), we also investigated whether the neutralization of IL-8 produced by these cells would affect their proliferation. Endometrial stromal cells in serum-free medium for 24 h were treated with various concentrations of mouse antihuman IL-8 neutralizing antibody (0.01–100 μg/mL) or with nonspecific mouse IgG (control) for 24 h, and [^{3}H]thymidine uptake was measured. There was a concentration-dependent inhibition of cell proliferation with anti-IL-8 neutralizing antibody (to 30% of the control at 1 μg/mL; $P < 0.01$ between control and concentrations above 0.1 μg/mL) (Fig. 2). Similar experiments performed with the MTT colorimetric assay for cellular proliferation revealed the same results.

Effect of IL-8 antisense oligonucleotides on IL-8 production and cell proliferation of endometrial cells

To determine the effect of IL-8 in regulating endometrial stromal cell proliferation, specific antisense and scrambled oligonucleotides were added to endometrial stromal cells in culture. We first confirmed the entry of both sense and scrambled oligonucleotides into endometrial stromal cells. Cells were treated with biotinylated oligonucleotides, and their intracytoplasmic localization was confirmed by immunohistochemistry (data not shown). We then determined the optimum conditions for the effective inhibition of endometrial stromal cell IL-8 production by antisense oligonucleotides. Confluent cells were treated with IL-8 antisense oligonucleotides (0–50 μM) in the presence of various FBS concentrations (0–10%; vol/vol). The maximal effect of antisense oligonucleotides was observed in the presence of 1% FBS. Because oligonucleotides at a concentration of 50 μM had a cytotoxic effect, concentrations between 1–20 μM were used in the following experiments. We also evaluated the optimum time interval between oligonucleotide treatment and the addition of FBS (1%; vol/vol) to cultured cells (0–6 h). A 2-h interval was the most effective time interval and was used in the following experiments.

To evaluate the effect of IL-8 antisense oligonucleotides on the IL-8 production by endometrial stromal cells in culture, confluent cells were treated with serum-free media for 24 h and were then subjected to different concentrations of IL-8 antisense and scrambled oligonucleotides (1, 5, and 20 μM). FBS (1%; vol/vol) was added 2 h later. IL-8 antisense oligonucleotides at these concentrations caused a decrease in IL-8 protein production ($P < 0.01$) (Fig. 3).

We then investigated the effect of IL-8 antisense oligonucleotides on endometrial stromal cell proliferation. Preconfluent cells were incubated in serum-free medium for 24 h, then were treated with IL-8 antisense or scrambled oligonucleotides (1, 5, or 10 μM). FBS (1%; vol/vol) was added 2 h later, and cell proliferation was assessed 24 h later. IL-8 antisense oligonucleotides caused a dose-dependent decrease in endometrial stromal cell proliferation at 1 and 5 μM concentrations ($P < 0.001$) (Fig. 4).

To confirm the specificity of the antiproliferative effect of IL-8 antisense oligonucleotides, endometrial stromal cells in culture were treated with IL-8 antisense oligonucleotides at 1 μM concentration in the presence or absence of recombinant IL-8 (1 ng/mL). IL-8 antisense oligonucleotides caused a decrease in the proliferation of endometrial stromal cells in culture ($P < 0.01$), confirming previous findings. The addition of IL-8 reversed the inhibitory effect of the IL-8 antisense oligonucleotides on cell proliferation (Fig. 5).

Discussion

Cytokines and growth factors are proteins that are produced locally in a wide variety of tissues and work by paracrine or autocrine mechanisms. Although these peptides are

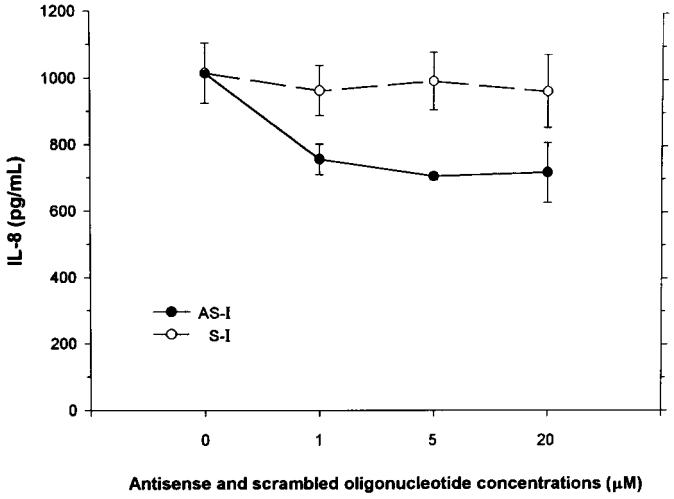

FIG. 3. Dose-dependent inhibition of IL-8 production by endometrial stromal cells in culture by IL-8 antisense oligodeoxynucleotides. Confluent cells were placed in serum-free medium for 24 h and were incubated for 2 h with culture medium containing various concentrations of IL-8 antisense (AS-I) or nonsense [scrambled (S-I)] oligonucleotides (0, 1, 5, 20 μM). After that, 1% FBS was added. Culture supernatants were collected 24 h later and IL-8 produced by cells was measured by ELISA. Data are mean ± SEM; $P < 0.01$ between AS-I and S-I (control) at 1 and 5 μM and $P < 0.05$ at 20 μM.

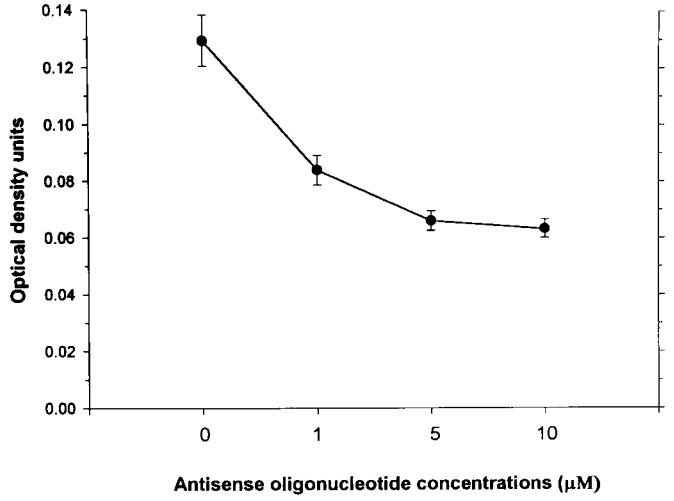

FIG. 4. Dose-dependent inhibition of endometrial stromal cell proliferation in culture by IL-8 antisense oligodeoxynucleotides. Confluent cells were placed in serum-free medium for 24 h and were incubated for 2 h with culture medium containing various concentrations of IL-8 antisense (AS-II) oligonucleotide (0, 1, 5, 20 μM). Next, 1% FBS was added. Cell proliferation was assessed by MTT colorimetric method. Data are mean ± SEM; $P < 0.001$ between control and treatments.

named after their originally observed biological action, they are generally involved in a wide range of other actions including stimulation of cell growth, inhibition of cellular proliferation, and alterations of cell functions. IL-8 is no exception to this. In addition to its chemotactic and activating properties for granulocytes and potent angiogenicity, IL-8 has been recently found to stimulate proliferation of various other cells. It acts not only as a mitogen but also as a chemoattractant in a wide range of responsive target cells such

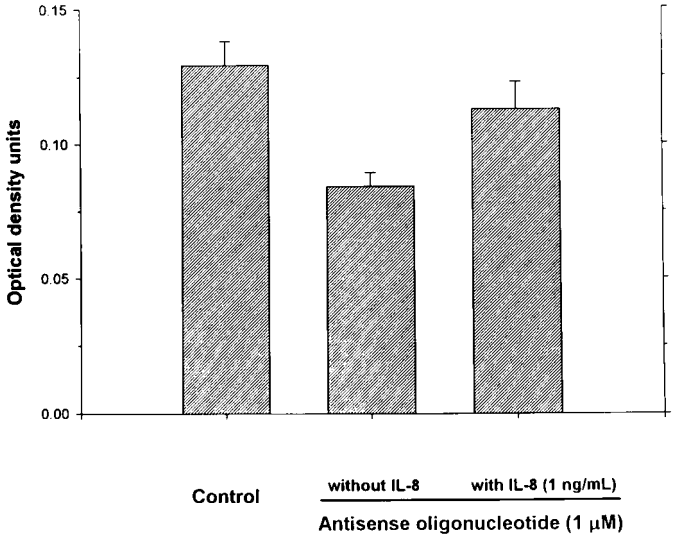

FIG. 5. Effect of recombinant IL-8 on IL-8 antisense oligonucleotide-induced inhibition of proliferation of endometrial stromal cells in culture. Confluent cells were placed in serum-free medium for 24 h and were incubated for 2 h with culture medium containing IL-8 antisense oligonucleotide (AS-II; 1 μM) in presence or absence of IL-8 (1 ng/mL). After that, 1% FBS was added. Cell proliferation was assessed by MTT colorimetric method. Data are mean ± SEM. IL-8 antisense oligonucleotide induced a decrease in proliferation of endometrial stromal cells in culture ($P < 0.01$). Addition of IL-8 reversed proliferation inhibitory effect ofIL-8 antisense oligonucleotide.

as endothelial cells (3), epidermal cells (10), melanoma cells (11), and smooth muscle cells (12).

We and others have recently shown that IL-8 is elevated in the peritoneal fluid of women with endometriosis (7, 16). We found that IL-8 is not only elevated in the peritoneal fluid of women with endometriosis compared with those without endometriosis, but the levels also correlate with the severity of the disease. In the peritoneal cavity, several tissues may account for the increased levels of IL-8. In addition to mesothelial cells (7), peritoneal macrophages, follicular fluid (17), and endometrial cells (9) themselves are potential sources of this cytokine. Thus, we hypothesize that elevated levels of peritoneal fluid IL-8 may play a role in the growth and maintenance of ectopic endometrial tissue not only by stimulating leukocytes to secrete growth factors and cytokines, but also by directly stimulating endometrial cell proliferation.

The first question that we asked was whether IL-8 might directly stimulate growth and proliferation of endometrial cells. Our data clearly demonstrate a direct proliferative effect of IL-8 on endometrial stromal cells as assessed by increased tritiated thymidine uptake. The mean concentration of IL-8 in the peritoneal fluid of women with moderate or severe endometriosis is 0.5 ng/mL (7). In the present study, we observed significant increases in the *in vitro* endometrial cell proliferation rate at IL-8 concentrations compatible with this *in vivo* concentration. Because we have previously shown that endometrial cells themselves produce IL-8 (9), we used neutralizing antihuman IL-8 antibody to eliminate the possible autocrine effect of IL-8 on endometrial cells. We demonstrated a concentration-dependent inhibitory effect of anti-IL-8 antibody on endometrial stromal cell proliferation. Such

a finding suggests that an elevation in peritoneal fluid IL-8 might create an environment favorable for the implantation and/or growth of ectopic menstrual debris. On the other hand, we have used two indirect methods to assess the cell proliferation, each with some limitations. Namely, small increases in thymidine uptake may occur due to causes other than cell proliferation, and false negative results in MTT assay may occur due to induction of certain enzymes in culture, independent of cell proliferation.

To further evaluate the effect of IL-8 in endometrial stromal cell proliferation, we used two different antisense oligonucleotides and showed that in addition to decreasing IL-8 production by the endometrial stromal cells, these oligonucleotides also decreased the cell proliferation. The strongest support for this claim comes from the finding that the inhibition of cell proliferation is reversed by the addition of recombinant IL-8 to the cell culture. These findings not only confirm the proliferative effect of IL-8 in endometrial cells, but also suggest antisense oligonucleotides worthy of future consideration as possible therapeutic agents in severe endometriosis.

Progestins (*e.g*. in oral contraceptives) are the most commonly used medications in the long-term management of endometriosis. We and others have investigated the effect of progestins on IL-8 expression in endometrial stromal cells. In our study progesterone (10^{-7} M) increased the IL-8 messenger RNA levels but did not affect the amount of IL-8 protein produced by these cells (18). In the study by Kelly *et al*. (19) progestins at higher concentrations (10^{-6} M) reduced the IL-8 production by endometrial explants, a finding that supports our hypothesis.

In conclusion, in this study we examined the possible role of IL-8 in the growth of endometrial cells. The results of our *in vitro* endometrial stromal cell proliferation experiments demonstrate a direct effect of IL-8 on endometrial cell proliferation. We propose that IL-8 may act as an autocrine/paracrine growth factor in the endometrium, and speculate that it may also play a role in the development or propagation of endometriosis.

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