Human endometrial interleukin-6 (IL-6): in vivo messenger ribonucleic acid expression, in vitro protein production, and stimulation thereof by IL-1β*

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Objective: To investigate human endometrial interleukin-6 (IL-6) expression and effects thereon by IL-1β.

Design: Prospective.

Setting: Academic medical center.

Patient(s): Endometrial biopsy specimens from normal volunteers (n = 20) at four specific menstrual stages were used for in vivo study. Endometrial specimens for in vitro study were obtained from patients (n = 19) undergoing gynecologic surgery.

Intervention(s): Time and dose-response treatment of endometria with IL-1β in tissue culture.

Main Outcome Measure(s): In vivo IL-6 messenger RNA expression by Northern analysis and in vitro endometrial IL-6 protein production by assay of the conditioned media.

Result(s): Midsecretory and late secretory phase endometria expressed more IL-6 messenger RNA than late proliferative phase endometria in vivo. Similarly in vitro, in pg/mg endometrium per hour secretory endometria IL-6 protein production, 25.7 ± 7.1 (mean ± SEM), exceeded that of proliferative endometria, 4.7 ± 1.0. With IL-1β treatment, secretory endometria IL-6 protein production exceeded that of proliferative endometria. Interleukin-1β stimulated endometrial IL-6 protein production in time- and dose-dependent manners.

Conclusion(s): Human endometrial IL-6 expression varies with the menstrual cycle, occurs more highly in secretory endometria, and in vitro is stimulated by interleukin-1β. Human endometrial IL-6 may therefore mediate some actions of IL-1β involving the endometrium and trophoblast.

Key Words: Endometrium, interleukin-1, interleukin-6

Interleukin-6 (IL-6), a 26-kd glycoprotein, is a pleiotropic cytokine produced by several different types of immune and nonimmune cells, including monocytes, lymphocytes, fibroblasts, and endothelial cells (1). Evidence for IL-6 acting physiologically in several tissues has been reported (2), and studies of IL-6 structure, function, and expression suggest that IL-6 acts at the endometrial-trophoblast interface. Regarding structure, IL-6 is a member of a family of structurally similar cytokines that share similar receptor mechanisms, including the signal transduction protein, gp 130 (3). A key member of this cytokine family, leukemia inhibitory factor, was found to inhibit the differentiation of embryonic stem cells, and later maternal expression of leukemia inhibitory factor was shown to be necessary for implantation in the murine model (3). Regarding IL-6 actions, in association with the IL-6 soluble receptor, IL-6 inhibits embryonic stem cell differentiation as does leukemia inhibitory factor (4). In addition, IL-6 acts through the IL-6 receptor to stimulate trophoblast hCG production (5). Regarding in vivo expression, however, IL-6 messenger RNA (mRNA) has been detected in the human endometrium only by reverse
transcriptase-polymerase chain reaction (PCR) (6), a highly sensitive technique prone to contamination artifact. Interleukin-6 protein immunolocalizes to the endometrial epithelium (6), but no studies have demonstrated across the menstrual cycle quantitative differences in IL-6 mRNA or protein expression by endometrial tissue, in vivo or in vitro.

Interleukin-6 is a mediator of IL-1 actions in several settings (7), and preliminary evidence suggests that IL-6 may have a similar role at the endometrial-trophoblast interface. In vitro, IL-1 stimulates IL-6 production by isolated endometrial epithelial cells (8), and IL-1 stimulates IL-6 and leukemia inhibitory factor production by isolated endometrial stromal cells (9, 10). Interleukin-1β stimulates IL-6 production by placental villous core mesenchymal cells (11), and IL-1 stimulates trophoblast hCG production through stimulating production of IL-6 (5). In addition, the recent finding that IL-1 is necessary for implantation in the murine model (12) emphasizes the need to understand the mechanisms by which IL-1 may act on the embryo or endometrium.

We suspect that, like leukemia inhibitory factor, IL-6 is supportive of implantation and does so in part by mediating IL-1 actions. As such, we hypothesize that human endometrium expresses IL-6, that this expression increases with the transition from proliferative to secretory status, and that this expression is, in part, under the regulation of locally produced IL-1. Consequently, we began investigating this hypothesis by quantitatively studying human endometrial IL-6 mRNA expression in vivo and human endometrial IL-6 protein production, baseline and in response to IL-1β, in vitro.

**MATERIALS AND METHODS**

**Specimens and Experimental Design**

Human endometria were obtained using two protocols approved by the Medical College of Virginia-Virginia Commonwealth University Committee for the Conduct of Human Research. Specimens for RNA analysis were obtained from consenting volunteers. Inclusion required age of 18 to 40 years, regular menstrual cycles of length 25 to 32 days, no history of infertility or endometrial pathology, no use of reproductive hormone medications, and a normal pelvic examination immediately before participation. Endometrial Pipelle (Unimar, Wilton, CT) biopsy was performed once on each participant at one of four times in the menstrual cycle: late proliferative, on cycle day 11 or 12; early secretory, on the 3rd or 4th day after the day of the LH surge as detected using a urinary LH detection kit (Clear Plan Easy; Whitehall Laboratories, Madison, NJ); midsecretory, on the 7th or 8th day after the day of the LH surge; and late secretory, on the 11th or 12th day after the day of the LH surge. Blood for serum P was obtained on the 7th to 8th day after the day of LH surge during the menstrual cycle in which the endometrial biopsy was performed. Serum P > 3 ng/mL was considered ovulatory. Specimens for RNA studies were snap frozen in liquid nitrogen and stored at −80°C. Portions of all specimens, including those specimens for RNA studies and those obtained as below for protein studies, were fixed in Formalin and processed for routine histology and menstrual dating using Noyes’ criteria. For the RNA studies, specimen collection continued until five specimens for each group had been acquired from ovulatory cycles as confirmed by serum P and histology.

For in vitro studies, endometria were obtained from patients with consent by Pipelle (Unimar) biopsy. Specimens were obtained from patients who were 18 to 40 years old, not on reproductive hormone medication, having regular menses of cycle length 25 to 32 days, and who were undergoing under anesthesia just before beginning gynecologic surgery unrelated to and not otherwise requiring sampling of the endometrium, such as tubal ligation, tubal anastomosis, and treatment of vulvar conditions. Immediately after sampling, the endometrium was placed in minimal essential medium alpha (MEMα) at 37°C and transported to the tissue culture laboratory where experiments were immediately begun. For the IL-6 protein studies, endometria were cultured in short-term, 18-hour tissue culture using slight modifications of methods previously published (13, 14). Briefly, freshly obtained endometrium was rinsed free of blood and clot by passing the specimen three times through fresh MEMα. It was then cut into 1 × 2 × 2 mm fragments and divided into 75- to 85-mg portions that were placed into 9.6-cm² six-well culture plates and cultured for 1 hour before continuing with the experiments. In all experiments, culture conditions were 6 mL of MEMα media with 1% antibiotic:antimycotic (GIBCO Laboratories, Grand Island, NY) per well at 5% CO₂-air atmosphere at 37°C with or without IL-1β treatments.

In time-course experiments, 75- to 85-mg portions of endometria were cultured with 0 or 10 ng/mL IL-1β and conditioned media were sampled at 1, 3, 6, 12, and 18 hours. In dose-response experiments, conditioned media were sampled at 18 hours after culturing the endometrial portions with IL-1β at 0, 0.01, 0.1, 1, and 10 ng/mL concentrations (5.8 × 10⁻¹³ to 5.8 × 10⁻¹⁰ molar IL-1β). Because two to six 75- to 85-mg portions of endometrium were obtained per endometrial specimen, the in vitro data collectively represent findings from endometria from 19 patients.

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whose endometrial datings were confirmed by histology. For these experiments, the sample size, n, ranged from four to seven where n represents the number of endometrial portions from different patients studied at each IL-1β dosage in dose-response studies or at each control or treatment group in time-course studies. Collectively then, 39 endometrial specimens were studied, but difficulty in obtaining more specimens prevented us from studying both IL-6 mRNA and protein expression in vitro, but led us to focus on the physiologic endpoint of IL-6 protein expression in vitro.

**Northern Analysis**

Total RNA was extracted from endometria by the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (15). Northern analysis was performed by fractionating 30 μg of total RNA per sample in a denaturing formaldehyde-1% agarose gel by electrophoresis. The RNA then was transferred to a charged nylon membrane (Nitran; Schlecter and Schuell, Keene, NH) and fixed by baking. The membranes were then hybridized with 1 × 10⁶ cpm/mL [³²P]deoxy-CTP-labeled IL-6 complementary DNA (cDNA) probe for 18 to 24 hours at 50°C, washed to high stringency, and quantitatively scanned for radioactive signal using a Betascope 603 blot analyzer (Betagen Corporation, Waltham, MA). The membrane then was exposed to Kodak XAR radiographic film (Eastman Kodak, Rochester, NY) with intensifying screens at −80°C for 96 hours. The membrane then was stripped, exposed to film to confirm radioactive probe removal, rehybridized to the 18S ribosomal RNA (rRNA) probe, and quantitatively scanned for signal. The signal obtained by quantitative scanning of the membrane for IL-6 mRNA was normalized to that obtained for 18S rRNA and expressed as the ratio of IL-6 to 18S RNA signals. The probes used in this study were a human IL-6 specific 1.16 kb cDNA insert from pCSF309 (ATCC, Rockville, MD) and a 0.75-kb human 18S rRNA cDNA insert from pN291III (ATCC). The IL-6 probe was radiolabeled with [³²P]deoxy-CTP, using random primer oligolabeling (Pharmacia LKB Bio-technologies, Inc., Piscataway, NJ), and the 18S rRNA probe was endabeled with [³²P]deoxy-CTP using terminal deoxynucleotidyl transferase (GIBCO).

**Assays**

Interleukin-6 protein concentrations in conditioned media were determined using an ELISA that has been described previously and was developed from commercially available reagents including mouse monoclonal anti-human IL-6 (Biosource International, Camarillo CA), recombinant human IL-6 (R and D Systems, Minneapolis, MN), goat polyclonal anti-human IL-6 (R and D Systems), and peroxidase-conjugated mouse anti-goat IgG (11). The assay range and sensitivity was 0.2 to 60 ng/mL. Samples that were diluted serially when compared with the standard curve showed parallelism validating the assay. Within-assay coefficients of variation at 1 and 20 ng/mL were 12% and 14%. Interassay coefficients of variation at 1 and 20 ng/mL were 14% and 16%. Time-course data were expressed as pg IL-6/mg endometrium, and dose-response data were expressed as picograms IL-6/mg endometrium per hour. Serum P was determined using a commercially available RIA for P (Diagnostic Products Corporation, Los Angeles, CA).

**Statistical Analyses**

Statistical analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA), which applies tests for equal variance and distribution normality to data analyzed parametrically. Data not meeting parametric test assumptions of equal variances and normal distributions therefore were analyzed with nonparametric tests. Because of unequal variances, two group comparisons were made with the Mann-Whitney rank sum test. Multigroup comparisons were made with analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test or with ANOVA by ranks with Dunn’s post hoc test as appropriate. P values < 0.05 were considered significant.

**RESULTS**

By Northern analysis, a single IL-6 mRNA band of approximately 1.3 kb was detected in vivo in 20 of 20 endometria. Expression was higher in the secretory endometria, reaching levels significantly higher in both the midsecretory and late secretory phases as compared with that of the late proliferative phase (P<0.05) (Fig. 1). Consistent with the mRNA findings, IL-6 protein was produced by both proliferative and secretory endometria and more so in the latter (Fig. 2). Over 18 hours incubation, production rates were 4.7 ± 1.0 and 25.7 ± 7.1 pg/mg endometrium per hour (mean ± SEM) for proliferative and secretory endometria, respectively (P<0.01).

Interleukin-1β stimulated endometrial IL-6 protein production in time- (P<0.01) and dose-dependent manners (P<0.01). Using proliferative endometria, IL-6 protein production from specimens treated with IL-1β (10 ng/mL) by 12 hours of incubation exceeded IL-6 protein production from untreated specimens (P<0.01), and mean IL-6 protein levels from treated specimens were 5.6-fold those of untreated specimens by 18 hours incubation (P...
< 0.01) (Fig. 3). Regarding the effect of dosage, treatment with IL-1β at 0 to 10 ng/mL increased proliferative endometria IL-6 protein production over 18 hours in a dose-responsive fashion with significance occurring at doses ≥ 0.1 ng/mL IL-1β (P < 0.05) (Fig. 3). Using secretory endometria, IL-6 protein production by specimens treated with IL-1β (10 ng/mL) by 6 hours of incubation exceeded that of untreated specimens (P < 0.05), and mean IL-6 protein levels from treated specimens were fourfold those of untreated specimens by 18 hours incubation (P < 0.01) (Fig. 4). Regarding the effect of dosage, treatment with IL-1β at 0 to 10 ng/mL increased secretory endometria IL-6 protein production over 18 hours in a dose-responsive fashion with significance occurring at doses ≥ 1.0 ng/mL IL-1β (P < 0.05) (Fig. 4). In addition, just as secretory endometria IL-6 protein production exceeded that of proliferative endometria in the absence of IL-1β treatments, in the presence of IL-1β (10 ng/mL) treatments, the secretory endometria IL-6 protein production rate, 104.5 ± 24.6, exceeded that of proliferative endometria, 26.5 ± 3.8 (P < 0.01) (Fig. 2).

**DISCUSSION**

We hypothesized that human endometrium expresses IL-6, that this expression increases with secretory development of the endometrium, and that this expression occurs partly under IL-1 regulation. The results presented in this report support this hypothesis. Interleukin-6 mRNA and IL-6 protein were expressed by both proliferative and secretory endometria. Compared with proliferative endometria, these expressions were greater in secretory endometria, and both proliferative and secretory endometrial IL-6 protein productions in vitro were stimulated by IL-1β in time- and dose-dependent manners. These findings of IL-1β stimulating endometrial IL-6 production are supported by previous work. Both IL-1β mRNA expression and IL-1 type 1 receptor expression in vivo are increased in secretory endometria as compared with proliferative endometria (16, 17), findings that are temporally consistent with the hypothesis that increased secretory endometria IL-6 production results from increased locally produced IL-1β acting through its receptor. In addition, our results are further consistent with IL-1β acting physiologically through a classic ligand-receptor interaction. Our results demonstrated significantly increased endometrial IL-6 production in response to $5.8 \times 10^{-12}$ molar IL-1β (proliferative endometria) or $5.8 \times 10^{-11}$ molar IL-1β (secretory endometria). The disassociation constant (Kd) of the IL-1 receptor is reported to vary from 2 to $6 \times 10^{-10}$ molar (18), and Tabibzadeh et al. (19) have demonstrated endometrial gland IL-1 receptor-specific
binding with a Kd of 1.9 to 8.2 $\times 10^{-11}$ molar. Collectively, our results thus show increased endometrial IL-6 production at IL-1β concentrations less than or equal to the IL-1 receptor Kd, findings consistent with IL-1β acting physiologically through a classic ligand-receptor interaction and with IL-1β having a similar in vivo role.

Our findings represent the first work demonstrating in vivo endometrial IL-6 mRNA expression using the well-established method of Northern analy-

Figure 3 Effects of IL-1β on proliferative endometria. (A), Time-course study of endometria IL-6 protein production (mean ± SEM) during 18 hour cultures. By 12 hours of incubation, production in response to IL-1β (10 ng/mL) exceeded that of control endometria (**P < 0.01, by Mann-Whitney rank sum test) and continued through 18 hours. (B), Dose-response study of IL-6 protein production rates (mean ± SEM) in response to 0 to 10 ng/mL IL-1β by endometria in 18-hour cultures. A dose-dependent increase in IL-6 protein production is seen with a significant increase occurring by the 0.1 ng/mL IL-1β dose group (*P < 0.05, as compared with the 0 ng/mL IL-1β dose group; *P < 0.05, as compared with the 0, 0.01, and 0.1 ng/mL IL-1β dose groups; all by ANOVA with Student-Newman-Keuls post hoc test).

Figure 4 Effects of IL-1β on secretory endometria. (A), Time-course study of endometria IL-6 protein production (mean ± SEM) during 18-hour cultures. By 6 hours incubation, production in response to IL-1β (10 ng/mL) exceeded that of control endometria (**P < 0.01) and endometrial IL-6 production (mean ± SEM) during 18-hour cultures. By 6 hours incubation, production in response to IL-1β (10 ng/mL) exceeded that of control endometria (**P < 0.01). (B), Dose-response study of endometria IL-6 protein production rates (mean ± SEM) in response to 0 to 10 ng/mL IL-1β in 18-hour cultures. A dose-dependent increase in IL-6 protein production is seen with a significant increase occurring by the 1 ng/mL IL-1β dose (*P < 0.05, for the 1 and 10 ng/mL dose groups as compared with the 0 ng/mL dose group, by ANOVA by ranks with Dunn's post hoc test).
sis, and this is the first study demonstrating quantitatively both IL-6 mRNA and IL-6 protein expression by the human endometrium and that these expressions are increased with secretory endometrial development. Prior studies involving human endometrial IL-6 expression have used methods of reverse transcriptase-PCR, immunohistochemistry, and isolated endometrial stromal or epithelial cell culture (6, 20). The relevance of these prior studies are strengthened markedly by our report of in vivo human endometrial IL-6 mRNA expression using Northern analysis, a method much less sensitive and less prone to contamination artifact than highly sensitive reverse transcriptase-PCR. The Northern analysis findings also suggest that cells within the endometrium produce IL-6 in vivo and that endometrial epithelial and stromal cells are not just sites of IL-6 immunolocalization or just in vitro IL-6 production, findings previously reported as we have noted. Confirmation of sites of in vivo IL-6 production within the endometrium will, however, require in situ hybridization studies.

Our results demonstrated functionally responsive tissue, given the time- and dose-responsive effects of IL-1 and the consistent findings in secretory endometria of both increased IL-6 mRNA expression in vivo and increased IL-6 protein production in vitro. And, because endometrial behavior reflects different cell types and their interactions, we chose to study endometrial tissue function as a whole, both in vivo and in vitro. Our results of increased secretory as compared with proliferative endometrial IL-6 expression do, however, differ from those of Laird et al. (8) in which in vitro isolated endometrial epithelial cell IL-6 protein production was greater by proliferative as compared with secretory phase cells. We do not believe these differences in IL-6 production by an isolated endometrial cell type as compared with that by endometrial explants to be discrepant. It clearly is recognized that endometrial functions change across the menstrual cycle and that endometrial function partly reflects interactions between the several cell types. As an example in support of the explant (and thus interacting multiple cell type) approach, it has been shown that P suppression of endometrial epithelial cell metalloproteinase is dependent upon P-induced production of endometrial stromal cell transforming growth factor-β (21). In addition, other in vitro endometrial explant function studies have been reported (14, 15).

Previous evidence suggests that endometrial IL-6 production is regulated by reproductive steroids and cytokines. Estradiol-17β (E2) inhibited IL-6 production, whereas IL-1α, IL-1β, tumor necrosis factor, and interferon-γ stimulated IL-6 production by freshly explanted human endometrial stromal cells (22). Estradiol and P also modulated endometrial epithelial cell IL-6 production (20), and this epithelial cell IL-6 production was stimulated by IL-1 (8). The well-recognized pattern of concentrations of serum E2 and P during the menstrual cycle and the endometrial responsiveness to these steroids suggest that these steroids are ultimately responsible for regulating endometrial IL-6 production. Our findings of increased secretory endometrial IL-6 expression support this concept. However, because of the recent demonstration that actions of IL-1 are necessary for implantation (12), we studied further the relationship between IL-1β and endometrial IL-6 production. Still, our findings of endometrial IL-6 protein production in response to IL-1β support IL-1β as being another regulatory factor for endometrial IL-6 production.

Evidence for IL-6 acting physiologically in several tissues has been reported (2), and our findings add human endometrium as another such tissue. Potential functions of IL-6 may involve the endometrium primarily and/or endometrial-embryo interactions, regardless of whether IL-6 is mediating actions of IL-1 or acting primarily. That IL-6 may have functions in the endometrium itself is suggested by IL-6 having stimulatory effects on proliferation of cells from other tissues (1), by IL-6 mRNA expression in proliferative endometrium as we report, and by its immunolocalization to stromal cells, the nonglandular component of the endometrium (6). That IL-6 may function in endometrial-trophoblast interactions is suggested by several findings, including the increased IL-6 expression in secretory as compared with proliferative endometria that we report here, the stimulation of trophoblast hCG production by complexes of IL-6 and IL-6 soluble receptor (23), and the expression of the IL-6 receptor by human blasto­cysts (24). In addition, our findings of IL-1β stimulation of endometrial IL-6 production and recent findings suggestive of early trophoblast production of IL-1β (25) raise the possibility that endometrial IL-6 production may be stimulated in response to IL-1β from either the endometrium or trophoblast itself.

In summary, we have demonstrated the expression of IL-6 mRNA in human proliferative and secretory endometria in vivo and that this expression is higher in secretory endometria. Also, we have demonstrated a similar pattern of endometrial IL-6 protein production in vitro and that endometrial IL-6 protein production is stimulated by IL-1β in time- and dose-dependent manners. Collectively, these findings suggest that IL-6 has physiologic functions in the endometrium where IL-6 production is partly regulated by IL-1β. In addition, these findings suggest that, in the endometrium and at the endome-
trial-trophoblast interface, endometrial IL-6 may mediate, in part, actions of locally produced IL-1β.

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