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Interleukin-8 in the Human Endometrium*

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ABSTRACT

We have previously shown that interleukin-8 (IL-8), a cytokine with neutrophil chemotactic/activating and T cell chemotactic activity, is produced by human endometrial stromal and glandular cells in culture. The present study investigated the temporal and spatial expression of IL-8 messenger ribonucleic acid (mRNA) and protein in the human endometrium. Endometrial tissue (n = 52) was obtained from human uteri after hysterectomy conducted for reasons other than endometrial disease or from endometrial biopsies. The day of the menstrual cycle was established from women's menstrual history and was confirmed by histology. Half of the tissues (n = 26) were snap-frozen in liquid nitrogen, cellular RNA was extracted, and Northern blots were hybridized with a riboprobe complementary to a specific sequence of IL-8 mRNA. The remaining tissues (n = 26) were processed for frozen sections, and immunohistochemistry was performed using mouse antihuman IL-8 antibody. Comparison of IL-8 mRNA levels throughout the menstrual cycle revealed that late secretory and early to midproliferative phase IL-8 expression was significantly greater than midcycle expression ($P < 0.02$). Analysis of the IL-8 immunohistochemistry revealed that IL-8 protein is found in the surface epithelium and glands throughout the menstrual cycle. There was no detectable immunoreactive IL-8 in the stromal cells. We conclude that IL-8 is produced in the human endometrium *in vivo*, and the variations of IL-8 mRNA throughout the menstrual cycle suggest that sex hormones may regulate its gene expression. We speculate that IL-8 may modulate the timely recruitment of neutrophils and lymphocytes into the endometrium. (*J Clin Endocrinol Metab* **83:** 1783–1787, 1998)

HUMAN endometrium is comprised of hormonally responsive glandular epithelium, stroma, and large numbers of bone marrow-derived cells (*i.e.* leukocytes) that become functional cellular components of this tissue in both health and disease. The number and type of leukocytes in the human endometrium vary in a predictable manner with the ovulatory cycle, suggesting that there is some measure of hormonal (endocrine) and local (paracrine) control of leukocyte migration to and/or replication in this tissue (1). In addition, it is known that the endometrium is a site of synthesis of multiple cytokines that could act to regulate the migration, replication, and/or function of these leukocytes.

The endometrium is involved in multiple unique functions. This tissue is responsive to sex steroid hormones, undergoes extraordinary growth in a cyclic manner, and is shed and regenerated nearly 450 times in the lifetime of women. Endometrium/decidua is capable of blastocyst implantation, immunological tolerance, regulation of trophoblast invasion, infectious agent control, and efficient disposal of blood and desquamated cellular debris with menstruation. To accomplish these many diverse functions, it seems reasonable to suspect that multiple mechanisms must be operative to recruit and to engage the functions of various leukocytes in a timely and specific manner. The validity of this deduction is supported by the large number of leukocytes of various types that normally are present in a predictable pattern in endometrial/decidual tissues. Much experimentation has been conducted to characterize and evaluate the function of leukocytes in endometrium and to define the contributions of these cells to reproductive function. For example, large numbers of neutrophils infiltrate the endometrium immediately before the onset of menstruation, and the number of uterine large granular lymphocytes increases strikingly in the endometrium after ovulation at the expected time of implantation and during early placentation (2).

One of the factors thought to play a role in the recruitment of leukocytes to endometrium is interleukin-8 (IL-8), a cytokine with neutrophil chemotactic/activating and T cell chemotactic activity both *in vivo* and *in vitro* (3, 4). It is synthesized as a 99-amino acid precursor and secreted as a 72-amino acid peptide after successive removal of amino-terminal residues. Its known actions include chemotaxis and activation of neutrophils, expression of surface adhesion molecules on neutrophils, angiogenesis (chemotaxis of endothelial cells) (5), and mitogenesis of epidermal (6), melanoma (7), and vascular smooth muscle cells (8). IL-8 is produced by a number of cell types, including peripheral blood monocytes (9), endothelial cells (10), fibroblasts (11), neutrophils (12), keratinocytes (11), synovial cells (13), and cells derived from human decidua and chorion laeve tissues (14). We have previously shown that IL-8 is produced by endometrial stromal and epithelial cells in culture and that this production is regulated by IL-1α and tumor necrosis factor-α (TNFα) (15). We and others have also shown that IL-8 levels are increased in the peritoneal fluid of women with endometriosis compared to those in healthy women (16, 17).

In the present study, we investigated menstrual cycle-dependent expression of IL-8 messenger ribonucleic acid (mRNA) and protein in the human endometrium.

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Materials and Methods

Collection of tissues

Endometrial tissue (n = 55) was obtained from women who underwent hysterectomy conducted for reasons other than endometrial disease or from endometrial biopsies. Written informed consent was obtained from each woman before surgery, using consent forms and protocols approved by the human investigation committee of Yale University. The day of the menstrual cycle was established from the woman's menstrual history and was confirmed by histology.

Northern analysis

The tissues used for RNA extraction (n = 26) were frozen in liquid nitrogen and stored at −80 C before isolation of RNA. Total RNA was prepared by the guanidium isothiocyanate-cesium chloride ultracentrifugation method of Chirgwin *et al*. (18). Total RNA was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N^+ membrane (Amersham, Arlington Heights, IL), and cross-linked to the membrane by the use of UV light.

The sequence of the proximal 3′-untranslated region of the human IL-8 complementary DNA was obtained from GenBank (accession no. Y00787). Two complementary oligonucleotides were synthesized by the Keck Biotechnology Resource Laboratory at Yale University. The strands were annealed to give double-stranded DNA. A 70-bp region was cloned into pGEM plasmid. Plasmids containing the sequence from the 3′-untranslated regions of IL-8 were used for transformation. Plasmid DNA was isolated by alkaline lysis method, purified over Qiagen columns (Qiagen, Chatsworth, CA), and linearized with *Eco*RI or *Hin*dIII. Linearized plasmid DNA was ethanol precipitated and used as template for generation of riboprobes. Radiolabeled RNA probes were generated by *in vitro* transcription. Sense and antisense probes were generated with the appropriately digested template and the corresponding RNA polymerase (T7 or SP6) and labeled with [α-^{32}P]UTP. Hybridization was performed overnight at 60 C in buffer comprised of formamide (50%, vol/vol), 1 × standard saline citrate (SSC), 5 × Denhardt's buffer, transfer RNA (0.2%, wt/vol), and ^{32}P-labeled riboprobe (2×10^6 cpm/mL). The membrane was washed twice at 68 C for 30 min each time in 0.1 × SSC-SDS (0.1%, wt/vol). Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) was exposed overnight at −70 C.

The autoradiographic bands were quantified by using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Each IL-8 band was normalized using the value for the corresponding glyceraldehyde-3-phosphate dehydrogenase, thus correcting for any variation in the amount of mRNA applied to each lane.

Immunohistochemistry

Endometrial tissue samples (n = 26) were obtained and snap-frozen in OCT (Tissue Tek, Sakura, Torrance, CA). Serial 6- to 8-μm cryosections were placed on poly-L-lysine-coated glass microscope slides and fixed in acetone for 10 min at 4 C. Sections were rinsed twice in phosphate-buffered saline (PBS; pH: 7.4) for 5 min each time and in PBS with 0.1% BSA (PBS-BSA; 0.1%, wt/vol) for 5 min. Slides were then incubated with 2% blocking horse serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature in a humidified chamber. Excess serum was drained, and primary antibody (murine monoclonal antihuman IL-8, clone: Nap II, IgG1; 10 μg/mL; Bender Med Systems, Vienna, Austria) in PBS-BSA 0.1% was directly added to the sections and incubated overnight at 4 C in a humidified chamber. The specificity of this antibody was validated by incubation with an excess amount of recombinant IL-8 that eliminated the staining. For a negative control, nonspecific mouse IgG was used at same concentration. Endogenous peroxidase activity was quenched with 0.6% H_2O_2 in PBS (vol/vol) for 15 min. Sections were rinsed, then biotinylated horse antimouse antibody (1.5 mg/mL; Vector Laboratories) was added at a 1:200 dilution for 45 min at room temperature. The antigen-antibody complex was detected using an avidin-biotin-peroxidase kit (ABC, Vector Laboratories). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical Co., Milwaukee, WI)-hydrogen peroxide (0.5 mg in 0.03% H_2O_2 in PBS) was used as the chromogen, and sections were counterstained with hematoxylin, then mounted with Permount (Fisher Chemicals, Springfield, NJ).

Immunohistochemistry staining was scored in a semiquantitative fashion, incorporating both the intensity and the distribution of specific staining (19). The evaluations were recorded as percentages of positively stained target cells in each of four intensity categories, which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), and 3+ (intense). For each tissue, an HSCORE value was derived by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of the staining [HSCORE = ΣP_i (i + 1), where i is the intensity scores, and P_i is the corresponding percentage of the cells].

Statistical analysis

Because the levels of IL-8 mRNA and immunohistochemistry scores in the endometrium were not normally distributed, they were analyzed with nonparametric NAOVA by ranks (Kruskal-Wallis test). Statistical calculations were performed using Statistical Package for Social Sciences version 6.0 for Windows (SPSS, Chicago, IL).

Results

Menstrual cycle-dependent changes in IL-8 mRNA in the endometrium

Twenty-six endometrial samples were evaluated. These were grouped according to menstrual cycle phases: early proliferative [days 1–5 of the cycle; n = 5 (2 of them menstrual)], midproliferative (days 6–10; n = 4), late proliferative (days 11–14; n = 4), early secretory (days 15–18; n = 5), midsecretory (days 19–23; n = 3), and late secretory (days 24–28; n = 5). The levels of IL-8 mRNA were highest in samples obtained from late secretory and early to mid proliferative phases. The level was lowest in the late proliferative phase and gradually increased during the secretory phase. Overall, the mean IL-8 mRNA level (measured by laser densitometry) in the late secretory and early to midproliferative phase samples was significantly higher than the level observed in the middle of the cycle ($P < 0.02$; Fig. 1). In three

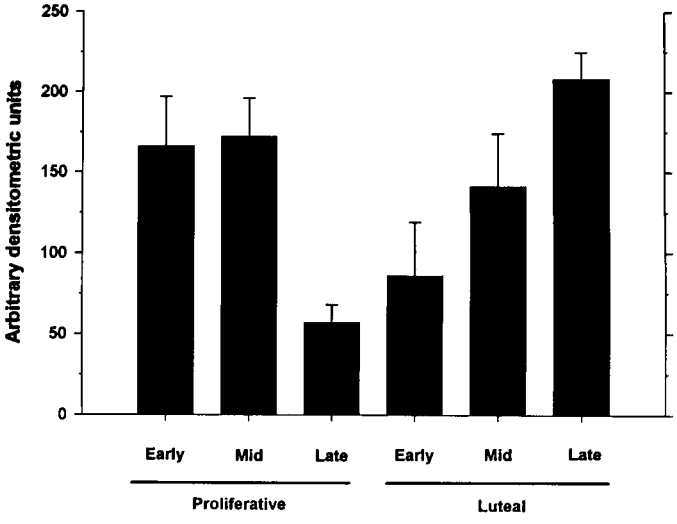

FIG. 1. Northern analysis of IL-8 mRNA in human endometrial tissue. Total RNA (20 μg/lane) isolated from endometrial tissues was evaluated. Samples are arranged according to menstrual cycle phase. *Graphic bars* represent the mean value of densitometric units for each group normalized to G3PDH. *, $P < 0.02$ for late secretory and early to midproliferative *vs.* midcycle.

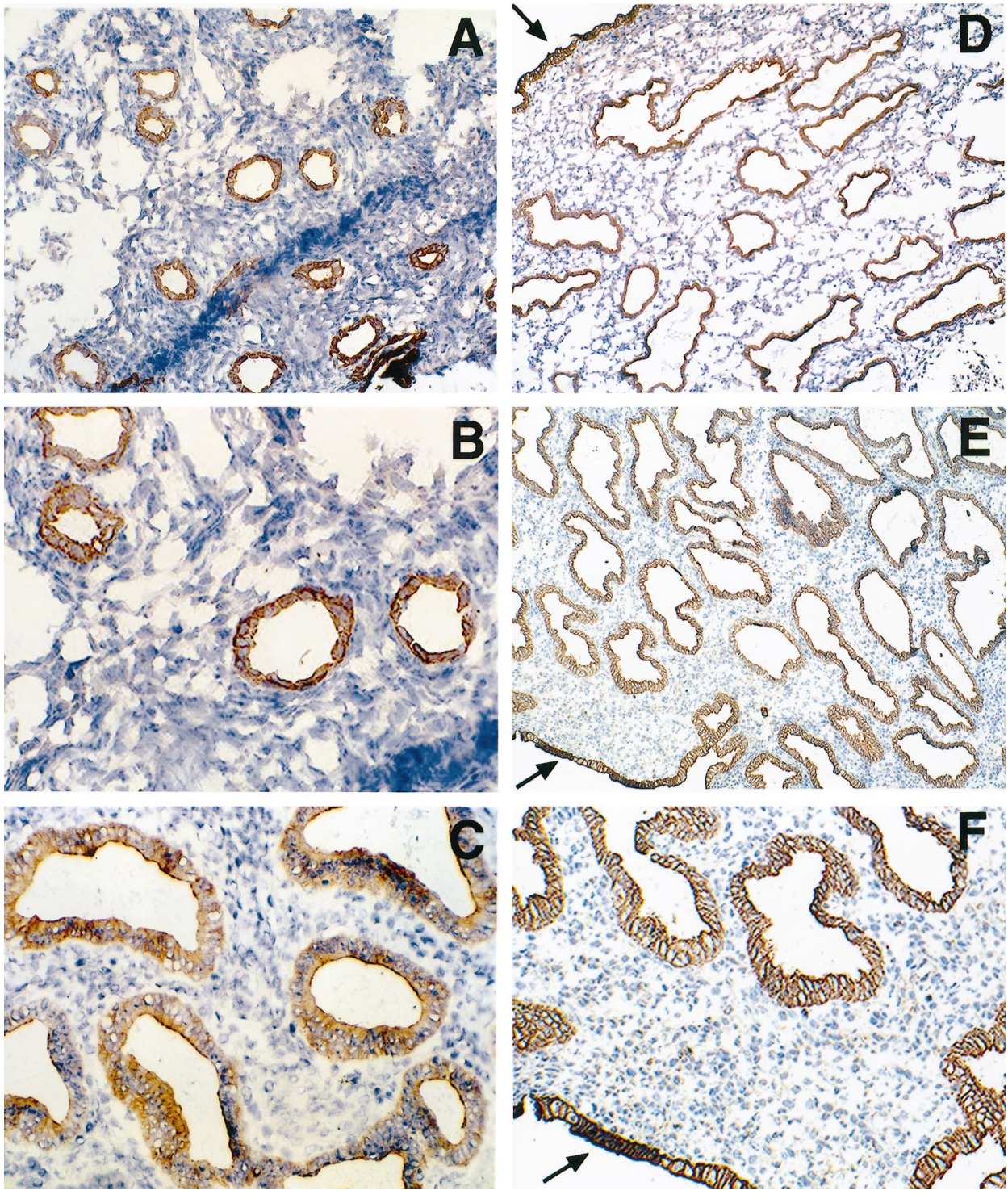

FIG. 2. Examples of endometrial tissue from proliferative (A–C) and secretory (D–F) phases of the menstrual cycle immunostained with murine monoclonal antihuman IL-8 antibody. B, High power view of A; F, high power view of E. Epithelial cells in glands and the surface epithelium are positively stained. The staining is membranous and cytoplasmic, the latter showing a punctate pattern. Glandular epithelial cells of the stratum functionalis show stronger staining than those of the stratum basalis. *Arrows* indicate the surface epithelium.

additional samples (two from proliferative and one from secretory phase), we evaluated IL-8 mRNA levels in freshly separated glands and stroma. IL-8 mRNA was detected in the glands but was undetectable in the stroma by Northern analysis (data not shown).

Endometrial immunohistochemistry for IL-8

Twenty-six endometrial samples different from those used in Northern analysis were evaluated by immunohistochemistry. The distribution according to menstrual cycle phase

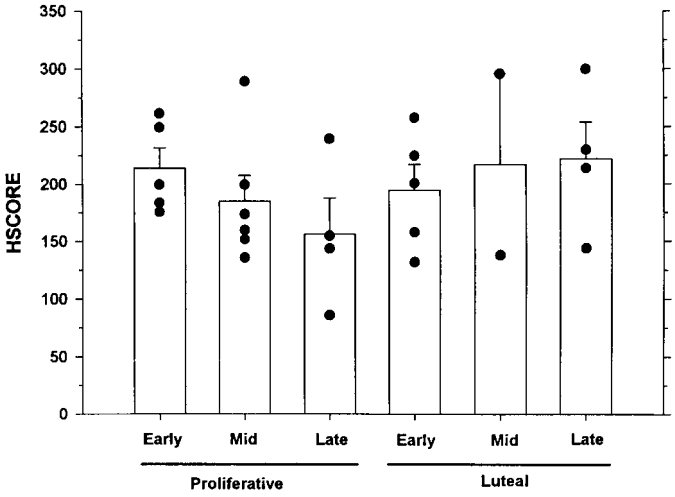

FIG. 3. The distribution of immunostaining intensity in endometrial glands with murine monoclonal antihuman IL-8 antibody according to the menstrual cycle phase. P = NS.

was early proliferative (n = 5), midproliferative (n = 6), late proliferative (n = 4), early secretory (n = 5), midsecretory (n = 2), and late secretory (n = 4). Immunohistochemistry of endometrial sections revealed staining of the glands and surface epithelium throughout the menstrual cycle. The staining was both membranous and cytoplasmic. The cytoplasmic staining showed a punctate pattern. The glandular epithelial cells of the stratum functionalis showed stronger staining than those of the stratum basalis. We did not observe any detectable staining in the stromal cells, although blood vessel walls were mildly positive. There was no staining in the negative controls (Fig. 2). When staining intensities were compared according to menstrual cycle phase, a distribution similar to the mRNA expression was observed, although the difference was not statistically significant (Fig. 3).

Discussion

Throughout the menstrual cycle, there are striking changes in the endometrium. The relative proportion of glandular epithelium and stromal tissue, the length and tortuosity of spiral arterioles, the expression of various proteins, and the number and type of leukocytes fluctuate in a predictable manner. Clearly, endocrine (estrogen and progesterone) and paracrine factors are involved in the modulation of these tissue-specific changes, and it is likely that chemotactic factors (*e.g.* IL-8, transforming growth factor-β, monocyte chemotactic protein-1, and growth-regulated gene-α) may be involved in the recruitment of the bone marrow-derived cells or their progenitors (*i.e.* neutrophils, uterine large granular lymphocytes, T cells, and monocytes) to the endometrium during menstrual cycle. The tissue concentration of IL-8 is important in determining whether neutrophils, lymphocytes, or both are recruited predominantly to a specific tissue. At low concentrations of IL-8, T lymphocytes are 2–10 times more sensitive to the chemotactic effect of this cytokine than are neutrophils (4). IL-8 is also mitogenic and angiogenic, two crucial events for the cyclic regenerating endometrium.

In this study, we found that IL-8 mRNA expression in the endometrium peaks during the late secretory and early to midproliferative phase. Neutrophils are known to invade the endometrium before menstruation, where these cells are probably involved in the degradation and digestion of sloughing endometrial tissue. The up-regulation of IL-8 expression in the late secretory phase may regulate this recruitment of neutrophils. We speculate that the IL-8 found in the endometrium during the early and midproliferative phase may be involved in the neovascularization of the growing endometrium, a known effect of IL-8 (5).

IL-8 protein was localized throughout the menstrual cycle to endometrial epithelial and glandular cells, but was absent in stromal cells. We have previously shown that endometrial glandular cells in culture produce constitutively large amounts of IL-8 and that this production is not regulated by IL-1α or TNFα. In the present study, immunohistochemistry results are consistent with our previous findings, revealing the endometrial glandular expression of IL-8 protein throughout the menstrual cycle. However, we could not detect IL-8 expression in endometrial stromal cells by immunohistochemistry, although we had previously shown that endometrial stromal cells in culture produce low amounts of IL-8, which is up-regulated to a significant extent by IL-1α or TNFα. As the bioavailability of most proteins is determined by a balance between their synthesis and degradation, we speculate that the rapid degradation of IL-8 in the stroma may be one of the reasons for the absence of IL-8 protein in these cells. Aminopeptidase N is a cell surface metalloprotease that degrades and inactivates IL-8 (20). In the human endometrium, the expression of aminopeptidase N is localized to the stroma and is not found in the glands (21). This would also explain why despite the high levels of IL-8 in endometrial glandular cells, the endometrium is not continuously invaded by granulocytes. IL-8 produced by the glandular cells may be degraded by the stroma; thus, its concentration around the vessels may not reach the levels necessary for granulocyte chemoattraction. Our findings are in contrast to a preliminary report by Critchley *et al.* (22). In their study, IL-8 immunostaining in the endometrium was limited to smooth muscle layer of arterioles. This finding may be due to the lack of sensitivity of the antibody used, because even the positive controls revealed immunostaining limited to perivascular location.

IL-8 protein expressed in endometrial epithelial cells may have many physiological roles. The immune cells chemoattracted to the surface are probably involved in the defense against pathogenic organisms. IL-8 is chemotactic for T lymphocytes at lower concentrations than required for neutrophils (4), consistent with the groups of T cells that are detected in the periglandular areas near the surface of the endometrium (23). Moreover, the secretion of IL-8 by way of the apical surface of the endometrial epithelial cells may subserve some function other than the recruitment of maternal leukocytes. Namely, an action of IL-8 on spermatozoa or on the blastocyst as well as a paracrine action on endometrial cells should be considered.

Collectively, findings from this and our previous studies suggest that 1) IL-8 is produced in the human endometrium and may modulate the recruitment of neutrophils and lymphocytes; 2) the amount of IL-8 mRNA in endometrium changes throughout the menstrual cycle, sug-

gesting that sex steroid hormones may act directly or indirectly to regulate IL-8 expression; 3) IL-8 in human endometrium is mainly localized in glandular cells, suggesting that it may have other functions besides the recruitment of maternal leukocytes. Our findings provide a basis for future investigations on the regulation of IL-8 expression by sex steroid hormones and paracrine-acting factors (*i.e.* those derived from implanting trophoblasts), on potential interactions between IL-8 of the surface epithelium and blastocyst, and on the localization and regulation of enzymes that degrade IL-8.

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