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Progestin Regulation of Interleukin-8 mRNA Levels and Protein Synthesis in Human Endometrial Stromal Cells

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The migration of leukocytes into the human endometrium is a normal occurrence that appears to be linked to the hormonal events of the ovarian cycle. In addition, the appearance of large numbers of leukocytes in decidua accompanies normal human pregnancy and puerperal endometrial repair during uterine involution. These leukocytic investments in endometrial-decidual function are also affected by external stimuli, e.g. infection. The formation of cytokines and the response of the uterine endometrium and decidua to cytokines is likely to be an important function of this tissue before, during and soon after pregnancy. Because interleukin-8 (IL-8) is a chemo-attractant activating factor for neutrophils and T cells, the possibility was considered that IL-8 may participate in endometrial leukocytic infiltration in a manner that is hormonally regulated. Previously, we found that IL-8 mRNA is present in the endometrium and decidual tissues; and, using human endometrial stromal cells in monolayer culture as a model system, we found that IL-1 and tumor necrosis factor-α act in a time- and concentration-dependent manner to increase IL-8 mRNA and the accumulation of immunoreactive (ir)IL-8 protein. In this study, we found that progesterone and a synthetic progestin, medroxyprogesterone acetate (MPA) act to enhance the action of IL-1 to increase the level of IL-8 mRNA; this action of progesterone/MPA appears to be affected principally by the stabilization of IL-8 mRNA, together with a small increase in IL-8 gene transcription. Copyright © 1996 Published by Elsevier Science Ltd.

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INTRODUCTION

The endometrium of ovulatory women undergoes morphological and functional changes in response to ovarian steroid hormones. These changes in the endometrium are directed towards preparation of the tissue for blastocyst apposition and implantation. In the case of an infertile cycle, there is a decline in ovarian (corpus luteum) estrogen and progesterone production, which is followed by endometrial shedding, i.e. menstruation. During this ovarian-hormone-induced endometrial cycle, there are characteristic changes in the endometrial glands and stroma and an influx of bone marrow-derived cells into the endometrium/decidua [1]. Although the precise function of the leukocytes in endometrium/decidua is not known, potential functions include participation in the remodeling of the endometrium during various stages of the cycle and in the interactions of fetal and maternal tissues during pregnancy; possibly participating in local immunosuppression to protect against embryo rejection. The pattern of appearance suggests that hormonal factors are involved in regulating this trafficking pattern of leukocyte entry. A possible explanation for this phenomenon is that the production of leukocyte chemo-attractant proteins in endometrial cells is modulated by estrogen or progesterone, or both. One candidate for the recruitment of leukocytes to endometrium is interleukin-8 (IL-8), a polypeptide with neutrophil chemotactic/activating and T cell chemotactic activity both *in vitro* and *in vivo* [2]. Previously, it was found that IL-8 is produced by endometrial stromal cells [3]. The present study was undertaken to

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determine whether IL-8 gene expression in endometrial cells is regulated by progesterone.

MATERIALS AND METHODS

Isolation and culture of human endometrial stromal cells

Endometrial tissue was obtained from the uteri of women after hysterectomy conducted for reasons other than endometrial disease. Informed consent in writing for the use of these tissues was obtained from each woman prior to surgery. The consent forms and protocols used were approved by the Institutional Review Board of this University. Endometrial tissues, removed from the uterus within 1 h of hysterectomy, were placed in culture medium and transported to the laboratory. Endometrial glands and stromal cells were separated as decribed previously [4]. Briefly, the tissue was finely minced ($\sim 1\ mm^3$ pieces) and cells were dispersed by incubation in Hank's balanced salt solution that contained Hepes (25 mM), penicillin (200 U/ml), streptomycin (200 μg/ml), collagenase (1 mg/ml, 15 U/mg), and DNAse (0.1 mg/ml, 1500 U/ mg) for 20–30 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 were retained by the sieve whereas the dispersed stromal cells passed through the sieve into the filtrate. The stromal cells were pelleted (and washed twice) by centrifugation at $500 \times g$ for 10 min and suspended in Ham F12:Dulbecco minimal essential medium (1:1, v/v) that contained antibiotics-antimycotics (1%, v/v) and fetal bovine serum (10%, v/v). The stromal 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, the cells were passed by standard methods of trypsinization and plated in culture dishes (100 mm diameter or 24-well plates).

All experiments were commenced with cells after first passage within 1–5 days after confluence was attained, and all were conducted in F12:DMEM (1:1, v/v) medium with fetal bovine serum (10%, v/v). The cells were treated with test agents or appropriate vehicle [e.g. ethanol as a vehicle for progesterone and medroxyprogesterone acetate (MPA); final concentration 0.1%, v/v). At the end of experiments, the culture media (from 24-well plates) were collected and frozen at −70°C (for subsequent quantification of IL-8 by ELISA); cells (in 100-mm diameter plates) were used for quantification of total protein or for isolation of total RNA. Experiments were performed three or more times.

Preparation of total RNA and northern analysis

Total RNA was prepared by the method of Chirgwin *et al.* [5], size-fractionated by electrophoresis on 1% formaldehyde-agarose gels (5 μg/lane), transferred electrophoretically to Hybond-N^+ membrane (Amersham, Arlington Heights, IL), and linked to the membrane by heating *in vacuo*. Prehybridization was conducted for 16 h at 65°C in buffer comprised of NaCl (0.9 M), Tris–HCl (90 mM, pH 8.3), EDTA (6 mM), Denhardt solution (5 ×), SDS (0.1%), sodium pyrophosphate (0.1%, w/v), and salmon sperm DNA (0.2 mg/ml). Hybridization was conducted for 16 h at 65°C in the same buffer exactly as described previously [3] using an IL-8-specific oligonucleotide probe (5'-TGT TGG CGC AGT GTG GTC CAC TCT CAA TCA-3') end-labeled with [γ-^{32}P]ATP, which corresponds to a portion of exon 2 in the coding region of the IL-8 gene [6]. Blots were washed with 6 × SSC and SDS (0.1%, w/v) for 15 min at room temperature, twice with 2 × SSC and SDS (0.1%, w/v) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membranes was performed at − 70°C using Kodak X-Omat AR film. The presence of equal amounts of RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits or by analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, using a specific 24-mer oligonucleotide probe (5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' [7]) end-labeled with [γ-^{32}P]ATP. Radioactivity that corresponded to specific hybridization with IL-8 and G3PDH mRNAs was quantified by use of an Ambis radioanalytic imaging system and radioactivity in IL-8 mRNA is normalized to that in G3PDH mRNA.

Analysis of transcription

IL-8 gene transcription was assessed (in three separate experiments) using a modification of the "nuclear run-on" procedure described by Sasaki *et al.*, [8] exactly as we have done previously in these cells [3]. Briefly, cells were treated for 8 h with test agents, then homogenized using a Dounce homogenizer in buffer comprised of sucrose (0.25 M), Hepes (10 mM, pH 8.0), $MgCl_2$ (10 mM), dithiothreitol (2 mM), and Triton X-100 (0.1%, v/v). The nuclei were pelleted (and washed) by centrifugation at $600 \times g$ for 5 min, layered over sucrose (1.3 M in lysis buffer). After centrifugation at $10\,000 \times g$ for 10 min, the pelleted nuclei were suspended in buffer that consisted of Hepes (50 mM, pH 8.0), $MgCl_2$ (5 mM), EDTA (0.1 mM), dithiothreitol (2 mM) and glycerol (40%, v/v). For transcription assays, nuclei were incubated at 26°C for 20 min in transcription buffer comprised of Hepes (20 mM, pH 8.0), $MgCl_2$ (5 mM), NH_4Cl (90 mM), $MnCl_2$ (0.5 mM), glycerol (16%, v/v), EDTA (0.04 mM), dithiothreitol (2 mM), ATP, CTP and GTP (0.4 mM each), and [α-^{32}P]UTP (0.25 mCi, 3000 Ci/mmol); the reaction was terminated by the addition of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN). After treatment with proteinase K, RNA was extracted into phenol/chloroform (1:1, v/v), was precipitated by the addition of

ammonium acetate (2.5 M) and ethanol (2 vols), then pelleted by centrifugation and dissolved in Tris–Cl (1 mM, pH 7.5), EDTA (0.5 mM), and SDS (0.1%, w/v). IL-8 cDNA [294 bp; 2 μg/dot; prepared by polymerase chain reaction (PCR) using sense (5'-ACT TCC AAG CTG GCC GTG GCT CTC TTG GCA-3') and antisense (5'-TGA ATT CTC AGC CCT CTT CAA AAA CTT CTC-3') primers corresponding to a translated portion of IL-8 mRNA [6]] or λ DNA (EcoRI digest; 2 μg/dot) was transferred to nylon membrane using a dot-blot apparatus and linked by heating the membrane at 80°C *in vacuo*. Prehybridization was conducted with salmon sperm DNA; hybridization was conducted with equal amounts of newly-transcribed [^{32}P]RNA for 36 h at 45°C. At the end of the hybridization procedure, the membranes were washed with 2× SSC and SDS (0.1%, w/v) for 30 min at 65°C then incubated in 2× SSC containing RNase A (10 μg/ml) and RNase T (1 μg/ml) at 37°C for 30 min. Membranes then were incubated in 2× SSC and SDS (0.1% w/v) containing proteinase K (100 μg/ml) at 37°C for 20 min, and washed in 2× SSC and SDS (0.1%, w/v) at 65°C for 30 min. DNA–RNA hybridization was evaluated by autoradiography and quantified by the use of an Ambis radioanalytical imaging system or by scintillation counting.

IL-8 ELISA

Immunoreactive IL-8 in culture media of endometrial stromal cells was quantified using an ELISA from R & D Systems (Minneapolis, MN). There is no detectable crossreactivity with other known cytokines (e.g. IL-1α, IL-1ß, IL-2, IL-6, TNF-α) in this assay. The sensitivity for IL-8 was 0.47 μg/100 μl sample. The intra-assay coefficient of variation was 7.95%.

Reagents

Human recombinant IL-1α was from Collaborative Research Corp. (Bedford, MA) and Amgen Biologicals (Thousand Oaks, CA), respectively. Actinomycin-D, progesterone and MPA were purchased from Sigma Chemical Co. (St Louis, MO).

RESULTS

Modulation of IL-8 mRNA levels and IL-8 protein secretion by progestins

To evaluate the possibility that progestin modulates IL-8 expression in endometrial stromal cells, the cells were pretreated with progesterone (10^{-7} M) for various times before cytokine treatment. In cells pretreated with progesterone for 14–17 days to induce decidualization [9], IL-1α (10 U/ml) treatment for 6, 18, or 24 h induced higher levels of IL-8 mRNA than in cells not pretreated with progesterone (Fig. 1). Progesterone pretreatment for shorter times (1 and 2 h) also led to increased IL-8 mRNA levels with IL-1 stimulation

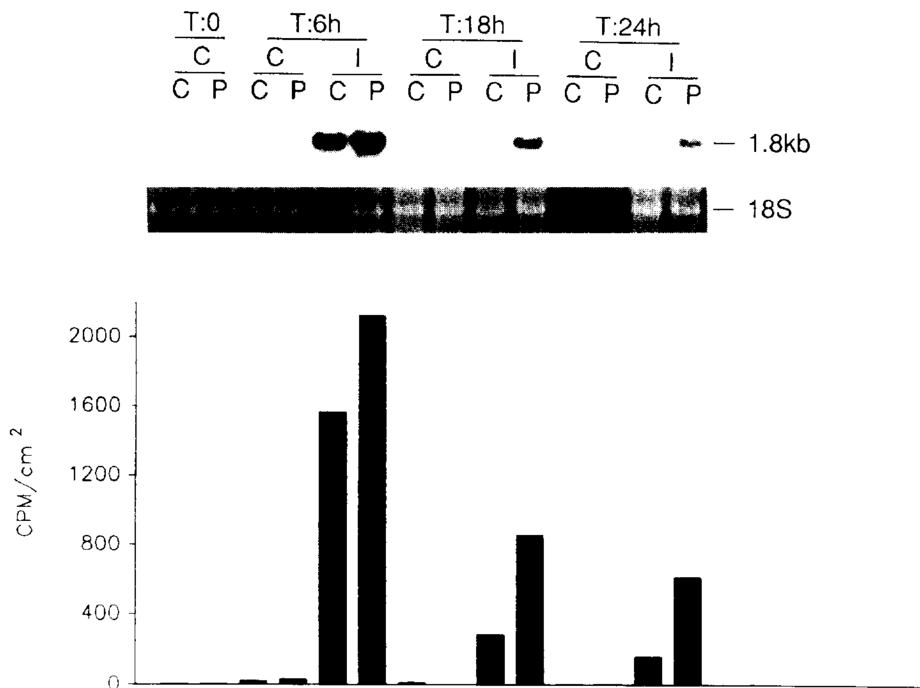

Fig. 1. Effect of long-term progesterone pretreatment. Confluent endometrial stromal cells in culture were pretreated with or without progesterone (10^{-7} M) for 17 days to facilitate decidualization. Then progesterone pretreated and non-pretreated cells were incubated for 6, 18 and 24 h in culture medium containing IL-1α (10 U/ml). At the end of the incubation period, the culture media were removed, total RNA was prepared; IL-8 mRNA was detected by northern analysis of total RNA (5 μg/lane). C, control; P, progesterone; I, IL-1α.

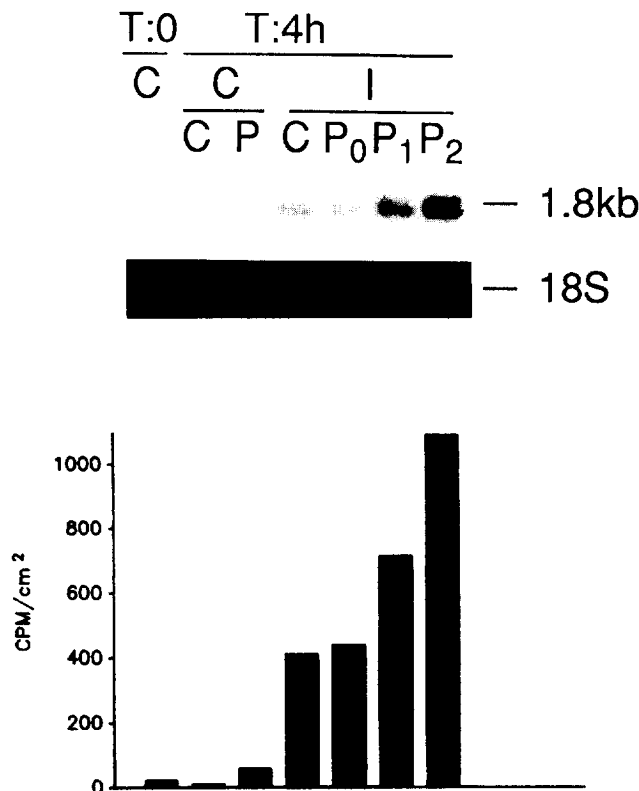

Fig. 2. Effect of short-term progesterone pretreatment. Confluent endometrial stromal cells in culture were pretreated with or without progesterone (10^{-7} M) for 1 or 2 h. Then cells were incubated for 4 h in culture medium containing IL-1α (10 U/ml) beginning at 0 h. At the end of the incubation period, the culture media were removed and IL-8 mRNA was detected by northern analysis of total RNA (5 μg/lane). C, control; I, IL-1α; P, progesterone; P_0, progesterone added to media at the same time as IL-1α; P_1, progesterone added to media 1 h before IL-1α; P_2, P added to media 2 h before addition of IL-1α.

when compared with those not treated with progesterone (Fig. 2). Similarly, MPA (10^{-7} M) pretreatment for 1 h also induced higher levels of IL-8 mRNA than in cells that were non-pretreated with MPA (Fig. 3). Treatment of endometrial stromal cells with estradiol 17-ß ($1–5 \times 10^{-8}$ M for times ranging from 4 to 48 h) had no discernible effect on IL-8 mRNA levels.

In another experiment, endometrial stromal cells were pretreated with progesterone (10^{-7} M) for 2 h before IL-1α treatment (1 U/ml). Culture media were collected at 3 h intervals for 33 h and immunoreactive IL-8 was quantified by ELISA (Fig. 4). The amount of IL-8 produced by progesterone-treated cells was not greater than that produced by non-treated cells. If an increase in IL-8 protein production in cells pretreated with progesterone were to accompany the increase in IL-8 mRNA, we would have expected to find such a difference after ~ 9 h and for some time (possibly a short time) thereafter. This estimate was based on the time courses of progesterone and IL-1 action on IL-8 mRNAs. At some times, the levels of immunoreactive IL-8 in the medium of cells pretreated with progesterone were lower (or greater) than that in controls, but these were not consistent time-dependent differences. Thus, we conclude that the increase in IL-8 mRNA in progesterone-treated cells is not accompanied by an increase in the amount of IL-8 protein that is secreted into the medium, as detected by immunoassay.

Regulation of IL-8 nuclear transcription

Nuclear run-on analysis was used to evaluate the effects of IL-1 and progesterone on IL-8 gene transcription. Constitutive transcription of IL-8 was demonstrated in nuclei from unstimulated cells as we found previously [3]. Progesterone (10^{-7} M) treatment for 8 h before isolation of nuclei (2 h prior to IL-1α treatment) was associated with a 10–20% increase in IL-8 transcription in both IL-1-treated and non-treated cells in the three studies conducted (Fig. 5).

Regulation of IL-8 mRNA stability by progesterone

To determine if the effect of progesterone was the result of alterations in IL-8 mRNA stability, we evaluated the degradation of IL-8 mRNA in cells that were or were not pretreated with progesterone for 2 h

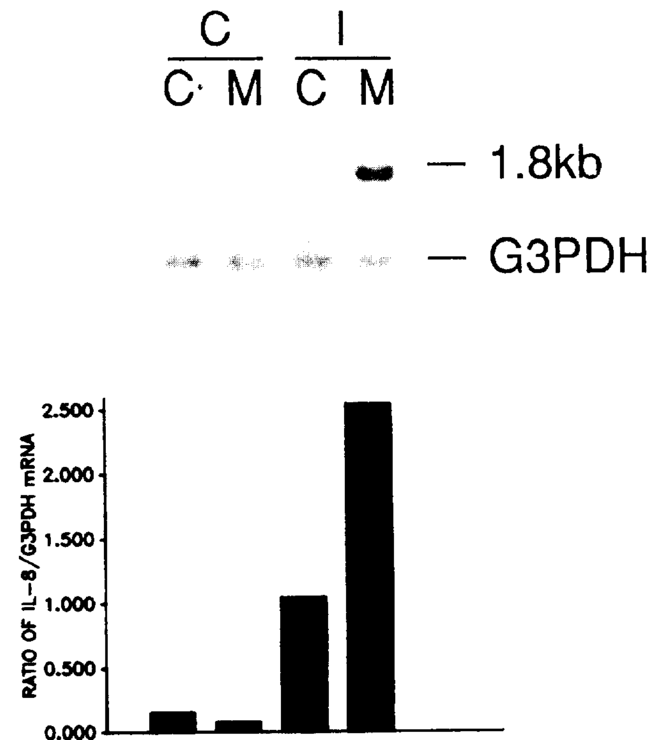

Fig. 3. Effect of medroxyprogesterone acetate (MPA) pretreatment. Confluent endometrial stromal cells in culture were pretreated with MPA (10^{-7} M) or vehicle (ethanol, 0.01%, v/v) for 1 h, then cells were incubated for 5 h in culture medium containing IL-1α (1 U/ml). At the end of the incubation period, the culture media were removed and IL-8 mRNA was detected by northern analysis of total RNA (5 μg/lane). C, control; M, medroxyprogesterone acetate; I, IL-1α.

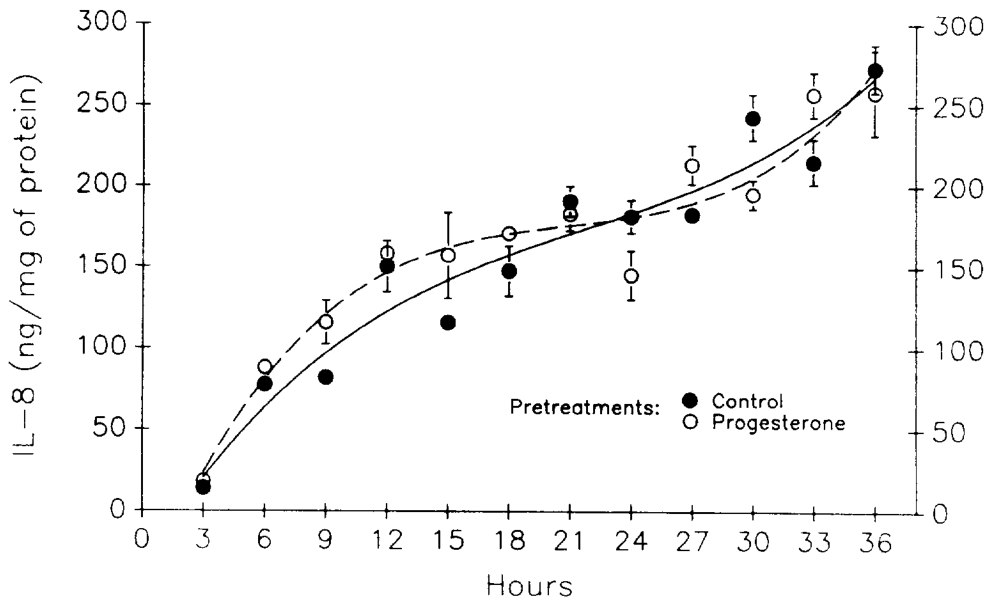

Fig. 4. Effect of progesterone pretreatment on immunoreactive IL-8 production. Confluent endometrial stromal cells in culture were pretreated for 2 h with progesterone (10^{-7} M), then cells were incubated for 3–6-h intervals up to 33 h in culture medium containing IL-1α (1 U/ml). Culture media were collected, and IL-8 was quantified by ELISA. Data are mean ± SEM for replicates of three.

prior to treatment with IL-1α (1 U/ml) for 6 h. The half-life of IL-8 mRNA in non-pretreated cells was approximately 45 min, whereas in progesterone-pretreated cells, it was 85 min (Fig. 6).

DISCUSSION

IL-8 is a chemotactic factor for neutrophils and lymphocytes. The local concentration of IL-8 is important in determining whether neutrophils or lymphocytes are recruited predominantly. At low concentrations, T lymphocytes are two to ten times more sensitive than neutrophils to the chemotactic effect of IL-8 [2]. It is likely that chemo-attractants, such as IL-8, are involved in regulating the proportions and locations of large granular lymphocytes and other leukocytes in the decidua during normal pregnancy and in response to infection by way of the elaboration of IL-1β and TNF-α. TNF-α production has been demonstrated in a number of extra-embryonic fetal tissues and in uterine decidua of normal pregnancy [10]; and IL-*1β* mRNA is present in decidual tissue, and the level of pro-IL-1β mRNA is increased in response to bacterial toxins or cytokines [11].

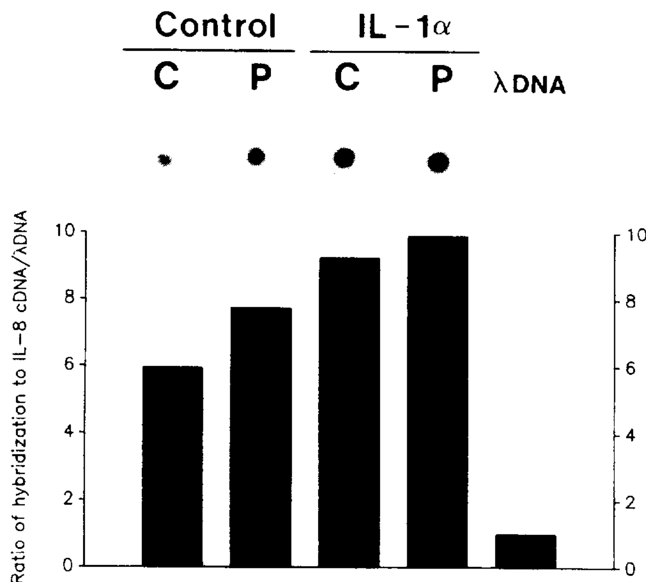

Fig. 5. Effect of IL-1α and progesterone on IL-8 transcription. Nuclei were isolated from endometrial stromal cells in culture after pretreatment with or without progesterone (10^{-7} M) for 2 h, then treatment with or without IL-1α (1 U/ml) for 6 h and transcription assays were performed. C, control; P, progesterone; I, IL-1α.

Previously [3], and again in this study, we found that IL-1α acted in endometrial stromal cells in culture to increase the levels of IL-8 mRNA and to increase the level of immunoreactive (ir)IL-8 protein secreted into the culture medium. Pretreatment of these cells with progesterone augmented (by two-fold) the effect of IL-1 on the levels of IL-8 mRNA in these cells. The effect of progesterone on induction of IL-8 gene transcription was minimal, whereas the half-life of IL-8 mRNA was prolonged by pretreatment with progesterone. The amount of immunoreactive IL-8 that accumulated in the medium, however, was not increased by progesterone pretreatment, suggesting that the translatability of IL-8 mRNA may be decreased or limiting under these circumstances. We find that IL-8 mRNA is present in decidual tissue obtained at mid-trimester and at term [3]. It is likely that other factors are involved in the regulation of IL-8 gene expression in the human endometrium/decidua. Progesterone, acting to prolong

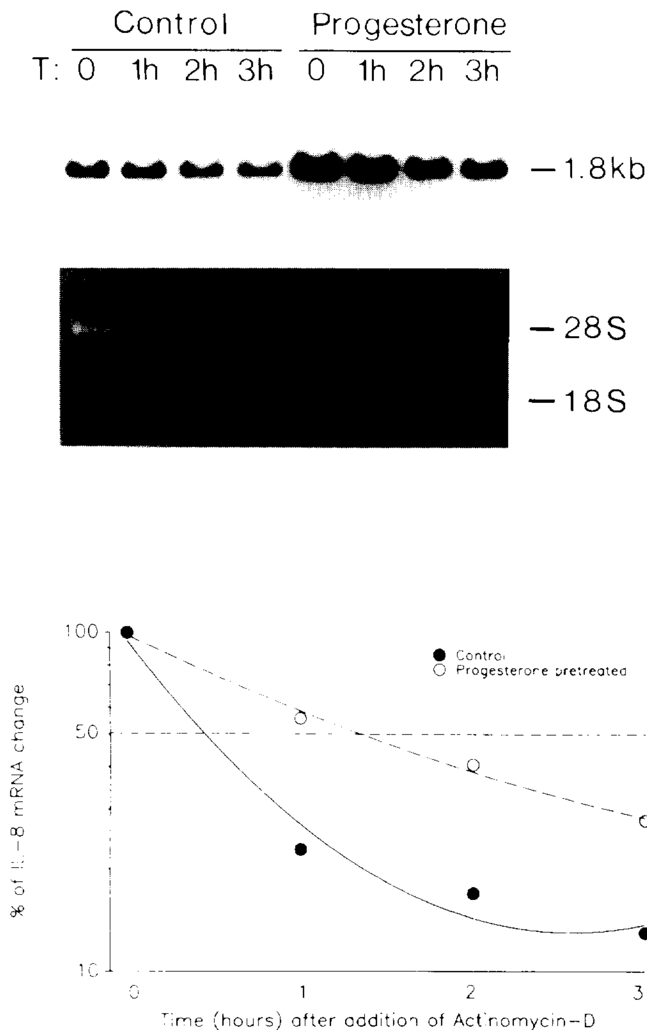

Fig. 6. Effect of progesterone pretreatment on IL-8 mRNA stability. Confluent endometrial stromal cells were pretreated with or without progesterone (10^{-7} M) for 2 h, then cells were incubated for 6 h in culture medium containing IL-1α (1 U/ml). The levels of IL-8 mRNA were determined before (time = 0) and at 30-min intervals after the addition of actinomycin-D (10 μg/ml). Ambis radioanalytical quantification of IL-8 mRNA after actinomycin-D treatment is also presented (graph); data are expressed as a percentage of the amount of IL-8 mRNA in the cells at time = 0. C, control; P, progesterone.

the half-life of IL-8 mRNA may participate in the regulation of IL-8 production. This may provide for an enhanced local defense mechanism against bacterial infections in decidua of pregnancy. It has been shown by others that neutrophil chemotactic activity in the rat vagina is hormonally controlled [12] and that germ-free mice have a normal vagina neutrophil response induced by progesterone [13].

Generally, IL-8 is not produced constitutively in significant amounts, but rather is induced in a variety of cell types after treatment with any of a number of cytokines or mitogens [14], [15]. Previously, we obtained evidence from nuclear run-on analyses that the IL-8 gene is transcribed constitutively in endometrial stromal cells in culture and that IL-8 mRNA has a short half-life [3].

The principal effect of progesterone to increase IL-8 mRNA levels appears to be by the prolongation of IL-8 mRNA half-life. Progesterone/progestins may act to induce the production of specific ribonuclease inhibitor proteins that limit the degradation of IL-8 mRNA.

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REFERENCES

1. Lea R. G. and Clark D. A.: Macrophages and migratory cells in endometrium relevant to implantation. *Bailliere Clin. Obstet. Gynecol.* **5** (1991) 25–59.
2. Larsen C. G., Anderson A. O., Appella E., Oppenheim J. J. and Matsushima K.: The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* **243** (1989) 1464–1466.
3. Arici A., Head J. R., MacDonald P. C. and Casey M. L.: Regulation of interleukin-8 gene expression in human endometrial cells in culture. *Mol. Cell. Endocrinol.* **94** (1993) 195–204.
4. Casey M. L., Smith J. W., Nagai K., Hersh L. B. and MacDonald P. C.: Progesterone-regulated cyclic modulation of membrane metalloendopeptidase (enkephalinase) in human endometrium. *J. Biol. Chem.* **266** (1991) 23041–23047.
5. Chirgwin J. M., Przybyla A. E., MacDonald R. J. and Rutter W. J.: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18** (1979) 5294–5299.
6. Strieter R. M., Kasahara K., Allen R., Showell H. J., Standiford T. J. and Kunkel S. L.: Human neutrophils exhibit disparate chemotactic factor gene expression. *Biochem. Biophys. Res. Commun.* **173** (1990) 725–730.
7. Maier J. A. M., Hla T. and Maciag T.: Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.* **265** (1990) 10805–10808.
8. Sasaki K., Cripe T. P., Koch S. R., Andrenone T. L., Peterson D. O., Beale E. G. and Granner D. K.: Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem.* **259** (1984) 15242–15251.
9. Chen G., Huang J. R., Mazella J. and Tseng L.: Long-term effects of progestin and RU 486 on prolactin production and synthesis in human endometrial stromal cells. *Human Reprod.* **4** (1989) 355–358.
10. Chen H. L., Yang Y. P., Hu X. L., Yelavarthi K. K., Fishback J. L. and Hunt J. S.: Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. *Am. J. Pathol.* **139** (1991) 327–335.
11. MacDonald P. C., Koga S. and Casey M. L.: Decidual activation in parturition: examination of amniotic fluid for mediators of the inflammatory response. *Ann. NY Acad. Sci.* **622** (1991) 315–330.
12. Jungi T. W., Besedovsky H. O., Sorkin E. and Schardt M.: Hormonal control of neutrophil chemotactic activity in the rat vagina. *Am. J. Physiol.* **233** (1976) R59–R65.
13. Beaver D. L.: The hormonal induction of a vaginal leukocytic exudate the germ-free mouse. *Am. J. Pathol.* **37** (1960) 769–773.
14. DeForge L. E. and Remick D. G.: Kinetics of INF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. *Biochem. Biophys. Res. Commun.* **174** (1991) 18–24.
15. Thorton A. J., Strieter R. M., Lindley I., Baggiolini M. and Kunkel S. L.: Cytokine-induced gene expression of a neutrophil chemotactic factor/IL-8 in human hepatocytes. *J. Immun.* **144** (1990) 2609–2613.
16. Mukaida N., Shiroo M. and Matsushima K.: Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J. Immun.* **143** (1989) 1366–1371.