0013-7227/96/$03.00/0
Endocrinology

Vol. 137, No. 9
Printed in U.S.A.

Interleukin-8 Expression and Modulation in Human Preovulatory Follicles and Ovarian Cells*

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ABSTRACT

Just before the time of ovulation, the number of neutrophils increases markedly in the thecal layer of the leading follicle. A preovulatory rise in chemotactic activity for neutrophils in human follicular fluid has also been detected. We hypothesized that interleukin-8 (IL-8), a neutrophil chemoattractant/activating factor and a potent angiogenic agent, may be an important modulator of leukocyte chemotaxis in ovulatory function. In this regard we investigated the expression and modulation of IL-8 in human follicular fluid samples from patients undergoing *in vitro* fertilization-embryo transfer therapy and in ovarian stromal and granulosa-lutein cell cultures.

The concentration of IL-8 in pre-hCG follicular fluid samples (n = 4) was 16 ± 12 (mean ± SEM) pg/ml, and that in post-hCG samples (n = 101) was 262 ± 45 pg/ml ($P = 0.001$). In post-hCG samples, the concentration of IL-8 in an individual follicle correlated with the size of that follicle ($r = 0.61$; $P = 0.02$). We also observed a correlation between serum IL-8 levels (22 ± 3 pg/ml) and follicular fluid levels (303 ± 143 pg/ml), with a 14-fold gradient ($r = 0.71$; $P = 0.01$) in 11 patients tested for both. IL-8 messenger RNA (mRNA) and the protein were expressed constitutively in ovarian stromal cell cultures, and the level was increased by IL-1α and tumor necrosis factor-α in a time- and concentration-dependent manner. hCG and LH induced higher levels of IL-8 mRNA expression and protein production. Granulosa-lutein cells also expressed IL-8 mRNA and protein, and the levels were increased by IL-1α and tumor necrosis factor-α. Importantly, progesterone suppressed both basal and IL-1α-stimulated IL-8 expression in stromal and granulosa-lutein cell types.

In summary, we found that IL-8 levels are elevated in periovulatory follicular fluid, and both granulosa-lutein and ovarian stromal cells express the mRNA and produce the protein. The modulation of IL-8 in these cell cultures by steroid and trophic hormones suggests that IL-8 may play an important role in the physiology of ovulation, such as timely follicular rupture and neovascularization of the corpus luteum. (*Endocrinology* **137:** 3762–3769, 1996)

SUCCESSFUL ovulation requires an integrated series of events that ultimately leads to the timely release of the mature oocyte from the follicle. Ovulation has many features in common with an inflammatory reaction, including the participation of leukocytes and classical inflammatory mediators, such as eicosanoids, histamine, and bradykinin (1). It is becoming increasingly evident that immune-ovarian cell interactions are essential components of the ovarian cycle, including ovulation (2). Indeed, the number and type of leukocytes in the human ovary vary in a predictable manner with the ovulatory cycle, and a marked preovulatory increase in neutrophil density has been observed in the theca concomitant with the LH surge (3). Once in and around the mature follicle, neutrophils may play a crucial role in timely follicular rupture by secreting specific proteolytic enzymes, PGs, and other paracrine factors. Experimental evidence suggests that leukocytes may play a role in ovulation; peripheral blood leukocytes added to the *in vitro* perfused rat ovary increased the number of LH-induced ovulations (4).

Although it has been shown that human follicular fluid contains chemotactic activity toward neutrophils (5, 6), specific chemotactic factors responsible for the recruitment and activation of neutrophils in and around the periovulatory follicles have not been identified. The most likely agents are peptide leukocyte chemoattractants. One such polypeptide is interleukin-8 (IL-8), a cytokine with neutrophil chemotactic and activating activity both *in vivo* and *in vitro* (7) and a potent angiogenic agent (8). The angiogenic property of IL-8 is relevant because neovascularization is a prominent feature of early luteinization. The rapid vascularization of the corpus luteum is believed to be guided by angiogenic factors detected in the follicular fluid (9), for which IL-8 may be a candidate. IL-8 is produced by a number of cell types, including monocytes (10), endothelial cells (11), fibroblasts (12), mesothelial cells (13), and endometrial stromal cells (14). We postulated that IL-8 may be an important modulator of periovulatory events by not only attracting and activating neutrophils that will play a role in timely follicular rupture, but also by stimulating new blood vessel formation for a healthy corpus luteum. Our hypothesis was that neutrophil migration in and around the preovulatory follicle may be hormonally regulated, and this regulation may occur through local modulation of IL-8 expression. In the present study we investigated IL-8 concentrations in human follicular fluid. Using granulosa-lutein cells and ovarian stromal cells in culture, we also evaluated the modulation of IL-8 gene expression by cytokines and hormones.

Received March 28, 1996.
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* This work was supported by NIH Grant 1-K08-HD-01041 (to A.A.).

Materials and Methods

Follicular fluid and tissue collection

Follicular fluids and cells for granulosa-lutein cell culture were obtained from patients (aged 24–43 yr) undergoing *in vitro* fertilization (IVF)-embryo transfer therapy at the Yale University IVF Program. Writ-

ten informed consent was obtained from each woman before the procedure; consent forms and protocols were approved by the human investigation committee of this university. A standard IVF protocol was used. Briefly, GnRH agonist (leuprolide acetate, Lupron, Tap Pharmaceuticals, Deerfield, IL) was administered 1 mg/day, sc, starting in the midluteal phase of the preceding cycle or on the first day of the stimulation cycle. Stimulation with human menopausal gonadotropin (hMG; Pergonal, Serono Laboratories, Norwell, MA) or FSH (Metrodin, Serono Laboratories) was initiated when there was no sonographic evidence of ovarian follicular activity, and the serum estradiol level was less than 50 pg/ml (conversion factor to Systeme International units, 3.671) and was continued until estradiol levels reached 500 pg/ml or greater and at least two follicles 17 mm or larger in diameter were present. At that time, 10,000 mIU hCG (Profasi, Serono Laboratories) were administered, and Lupron and hMG were discontinued. Oocyte retrieval by transvaginal ultrasound guidance was performed approximately 34 h after hCG administration. After removal of the cumulus-oocyte complexes, follicular fluids were centrifuged at 600 × *g* for 20 min. The cell-free supernatants were then aliquoted into polypropylene microfuge tubes and stored at −80 C until assayed. Cell pellets were used for culture of granulosa-lutein cells. The four samples of follicular fluid collected before hCG administration were obtained from King Faisal Specialist Hospital and Research Center (Riyadh, Saudi Arabia), shipped on dry ice, and stored at −80 C until assayed together with other samples. Consent forms and protocols were approved by the human investigation committee of that institution. These samples were obtained from patients undergoing controlled ovarian hyperstimulation with GnRH agonist and hMG for timed intrauterine insemination. Patients underwent ultrasound guided follicular reduction (of follicles of 16 mm or larger in diameter) just before hCG administration to prevent ovarian hyperstimulation syndrome and/or multiple gestation.

Blood samples were collected from all women on the day of hCG administration and additionally from 11 women on the day of oocyte retrieval. The blood was centrifuged to separate serum, which was stored at −80 C until assayed.

Ovarian tissue was obtained from women of reproductive age undergoing hysterectomy with oophorectomy for reasons other than ovarian disease. Informed consent in writing for the use of these tissues was obtained from each woman before surgery. The consent forms and protocols used were approved by the human investigation committee of this university. The ovarian tissues were placed in culture medium and transported to the laboratory for dissection of the visible follicles and culture of ovarian stromal cells.

Isolation and culture of granulosa-lutein cells

Cell pellets obtained after the centrifugation of follicular fluids were resuspended in Hanks' Balanced Salt Solution (HBSS), and the cell suspension was gently layered over Ficoll (LSM, Organon Teknika, Durham, NC), then centrifuged at 500 × *g* for 20 min to pellet red blood cells. Cells at the interface were removed with a Pasteur pipette, resuspended in HBSS, and centrifuged at 500 × *g* for 10 min. Finally, the cell pellet was resuspended in Ham's F-12 medium-DMEM (1:1, vol/vol) containing antibiotics-antimycotics (1%, vol/vol) and FBS (10%, vol/vol). The average viability was 85%, as assessed by the dye exclusion test using trypan blue (Life Technologies, Grand Island, NY). Dispersed cells were counted using a hemocytometer; cell number varied from 10×10^6 to 30×10^6 cells/patient. Cell suspension was then diluted accordingly to a concentration of 5×10^5 viable cells/ml and plated in 6-well plates. The plates were incubated for 1–6 days at 37 C in a humidified atmosphere of 5% CO_2 in air, with daily replacement of medium.

In some experiments, granulosa-lutein cells were treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Amac, Westbrook, ME) to remove white blood cells. Briefly, isolated granulosa-lutein cells were suspended in 2 ml medium containing 30% FCS and incubated for 10 min at room temperature with anti-CD45 immunomagnetic beads. Then, the suspension was placed into a magnetic test tube rack (Bio-Mag, Lexington, MA) for 10 min at room temperature, resulting in the removal of immunobead-bound white blood cells from the cell suspension. After careful removal of the supernatant, the process was repeated twice under identical conditions, and purified granulosa-lutein cells were centrifuged, then resuspended in Ham's F-12 medium-DMEM. The viability was 95%, as assessed by the dye exclusion test using trypan blue. The granulosa-lutein cells were plated in 24-well plates at 5×10^4 cells/well density for experiments.

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). Cells were used for quantification of total protein or for isolation of RNA. Similar experiments were conducted on at least three different occasions.

Isolation and culture of ovarian stromal cells

Ovarian tissue cleared from visible follicles 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 deoxyribonuclease (0.1 mg/ml; 1500 U/mg) for 2 h at 37 C with agitation. The dispersed cells were filtered through a wire sieve (73-μm diameter pore) to remove undigested tissue pieces. The ovarian stromal cells were plated in Ham's F-12 medium-DMEM (1:1, vol/vol) containing 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, the stromal cells were passed by standard methods of trypsinization and plated in culture dishes (100-mm diameter or 24-well plates) as appropriate for the experimental design and allowed to replicate to confluence. Experiments were commenced 1–3 days after confluence was attained. Because we have previously shown in other cell types that serum stimulates IL-8 production (14), the confluent cells were treated with serum-free medium for 24 h before treatment with test agents was initiated.

At the end of each experiment, the culture media were collected and frozen at −80 C for quantification of IL-8 by ELISA. Cells were used for quantification of total protein or for isolation of RNA. Similar experiments were conducted on at least three different occasions.

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells were conducted using factor VII as a marker of endothelial cells, low mol wt cytokeratin as a marker of epithelial cells, vimentin as a marker of mesenchymal cells, aromatase as a marker of estrogen-producing cells, and HAM 56 as a marker of monocyte/macrophages (specific for CD56). Freshly isolated granulosa-lutein cells and cultured ovarian stromal cells at first passage were plated on 22-mm^2 coverslips in 35-mm wells. Cells were fixed in 4% paraformaldehyde and stored at −20 C. Cells were exposed to monoclonal murine antihuman antibodies [for factor VII (Dako, Carpinteria, CA; 1:100 dilution), low mol wt cytokeratin (Dako; 1:500 dilution), vimentin (BioGenex, San Ramon, CA; 1:30 dilution), HAM 56 (Dako; 1:150 dilution), and aromatase (generously donated by Dr. N. Harada, Fujida Health University, Toyoake, Aichi, Japan; 1:2000 dilution)]. An avidin-biotin developing system (Vectostain ABC kit, Vector Laboratories, Burlingame, CA) was used, as described.

In the granulosa-lutein cell cultures, CD56 marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 6–9%, 1–2%, and 0%, respectively. Nearly all cells (95–100%) were positive for vimentin. CD56 marker-positive cells represented less than 1% after treatment with anti-CD45 immunobeads. In confluent ovarian stromal cell cultures after first passage, CD56 marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 2–4%, 1–2%, and 5–10%, respectively. Nearly all cells (95–100%) were positive for vimentin. Approximately 10–15% of the cells were positive for aromatase. These data were compiled from preparations of cells from three different samples.

Preparation of total RNA and Northern analysis

Total RNA from cells in culture was extracted using Trizol (Life Technologies, Grand Island, NY) (15). Total RNA was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N^+ membrane (Amersham Corp., Arlington Heights, IL), and cross-linked to the membrane by use of UV light. Prehybridization was conducted for 5 h at 65 C in buffer comprised of NaCl (0.9 M), Tris-Cl (90 mM; pH 8.3), EDTA (6 mM), 5 × Denhardt's solution [polyvinylpyrrolidone (0.1%, wt/vol), BSA (0.1%, wt/vol), Fi-

coll 400 (0.1%, wt/vol)], SDS (0.1%), sodium pyrophosphate (0.1%; wt/vol), and salmon sperm DNA (0.2 mg/ml). Hybridizations were conducted for 16 h at 65 C in buffer that contained 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; the sequence of this probe corresponds to a portion of exon 2 in the coding region of the IL-8 gene (16). Thereafter, the blots were washed with 6 × standard saline citrate (SSC) and SDS (0.1%; wt/vol) for 15 min at room temperature, once with 2 × SSC and SDS (0.1%; wt/vol) 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 (Eastman Kodak, Rochester, NY). The presence of equal amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits and analysis of glyceraldehyde-3-phosphate dehydrogenase messenger RNA (mRNA), using a complementary DNA probe (Clontech Laboratories, Palo Alto, CA) radiolabeled with [α-^{32}P]deoxy-CTP by random hexamer priming. The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Each IL-8 band was normalized using the value for the corresponding glyceraldehyde-3-phosphate dehydrogenase mRNA, thus correcting for any variation in amounts of RNA applied to each lane. Similar experiments were conducted on three different occasions with cells prepared from different patients.

IL-8 immunoassay

Immunoreactive IL-8 in follicular fluid samples and culture supernatant 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. The sensitivity for IL-8 was 0.47 pg/100 μl sample. All of the follicular fluid samples were evaluated in a duplicate assay. Validation of its use for human follicular fluid was also performed: recombinant IL-8 was diluted in assay buffer and pooled follicular fluid, and parallelism was observed between the standard curve of buffer and follicular fluid dilutions. Each experiment was performed using three replicate wells for each condition, and supernatant from each well was tested in a single ELISA assay. Each experimental set-up was repeated on three or more occasions using cells obtained from three different patients. The intra- and interassay coefficients of variation were 7.95% and 10.2%, respectively.

Statistical analyses

Because the levels of IL-8 in follicular fluid were not normally distributed, they were analyzed with nonparametric ANOVA by ranks (Kruskal-Wallis). Individual groups were compared *post-hoc* with the nonparametric Mann-Whitney test. Data from the ELISAs were evaluated by ANOVA with Bonferroni *post-hoc* analysis for multiple comparisons. Correlation analyses were performed using Pearson or Spearman rank tests as appropriate. Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS), version 6.0 for Windows (SPSS, Chicago, IL).

Reagents

Culture media, antibiotics-antimycotics, estradiol, progesterone, human recombinant hCG, and FBS were obtained from Sigma Chemical Co. (St. Louis, MO). IL-1α, tumor necrosis factor-α (TNFα; both recombinant) were purchased from the R&D Systems. Human LH and FSH were obtained from the National Hormone and Pituitary Program, NIH.

Results

Immunoreactive IL-8 in follicular fluid

The mean concentration of IL-8 in follicular fluid samples obtained from women (n = 4) before the administration of hCG and before a detectable LH surge was 16 ± 12 pg/ml (mean ± SEM; range, 0–52). The mean diameter of aspirated follicles was 17 mm (range, 15–22 mm). In follicular samples obtained from women (n = 101) 34 h after hCG administration, the mean concentration of IL-8 was 262 ± 45 pg/ml (range, 26–3663), which was significantly higher than pre-hCG levels (P = 0.001; Fig. 1). There was only one sample from the pre-hCG group (52 pg/ml sample) that overlapped with the post-hCG group. When we measured IL-8 levels in individual follicles from four women after hCG administration, the concentration of IL-8 correlated with the size of that follicle (r = 0.61; P = 0.02; Fig. 2).

In 11 women from whom blood samples were taken on the day of follicular aspiration (34 h after hCG administration), we observed substantially higher (14-fold) follicular fluid IL-8 levels (303 ± 143 pg/ml) than serum IL-8 levels (22 ± 3 pg/ml). There was also a correlation between serum and

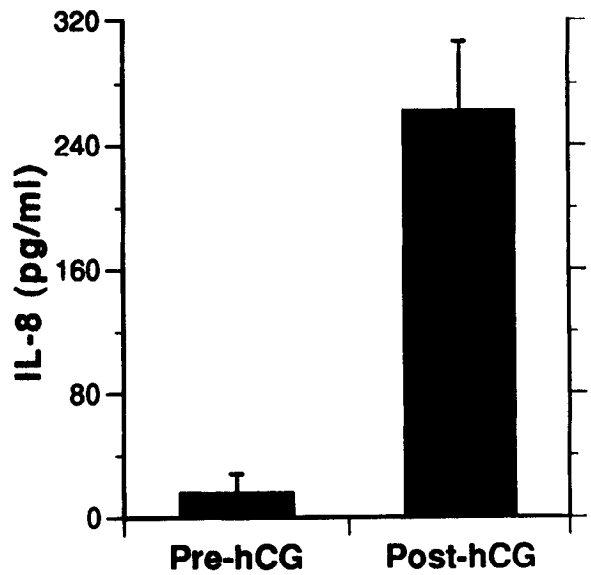

FIG. 1. Immunoreactive IL-8 concentrations in follicular fluid obtained before (n = 4) or after (n = 101) hCG administration (P = 0.001). Values are the mean ± SEM.

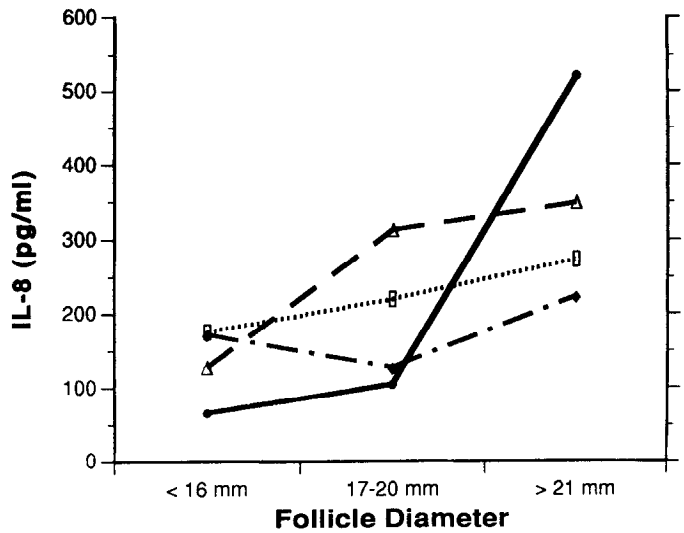

FIG. 2. Immunoreactive IL-8 concentrations in follicular fluid; correlation with follicle diameter. Follicular fluids from four representative patients were obtained after hCG administration.

follicular fluid IL-8 levels from individual patient samples ($r = 0.71$; $P = 0.01$).

Cytokine modulation of IL-8 production and mRNA expression in granulosa-lutein and ovarian stromal cells

In cultures performed after elimination of white blood cells by use of anti-CD45 immunobeads, granulosa-lutein cells maintained in culture medium that contained 10% FBS secreted IL-8 into the culture medium. Treatment of granulosa-lutein cells with IL-1α (10 and 100 U/ml) and TNFα (1 and 10 ng/ml) alone or in combination resulted in increased accumulation of IL-8 in the medium; these differences were significant by 8 h ($P = 0.001$; Fig. 3). The level of IL-8 mRNA was easily detectable in granulosa-lutein cells and increased in response to IL-1α and TNFα treatment 5- and 2-fold, respectively (data not shown).

Ovarian stromal cells maintained in serum-free culture medium secreted IL-8 (8.2 ± 0.9 ng/ml, or 166 ± 21 ng/mg total protein by 8 h). Treatment of ovarian stromal cells with IL-1α (10 U/ml) resulted in increased accumulation of IL-8 in the medium (22.5 ± 2.5 ng/ml, or 737 ± 97 ng/mg total protein by 8 h; $P = 0.001$). Similar findings were also observed after treatment with lower concentrations of IL-1α (0.1 and 1 U/ml) and TNFα (0.1 and 10 ng/ml) by 8 h (data not shown). The level of IL-8 mRNA was low, but detectable, in stromal cells, but increased markedly in response to IL-1α and TNFα treatment alone or in combination (Fig. 4). This increase was evident by 15 min (data not shown), and high levels of IL-8 mRNA were present by 4–6 h after commencement of either treatment, after which they began to decrease (Fig. 5, A and B). The increase in IL-8 mRNA levels in stromal cells was dependent upon the concentration of IL-1α (0.01–10 U/ml) or TNFα (0.01–1 ng/ml; Fig. 6, A and B).

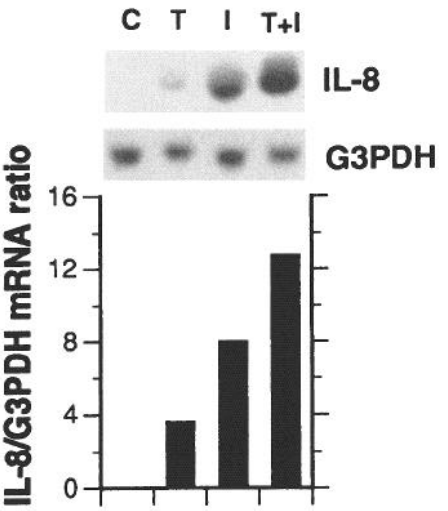

FIG. 4. Northern analysis of IL-8 mRNA in ovarian stromal cells treated with IL-1α and TNFα. Confluent ovarian stromal cells were incubated for 6 h with IL-1α (10 U/ml), TNFα (10 ng/ml), or both. Total RNA (10 μg/lane) was evaluated. C, Control; I, IL-1α; T, TNFα.

Steroid and trophic hormone modulation of IL-8 production and mRNA expression in ovarian stromal and granulosa-lutein cells

hCG treatment for 6 h of ovarian stromal cells in culture led to increases in the level of IL-8 mRNA (Fig. 7). Treatment of ovarian stromal cells with hCG (1–10,000 mIU/ml) for 8 h led to concentration-dependent increases (100–10,000 mIU/ml) in IL-8 protein secretion into the medium (Fig. 8). Treatment with LH (1–10,000 mIU/ml) for 6 h also resulted in increases in IL-8 mRNA, although the increase was less pronounced than that observed after hCG treatment (Fig. 9). Similar, but less pronounced, findings were observed with

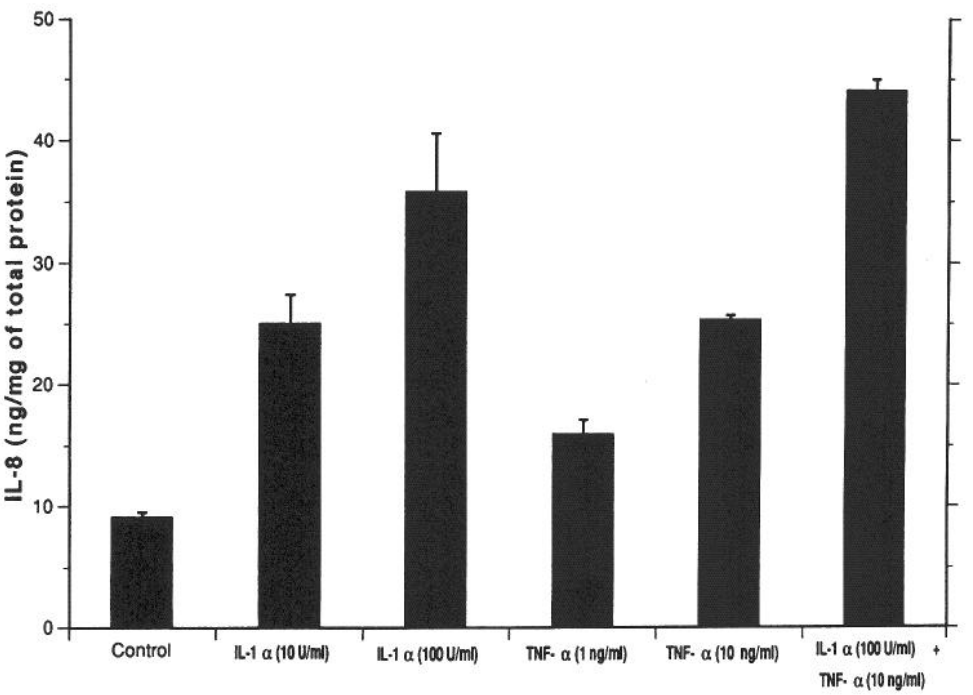

FIG. 3. Stimulation of immunoreactive IL-8 production by granulosa-lutein cells in culture by IL-1α and TNFα. Granulosa-lutein cells, purified using anti-CD45 immunobeads, were treated with medium alone (control) or containing IL-1α (10 and 100 U/ml), TNFα (1 and 10 ng/ml), or both for 8 h. The culture media were collected, and IL-8 was quantified by ELISA. Data are the mean ± SEM for three replicates.

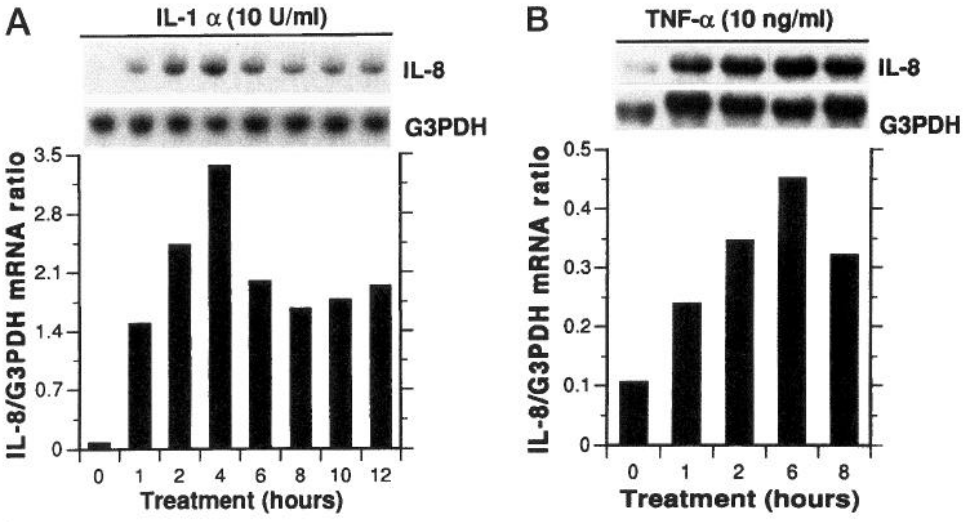

FIG. 5. IL-1α- and TNFα-mediated increases in IL-8 mRNA levels. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation with culture medium containing IL-1α (10 U/ml) for 1–12 h (A) or TNFα (10 ng/ml) for 1–8 h (B). At the end of the incubation period, the culture media were removed, and total RNA was prepared from the cells. IL-8 mRNA was evaluated by Northern analysis of total RNA (10 μg/lane).

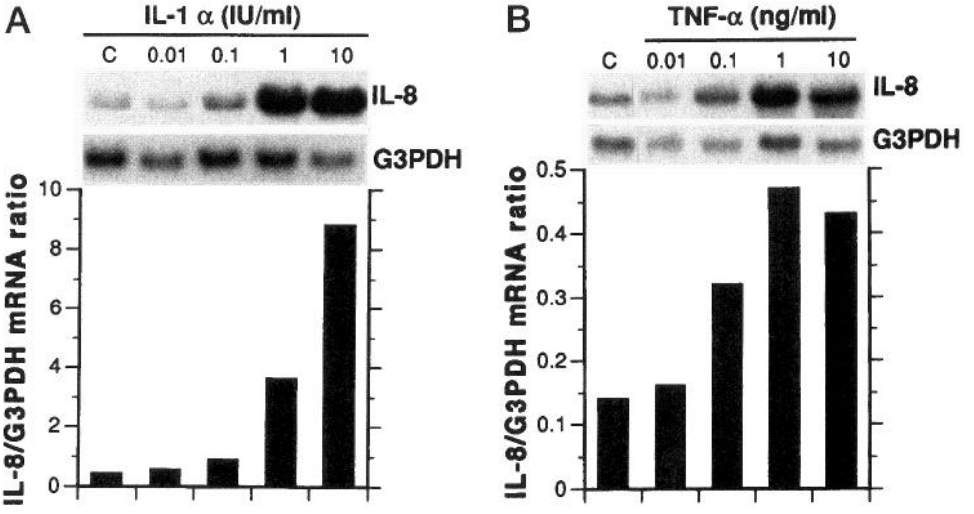

FIG. 6. Induction of IL-8 mRNA in ovarian stromal cells by IL-1α and TNFα: dose response. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 6 h in culture medium containing various concentrations of IL-1α (0.01–10 U/ml; A) or TNFα (0.01–10 ng/ml; B). At the end of the incubation period, total RNA was prepared from the cells. IL-8 mRNA was evaluated by Northern analysis of total RNA (10 μg/lane).

hCG treatment of granulosa-lutein cells in culture maintained in medium that contained FBS (data not shown). Treatment of stromal cells and granulosa-lutein cells with FSH (1–1,000 mIU/ml, up to 24 h of treatment) did not affect IL-8 mRNA expression or protein production (data not shown).

Ovarian stromal cells treated with progesterone (10–1000 ng/ml) for 24 h decreased IL-8 mRNA levels and IL-8 protein secretion into the medium in a concentration-dependent manner (Fig. 10, A and B). Similar findings were observed after progesterone treatment of granulosa-lutein cells (data not shown). Treatment of stromal cells and granulosa-lutein cells with estradiol (1–100 ng/ml, up to 24 h of treatment) did not affect IL-8 mRNA expression or protein production (data not shown).

Discussion

Successful ovulation requires an integrated series of events that ultimately leads to the timely release of a mature oocyte from the follicle. The LH surge initiates the final maturation of the oocyte and simultaneously triggers the cascade of events leading to follicular rupture and luteinization of granulosa cells. The observation that just before the time of ovulation there is a massive infiltration of granulocytes into the area surrounding the leading follicle in rat (17) and human (3) ovaries suggests a critical role for these leukocytes in periovulatory events. Although regulation of the traffic of granulocytes in and around the periovulatory follicles is not clearly understood, the temporal and spatial distributions would point to a hormonally dependent event.

In the human, preovulatory follicular fluid has been shown to contain neutrophil chemotactic activity (5, 6), which was significantly higher in conceptual cycles than in nonconceptual cycles. One of the candidates for this activity is IL-8, a chemoattractant and activating cytokine for neutrophils (7) and a potent angiogenic agent (8). We postulated that IL-8 may be involved in periovulatory events by not only attracting and activating neutrophils that would play a role in timely follicular rupture, but also by stimulating new blood vessel formation for a healthy corpus luteum. If IL-8 is truly involved in the periovulatory events, then the concentration of IL-8 should be increased in follicular fluids obtained after the LH surge (or the administration of hCG) compared to presurge follicular fluids. This is precisely the

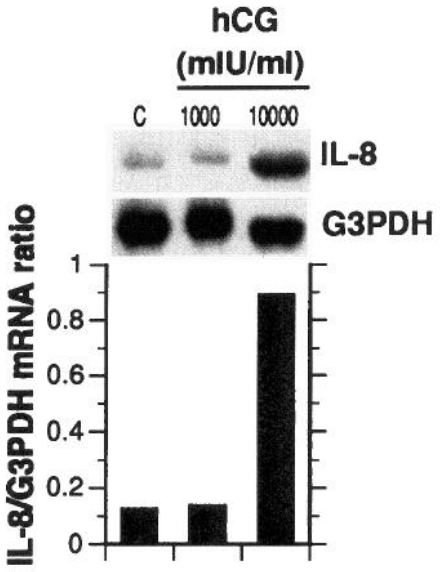

FIG. 7. Induction of IL-8 mRNA in ovarian stromal cells by hCG. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 6 h in culture medium alone or containing hCG (1,000 or 10,000 mIU/ml). At the end of the incubation period, total RNA was prepared from the cells. IL-8 mRNA was evaluated by Northern analysis of total RNA (10 μg/lane).

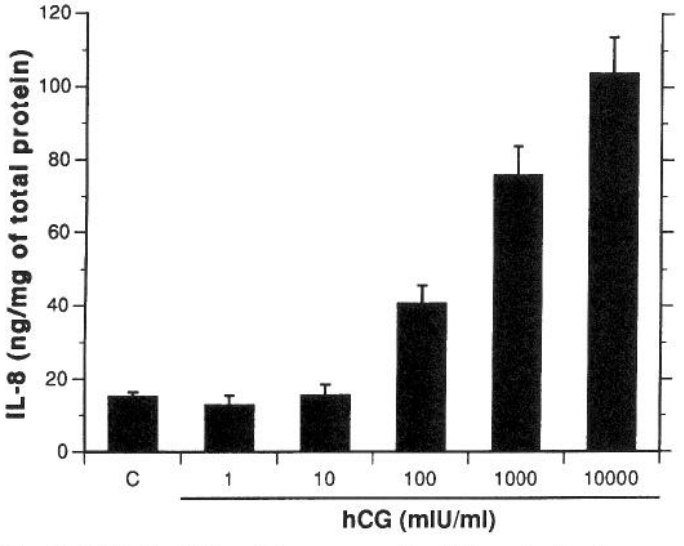

FIG. 8. hCG stimulation of immunoreactive IL-8 production by ovarian stromal cells in culture. Confluent ovarian stromal cells were treated with serum-free medium alone (C, control) or containing hCG (1–10,000 mIU/ml) for 8 h. The culture media were collected, and IL-8 was quantified by ELISA. Data are the mean ± SEM for four replicates.

finding of this study; IL-8 was found in greater quantity in follicles after the LH surge or after hCG administration, and the larger the size of the follicle, the higher the IL-8 concentration.

If IL-8 is a participant in the chemoattraction of granulocytes in and around the periovulatory follicle and also in the neovascularization of the early corpus luteum, then where does this cytokine originate? Peripheral blood contamination is one potential source, but the IL-8 concentrations observed in follicular fluid are substantially (14-fold) higher than lev-

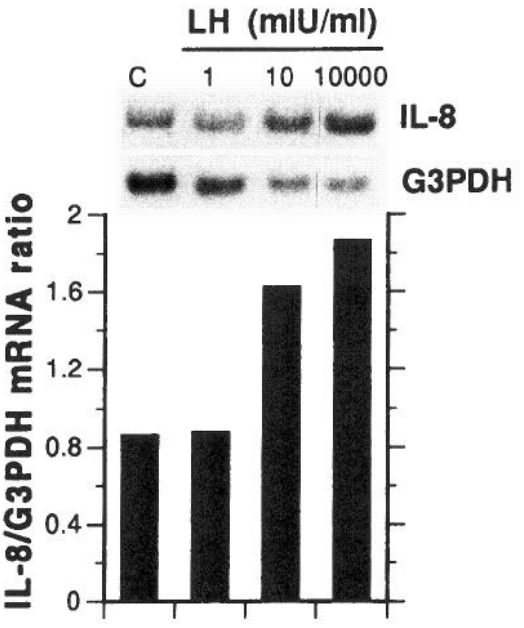

FIG. 9. Induction of IL-8 mRNA in ovarian stromal cells by LH. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 6 h in culture medium alone or containing LH (1, 10, or 10,000 mIU/ml). At the end of the incubation period, total RNA was prepared from the cells. IL-8 mRNA was evaluated by Northern analysis of total RNA (10 μg/lane).

els observed in serum. Therefore, the markedly elevated IL-8 concentrations should reflect the true follicular fluid levels and are not secondary to contamination by peripheral blood. Although granulosa-lutein cells predominate in follicular fluid, resident macrophages and monocytes comprise 5–15% of human follicular tissue cells (18), and these cells are a known source of IL-8. We found that after purification of granulosa-lutein cells by use of anti-CD45 immunobeads, the level of IL-8 production decreased, but was still present. Thus, both granulosa cells and macrophages may contribute to the follicular fluid IL-8 pool. Finally, another potential source is the thecal cell from the ovarian stroma. We found that cultured ovarian stromal cells express IL-8 mRNA and secrete IL-8 protein. Thus, sources of IL-8 are abundant in and around the follicle.

Another question we asked was how IL-8 secretion might be regulated. This study presents evidence that the expression of this chemoattractant cytokine from ovarian stromal and granulosa-lutein cells is modulated by other cytokines, such as IL-1 and TNFα. In stromal cells, we observed an additive effect of IL-1 and TNFα on IL-8 mRNA expression. Although we have seen a similar additive effect in granulosa-lutein cells before elimination of white blood cells by use of anti-CD45 immunobeads, no such effect was observed even at different doses after the purification step. This finding may represent a specific response of granulosa-lutein cells to these cytokines. An intraovarian IL-1 system complete with ligands, receptors, and a receptor antagonist in humans is now well established (19). TNFα also is found in preovulatory follicular fluid at the concentrations used in the present study (20). There are many potential sources of IL-1 and TNFα in and around the preovulatory follicle, such as resident mac-

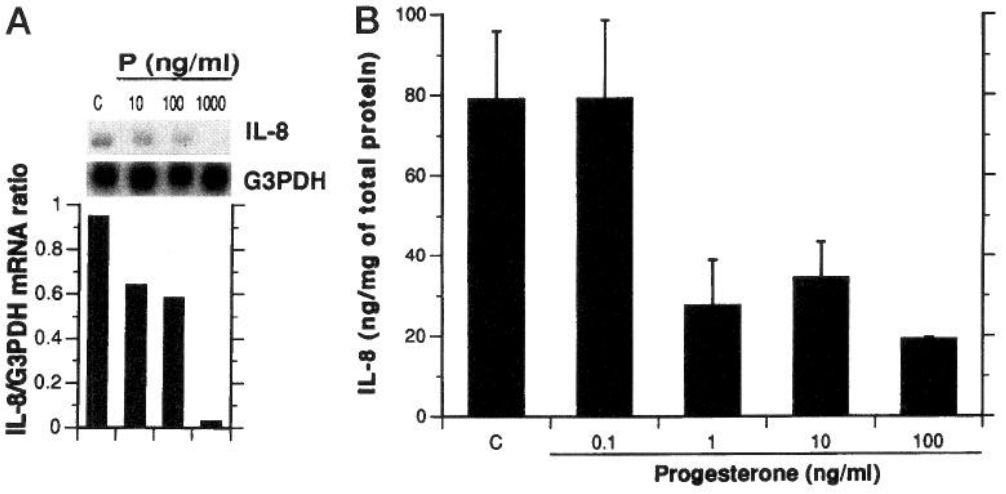

FIG. 10. Inhibition of IL-8 mRNA and protein production in ovarian stromal cells by progesterone. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 24 h in culture medium alone or with containing progesterone (10–1000 ng/ml). At the end of the incubation period, total RNA was prepared from the cells. IL-8 mRNA was evaluated by Northern analysis of total RNA (10 μg/lane; A). To quantify immunoreactive IL-8 production, confluent ovarian stromal cells were treated with serum-free medium alone (C, control) or containing progesterone (0.1–100 ng/ml) for 24 h. The culture media were collected, and IL-8 was quantified by ELISA. Data are the mean ± SEM for four replicates (B).

rophages, granulosa cells, and theca-interstitial cells. Thus, both cytokines may play some role in the constitutive secretion of IL-8 as well as stimulate enhanced production and secretion.

As our hypothesis is that granulocyte migration in and around the periovulatory follicle is hormonally regulated and that regulation occurs through modulation of the expression of IL-8, we also investigated the effects of trophic hormones and sex steroids on the expression of IL-8. We found that hCG/LH, at concentrations found in the preovulatory follicular fluids (10–1000 mIU/ml) (21–23), stimulates IL-8 mRNA expression and protein production from ovarian stromal cells and granulosa-lutein cells within 6–8 h. On the other hand, progesterone at 24 h inhibits IL-8 mRNA expression and protein production from these cells. The presence of progesterone receptor was demonstrated in both ovarian stroma and granulosa-lutein cells (24–26). Both of our findings are consistent with existing ovarian physiology. Granulocytes should arrive in and around the preovulatory follicle in response to the LH surge to release their secretory products, such as histamine, bradykinin, PGs, serotonin, and cytokines, all of which have a well documented role in the biochemistry of ovulation (27). Once ovulation occurs, they are no longer needed, so shortly thereafter these cells should decrease production or else risk early luteolysis. Our findings reflect exactly this theoretical picture. LH/hCG stimulates IL-8 production, which attracts and activates neutrophils to participate in the follicular rupture and neovascularization necessary for the newly forming corpus luteum. With the rise in local progesterone, however, IL-8 production is suppressed, and no further activation of neutrophils occurs.

In summary, we found that IL-8 levels are elevated in periovulatory follicular fluid and both granulosa-lutein and ovarian stromal cells express the mRNA and produce the protein. Modulation of IL-8 in these cell cultures by steroid and trophic hormones suggests that IL-8 may play an important role in the physiology of ovulation, such as aiding follicular rupture and corpus luteum neovascularization.

Acknowledgment

The authors thank Dr. Kamal Jaroudi for his contribution in obtaining follicular fluids.

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