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The human preovulatory follicle is a source of the chemotactic cytokine interleukin-8

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Mammalian ovulation has several similarities to local inflammatory reactions, involving participation of leukocytes and inflammatory mediators. In response to a preovulatory luteinizing hormone surge, there is an influx of leukocytes into the preovulatory follicle and uncharacterized chemotactic activity towards these cells has previously been reported in follicular fluid of several species, including the human. In the present study, we have investigated the presence and local production of interleukin-8 (IL-8), a potent leukocyte-chemotactic and neutrophil-activating cytokine, in the human preovulatory follicle. Immunoreactive IL-8 was present in the follicular fluid in all of 12 in-vitro fertilization (IVF) patients investigated. IL-8 concentrations in follicular fluid (1269 ± 245 pg/ml) were ~30-fold higher than in plasma (41 ± 14 pg/ml). Isolated granulosa cells in culture secreted large amounts of IL-8 protein. Basal secretion of IL-8 was dose-dependently enhanced by the presence of fetal calf serum and was further stimulated by the addition of the ovulation-associated cytokine IL-1β. Messenger RNA for IL-8 was detected by reverse transcription/polymerase chain reaction (RT-PCR) in all tested samples of granulosa cells of IVF patients (*n* = 8) and in all biopsies from preovulatory follicle walls obtained in natural cycles (*n* = 6). This is the first demonstration of IL-8 in the mammalian ovary. Local production, combined with high follicular fluid concentrations, suggests that this cytokine plays a role in cyclic ovarian events, such as ovulation.

Key words: chemokine/follicle/follicular fluid/human ovary/interleukin-8

Introduction

Immune cells and immunomodulatory cytokines have been shown to be active in several physiological processes in the reproductive tract (Robertson *et al.*, 1992). In the ovary, the immune system seems to play an integral part in physiological processes, such as ovulation and luteolysis (Brännström and Norman, 1993).

Animal studies have shown that large numbers of leukocytes are present in the follicle wall just prior to ovulation (Murdoch and McCormick, 1989; Gerdes *et al.*, 1992; Brännström *et al.*, 1993a), and the addition of leukocytes to in-vitro perfused rat ovaries gives rise to an increased rate of ovulation (Hellberg *et al.*, 1991). In humans, it has been shown that follicular fluid exerts chemotactic activity towards neutrophilic granulocytes and that the concentrations of this activity are related to the outcome of in-vitro fertilization (IVF) treatment (Herriot *et al.*, 1986). In addition, we have recently shown that the densities of macrophages and neutrophils in the human preovulatory follicle are high at the time of ovulation (Brännström *et al.*, 1994), and that neutrophil-depleted rats have a decreased rate of ovulation (Brännström *et al.*, 1995). Earlier reports have demonstrated the presence of the cytokines interleukin (IL)-1β, IL-2, IL-6 and tumour necrosis factor (TNF)-α in human preovulatory follicular fluids (Wang and Norman, 1992; Wang *et al.*, 1992; Machelon *et al.*, 1994) with concentrations being somewhat lower than in peripheral blood.

A possible substance inducing leukocyte infiltration and neutrophil activation in ovarian tissue is the cytokine IL-8, also called neutrophil activating peptide 1 (NAP-1). IL-8 belongs to a family of small chemotactic cytokines. It is synthesized as a 99 amino acid precursor and secreted in at least two different forms (77 amino acids, 72 amino acids), after cleavage of signal peptides. This cytokine is produced by a number of cell types, including monocytes (Matsushima *et al.*, 1988), endothelial cells (Strieter *et al.*, 1989), fibroblasts (Schröder *et al.*, 1990), epithelial cells (Fierer *et al.*, 1994), neutrophils (Au *et al.*, 1994) and cells derived from human endometrium (Kelly *et al.*, 1994). In addition to its chemotactic activity, IL-8 activates neutrophil functions, including induction of respiratory burst, exocytosis, transendothelial migration, and the expression of adhesion molecules (Schröder and Christophers, 1991). All of these functions could be of importance in several ovarian processes involving tissue remodelling such as ovulation and luteolysis (Brännström and Norman, 1993). Several of these processes may involve the cytokine IL-1, which recently has emerged as a factor regulating a number of intraovarian events (Hurwitz *et al.*, 1991; Brännström *et al.*, 1993b).

Since IL-8 may be the cause of both the infiltration and activation of leukocytes in the preovulatory follicle, the

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presence of this cytokine, its mRNA, and its regulation by IL-1 was investigated in the human preovulatory follicle.

Materials and methods

Patients and specimens

All patients had given their consent before being included in the study, which was approved by the Human Research Ethics Committee at the Faculty of Medicine, Göteborg University, Sweden.

Follicular fluids and blood samples were taken from 12 patients chosen at random who were participating in the IVF programme at the Reproductive Medicine Unit of the Department of Obstetrics and Gynaecology, Sahlgrenska Hospital. All patients had normal concentrations of follicle stimulating hormone (FSH). The women were treated intra-nasally with buserelin acetate (Suprefact; Hoechst AG, Frankfurt am Main, Germany; 1.2 mg/day) and were subsequently stimulated with human menopausal gonadotrophin (HMG) (Pergonal; Serono, Rome, Italy; 150–225 IU/day) or FSH (Fertinorm; Serono, 75–150 IU/day). Follicular puncture and aspiration of follicular fluid from follicles >15 mm in diameter were performed with ultrasound guidance 36–37 h after human chorionic gonadotrophin (HCG) (Profasi; Serono, 10 000 IU) was administered i.m. Two to six visually blood-free samples of follicular fluid were collected from each patient. The samples were centrifuged at 200 *g* for 10 min and the supernatants were frozen at –70°C until analysis. Blood samples were obtained from each patient within 10 min prior to the start of follicular aspiration and the plasma was frozen at –70°C until analysis.

Granulosa cells for culture were collected at the time of follicular aspiration from nine IVF patients. Cells were centrifuged at 200 *g* on an isotonic Percoll gradient (Pharmacia, Uppsala, Sweden) to exclude erythrocytes. The purified cell preparation was washed twice in medium 199 (M199; GIBCO, Paisley, UK), supplemented with $NaHCO_3$ (0.026 M), gentamicin (50 mg/ml), and bovine serum albumin (BSA, 0.1%). Cell viability was examined using Trypan Blue exclusion and was >90% in all experiments. Cells ($\sim 3\times 10^4$) were seeded in each well of a 24-well plate. For each patient, cells were cultured in M199 and BSA, with 0%, 5% or 10% fetal calf serum (FCS), at 37°C in an atmosphere of 5% CO_2 in air. The total volume of each well was 0.5 ml. In some experiments with 10% FCS, human recombinant IL-1β (Genzyme, Boston, MA, USA; 3 ng/ml) was added. All samples were run in duplicates. Supernatants were collected after 24 h and new media was added for a subsequent 24 h culture period. The supernatants were frozen at –70°C until analysis of IL-8 content. Granulosa cells for polymerase chain reaction-reverse transcriptase (PCR-RT) were collected from eight patients at the time of follicular aspiration. The cells were washed twice with PBS (pH 7.4) and used for extraction of total RNA (see below).

Follicular wall biopsies were taken from preovulatory follicles of six women with normal menstrual cycles, undergoing legal sterilization. The biopsies were immediately frozen in liquid nitrogen and stored in –70°C until further processing for isolation of total RNA. As a positive control, lipopolysaccharide (200 ng/ml) and interferon-γ (100 IU/ml) treated human macrophages were processed for extraction of total RNA in the same manner as the granulosa cells.

Immunoassays

IL-8 in follicular fluid, matched plasma, and supernatants from cultured granulosa cells were measured using a commercially available human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (Amersham International, Amersham, UK). This kit has previously been used and evaluated in our laboratory (Arnestad *et al.*, 1995). The range of the standard (recombinant human IL-8) curve was from 94 to 6000 pg/ml. According to the manufacturer, the cross-reactivities against several human recombinant (hr) cytokines [h(r)IL-1α, h(r)IL-1β, h(r)IL-2, h(r)IL-3, h(r)IL-4, h(r)IL-6, h(r)TNF-α, h(r)TNF-β, h(r)GM-CSF, h(r)GM-CSF, h-transforming growth factor (TGF)β1, pTGFβ1, p platelet-derived growth factor (PDGF), b fibroblast growth factor (FGF)-8(a)] were ≤ 5pg/ml.

Oestradiol in follicular fluid and plasma was analysed by a microparticle enzyme immuno assay (MEIA; Abbott Laboratories, Abbott Park, IL, USA). The sensitivity of the assay was 25 pg/ml.

Progesterone in follicular fluid was analysed by a direct immunofluorescence kit (DELFIA; Wallac Oy, Turku, Finland). The sensitivity of the assay was 251 pg/ml. The intra-assay variations in all assays were <5%, and inter-assay variations were <10%.

RNA extraction and RT-PCR

Total cellular RNA was extracted from tissue biopsies and cell suspensions using total RNA isolation reagent (Advanced Biotechnologies Ltd., Surrey, UK), in a modification of the guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Briefly, 50–100 mg frozen tissue, or 1×10^6 cells were homogenized and lysed in a 14 M solution consisting of guanidine salts and urea (1 ml solution/50–100 mg tissue). The RNA was extracted with chloroform (0.2 ml chloroform/1 ml lysate), precipitated with isopropanol (1 volume isopropanol/volume sample), and finally pelleted by centrifugation (12 000 *g*, 10 min, 4°C). Pellets were washed in 75% ethanol, air dried and resuspended in diethyl pyrocarbonate-treated water. RNA (1 μg) was transcribed into cDNA by incubation in 20 ml 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 50 pmol Oligo dT, 20 IU human placental ribonuclease inhibitor (RNAsin; Promega, Madison, WI, USA), and 200 IU Moloney murine leukaemia virus–reverse transcriptase (M-MLV RT, Promega, Madison, WI, USA). RT reactions were performed at 37°C for 1 h, followed by heating to 95°C for 5 min to inactivate the enzyme, and stored at 4°C. For PCR amplification of the cDNA products, 3 μl reaction product were mixed with 50 pmol 3′-specific IL-8 primer (5′-TTG CTT GAA GTT TCA CTG GC-3′) and 50 pmol 5′-specific IL-8 primer (5′-TCC AAA CCT TTC CAC CCC AA-3′), 25 pmol 3′-specific β-actin primer (5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) and 25 pmol 5-specific β-actin primer (5′-GTG GGG CGC CCC AGG CAC CA-3′), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1×PCR-buffer, 2.5 IU *Taq* DNA polymerase (Promega, Madison, WI, USA), and the final volume was adjusted to 50 μl. The reaction mixture was amplified with a thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) for 35 cycles. The following temperature profile was used: 94°C for 1 min (denaturation), 58°C for 2 min (annealing), and 72°C for 2 min (extension). PCR products were separated on ethidium bromide-stained gels (3%).

Statistical analysis

The follicular fluid concentrations of IL-8, oestradiol, and progesterone and plasma concentrations of IL-8 and oestradiol are expressed as the mean ± SEM. The values of IL-8 in granulosa cell culture are expressed as the mean ± SEM, where the means are calculated from duplicate means of nine patients. Statistical analysis was performed by means of non-parametric methods. When more than two groups were compared, the Kruskal–Wallis test was used for an overall test of significance. Test of differences between two specific groups has been performed using Wilcoxon's rank sum test. For correlation analysis we used Pearson's linear correlation analysis. Significance was assigned at $P < 0.05$.

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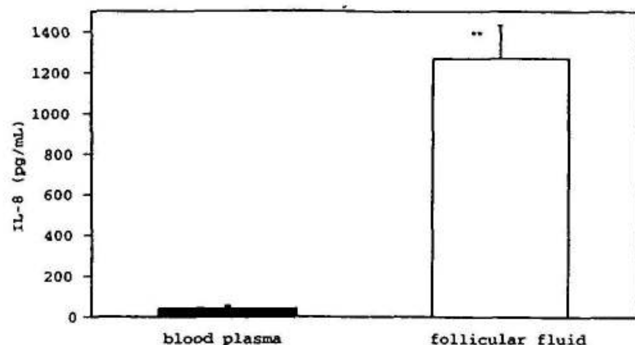

Figure 1. Concentrations of interleukin (IL)-8 in blood plasma and follicular fluid of in-vitro fertilization (IVF) patients at oocyte retrieval ($n = 12$). **Significantly ($P < 0.01$) higher than in blood plasma. Results are mean ± SEM.

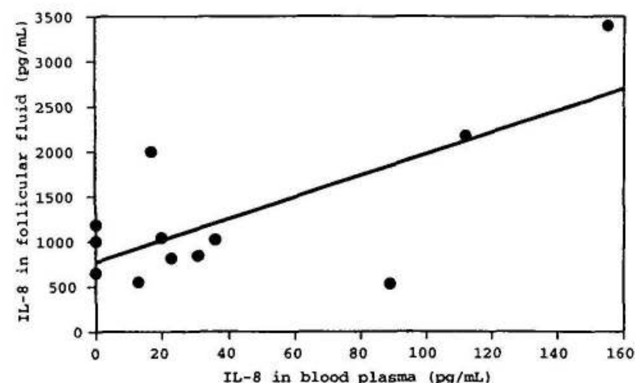

Figure 2. Correlation between interleukin (IL)-8 concentrations in follicular fluid and blood plasma ($n = 12$). Significant ($P < 0.01$) correlation exists; r = 0.711.

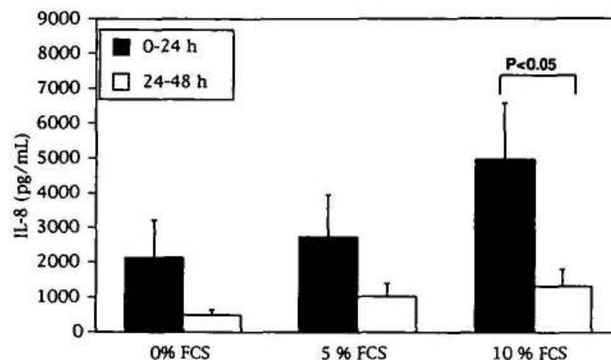

Figure 3. Concentrations of interleukin (IL)-8 in conditioned media of cultured human granulosa cells in the presence of increasing concentrations of fetal calf serum (FCS) ($n = 9$). Medium was changed after 24 h. Results are mean ± SEM.

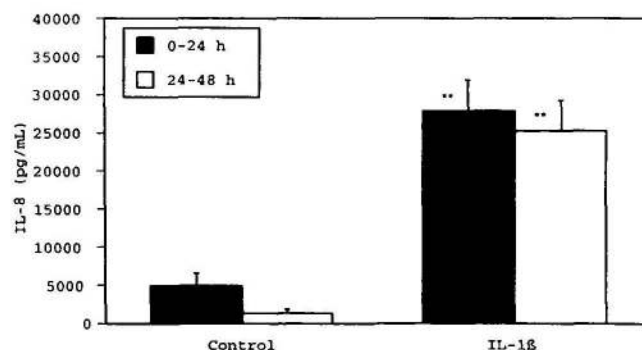

Figure 4. Concentrations of interleukin (IL)-8 in conditioned media of cultured human granulosa cells in the presence of 10% FCS ($n = 9$). Interleukin-1β was present at a concentration of 3 ng/ml. **Significantly ($P < 0.01$) higher than respective control group. Results are mean ± SEM.

Figure 5. Expression of interleukin (IL)-8 mRNA in granulosa cells collected from eight patients undergoing in-vitro fertilization (IVF) (lanes 1–8); lane 9 = positive control, lane 10 = DNA ladder, lane 11 = negative control, and lane 12 = contamination control. A 240 base pair (bp) fragment of DNA characterized the amplification product of IL-8 mRNA transcripts. A 540 bp β-actin fragment was coamplified as a control of uniform RNA/cDNA addition.

Results

Concentrations of IL-8 and steroids in plasma and follicular fluid

The mean concentrations of oestradiol in plasma and follicular fluid at follicular aspiration were 5.17 ± 0.87 nM and 1276 ± 162 nM, respectively, with the mean concentrations of IL-8 in plasma and follicular fluid being 41 ± 14 pg/ml and 1269 ± 245 pg/ml respectively. The mean concentration of progesterone in follicular fluid was 37.1 ± 3.5 μM.

There were ~30-fold ($P < 0.01$) higher concentrations of IL-8 in follicular fluid than in blood plasma of the same patient (Figure 1). A positive correlation was found between follicular fluid concentrations and blood plasma concentrations of IL-8 (Figure 2). No significant ($P > 0.05$) correlation was found between oestradiol concentrations in follicular fluid or blood plasma when compared to IL-8 concentrations in follicular fluid (data not shown).

Secretion of IL-8 from cultured granulosa cells

To examine a possible local production of IL-8, granulosa cells were cultured for two 24 h periods. The basal secretion of IL-8 from cultured granulosa cells was significantly higher ($P < 0.05$) during the first 24 h period (4981 ± 1603 pg/ml; in 10% FCS) compared to the second 24 h period (1324 ± 484 pg/ml; in 10% FCS). Secretion during both the first and second 24 h period was dose-dependently enhanced by the presence of FCS (Figure 3). Presence of the cytokine IL-1β (3 ng/ml) in cultures with 10% FCS markedly increased the secretion of IL-8, with a 6- and 19-fold increase over basal conditions during the first and second 24 h period, respectively (Figure 4).

IL-8 mRNA in granulosa cells and in follicular walls

A 240 base pair (bp) band, representing IL-8 mRNA, was detected by RT-PCR in granulosa cells from all eight patients tested (Figure 5), and in preovulatory follicular walls from all

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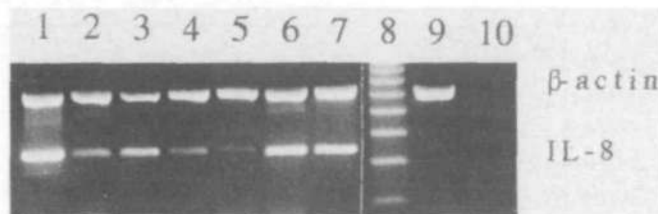

Figure 6. Polymerase chain reaction (PCR) products from preovulatory follicle walls of six patients (lanes 1–6); lane 7 = positive control, lane 8 = DNA ladder, lane 9 = negative control, and lane 10 = contamination control. The fragment of interleukin (IL)-8 is 240 bp, whereas the fragment of β-actin is 540 bp.

six patients (Figure 6). Positive control tissue (activated human macrophages) showed a band of predicted size. No signal was evident in negative control (granulosa cells without RT treatment).

Discussion

There is now compelling evidence of a link between cells and mediators of the immune system and those of the reproductive system (Robertson *et al.*, 1992). During the ovulatory process several subclasses of leukocytes and cytokines seem to act synergistically and in synchrony to promote rupture of the follicle apex and to remodel the ruptured follicle into a corpus luteum (Brännström and Norman, 1993). A key substance in the early steps of this cascade seems to be the multifunctional and pro-inflammatory cytokine IL-1. A complete IL-1 system has been detected in the human ovary (Hurwitz *et al.*, 1992). The expression of this cytokine is induced by luteinizing hormone (LH)/HCG in the equine CG-primed immature rat (Hurwitz *et al.*, 1991), and its direct importance in ovulation has been demonstrated both *in vitro* (Brännström *et al.*, 1993b) and *in vivo* (Simon *et al.*, 1994). IL-1 activates both lymphohaemopoietic and non-lymphohaemopoietic cells to increase the synthesis of the cytokine IL-8 (Schröder and Christophers, 1991), which in turn may be important in ovulation. Previous studies in the rat (Brännström *et al.*, 1993a) and human (Brännström *et al.*, 1994) have demonstrated that the chemotactically responsive leukocyte subtypes, neutrophilic granulocytes and macrophages, represent the majority of leukocyte subclasses in the ovulating follicle. Likewise, the corpus luteum seems to be partly controlled by locally acting leukocytes and cytokines (Brännström and Norman, 1993).

IL-8 is one of several chemokines, a family of chemotactic cytokines, which have the ability to stimulate directed movement of leukocytes according to a concentration gradient of the chemokine (Miller and Krangel, 1992).

The present study is the first demonstration of IL-8 in the ovarian tissue of any species. The concentrations of IL-8 were 30-fold higher in follicular fluid than in samples of plasma drawn at the same time. The markedly higher IL-8 concentrations in follicular fluid is somewhat different from previous studies where the concentrations of other interleukins (IL-1, IL-2, IL-6) and other cytokines [TNFα, granulocyte-macrophage colony stimulating factor (GM-CSF)] were lower than in blood (Jasper *et al.*, 1992; Wang and Norman, 1992; Wang *et al.*, 1992; Machelon *et al.*, 1994; Watanabe *et al.*, 1994). The concentrations of these other cytokines were still in the range known to have effects on ovarian cells in culture (Brännström and Norman, 1993), but a follicular origin could not be established since the concentration gradient indicated that the presence in follicular fluid might be due to filtration from blood. However, recently, local production in the human follicle of both IL-1 and IL-6 has been established (Hurwitz *et al.*, 1992; Machelon *et al.*, 1994) and in a subsequent study on IL-6 (Huyser *et al.*, 1994), concentrations of immunoreactive IL-6 found in follicular fluid were twice those found in serum.

Follicular fluid is essentially a serum-based fluid with similar protein composition as serum, but with higher concentrations of products secreted from the granulosa or theca cells (Shalgi *et al.*, 1973). Thus, the 30-fold concentration gradient of IL-8 in follicular fluid in relation to blood strongly indicates a local production of follicular fluid IL-8. The concentration of IL-8 in follicular fluid found in the present study is similar to that demonstrated to have chemoattractant properties on neutrophils *in vitro* (Yoshimura *et al.*, 1987). The high intrafollicular concentrations of IL-8 further highlight the intraovarian cyclic events as physiological inflammatory reactions (Espey, 1980), since high concentrations of IL-8 also have been found in classical inflammatory sites, such as the synovial fluid of rheumatoid arthritis patients (Seitz *et al.*, 1991) and in bronchial lavage fluids from patients with idiopathic pulmonary fibrosis (Carre *et al.*, 1991).

Since ovulation induction during assisted reproduction gives rise to follicular asynchrony, intrafollicular markers of follicular maturity have been sought (Pellicer *et al.*, 1987). In previous studies of chemotactic activity against neutrophils in follicular fluid, this activity was demonstrated to increase in follicular fluid in the late phase of natural cycles (Herriot *et al.*, 1987) and was related to the capability of the oocyte to produce a pregnancy-potent embryo in IVF cycles (Herriot *et al.*, 1986). It is possible that this chemotactic activity is at least partly due to follicular fluid IL-8, which may prove to be an indicator of follicular maturity.

We found no correlation between IL-8 concentrations in follicular fluid and the concentrations of progesterone or oestradiol, which agrees with previous studies on cytokines in human follicular fluid (Jasper *et al.*, 1992; Wang and Norman, 1992; Watanabe *et al.*, 1994; Huyser *et al.*, 1994). Also in agreement with our findings, one study showed lack of correlation between steroids in follicular fluid or serum and the follicular fluid-induced migratory response of human granulocytes (Herriot *et al.*, 1986). These results suggest that the regulation of ovarian production of IL-8 is steroid-independent and that IL-8 does not influence ovarian steroid production. However, a recently published report on IL-8 production from another reproductive tissue, choriodecidual cells, suggests that the release of IL-8 from these cells is modulated by progesterone (Kelly *et al.* 1994).

The secretion of IL-8 protein from cultured granulosa cells was measured and detectable concentrations were found in conditioned media from granulosa cells of all nine patients studied. The concentrations were of the same order as those previously observed for endometrial and decidual cells (Kelly

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et al., 1994), and likewise we found that increased IL-8 secretion occurred with increasing concentrations of serum. This could be due to the presence of well-known inducers of IL-8 production, such as IL-1 or TNFα in serum (Miller and Krangel, 1992). It could also be due to the presence of additional IL-8 mRNA-stabilizing factors in serum, as demonstrated by Arici *et al.* (1993). Since IL-1 is hormonally induced by LH/HCG in the preovulatory rat ovary (Hurwitz *et al.*, 1991), we tested the effect of this multifunctional cytokine on the induction of IL-8 in granulosa cells. During the first and second 24 h period, an ~6- and 19-fold induction of IL-8 secretion, respectively, was found in all patients tested. The IL-1β-induced stimulation of IL-8 production in granulosa cells was greater than previously reported in cultures of chorion cells (Dudley *et al.*, 1993).

To investigate whether IL-8 is locally produced in the follicle, we examined IL-8 mRNA expression in granulosa cells from IVF patients and in follicle walls from preovulatory follicles of natural cycles. All samples constitutively expressed IL-8 mRNA. Since IL-8 mRNA is known to have a very short half-life, it is possible that intraovarian IL-1 or TNFα could regulate the synthesis of protein by stabilizing the IL-8 mRNA, as previously demonstrated in other systems (Stoeckle, 1991).

Apart from the chemoattractant and neutrophil-activating properties of IL-8, the stimulation of basophils to increase the release of histamine and leukotrienes has been observed (Dahinden *et al.*, 1989). This is of interest in view of the accumulation of basophils around the ovulating follicle (Zachariae *et al.*, 1958), and the proposed importance of both histamine (Schmidt *et al.*, 1988) as well as leukotrienes (Reich *et al.*, 1985) in the ovulatory process.

Several lines of evidence now support a role for a regulated cytokine network in the ovulatory process, where leukocyte infiltration and activation may be involved in site-specific tissue degradation and subsequent tissue reorganization processes. In this study, we have demonstrated the presence of significant IL-8 concentrations in follicular fluid from preovulatory follicles. Furthermore, the capability of granulosa cells to synthesize IL-8 and its regulation by IL-1β have been demonstrated. These findings, in combination with the well known capability of IL-8 to induce leukocyte chemotaxis and neutrophil activation, may suggest a role for IL-8 in intraovarian events.

Acknowledgments

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