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-1b. 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)-1b, 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...
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 visibly 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 stored 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 NaHCO3 (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 (≤3×10⁶) 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% CO₂ 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 [hr(IL-1α, hr(IL-1β, hr(IL-2, hr(IL-3, hr(IL-4, hr(IL-6, hr(TNF-α, hr(TNF-β, hr(GM-CSF, hr(GM-CSF, h(Tranforming growth factor (TGF)β, pTGFβ), p platelet-derived growth factor (PDGF), b fibroblast growth factor (FGF)-8(a)] were ≤5 pg/ml.

Oestradiol in follicular fluid and plasma was analysed by a microplate 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; Wallace 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 1×10⁶ 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 KC1, 3 mM MgCl₂, 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’-TGT GAT GCC ATG TGG G-3’) and 50 pmol 5'-specific IL-8 primer (5’-TCC AAT CCT TTC CAC CCC AA-3’), 25 pmol 3'-specific β-actin primer (5’-CTC AAT GTC ACG AAC TAT GTC-3’) and 25 pmol 5'-specific β-actin primer (5’-GTC TGG GCC CCC AGG AAC CA-3’), 0.2 mM dNTP, 1.5 mM MgCl₂, 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.
IL-8 in the human preovulatory follicle

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.

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...
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 reproductuve 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 et al., 1992). The secretion 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.

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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., 1989) 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.

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